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Full Length Article

Nitrogen Biofertilizer Alleviates the Inhibitory Effect of Cadmium on Physiology and Nitrogen Assimilation in Maize Plants

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Abstract

The present study investigated the role of inoculation with Nitrobien biofertilizer (N-Bio, *Azospirillum* and azotobacter spp.) on the response of maize plants to cadmium-toxicity (applied as 2 and 10 m*M* $CdSO_4$). Cd-stress caused a significant reduction in the fresh and dry biomass of leaves and roots as well as a marked disturbance in the anatomical features of roots and stomatal structure and behavior. Cd-stress significantly depressed the total photosynthetic pigments, photochemical efficiency of PS II, total carbohydrates, and proteins content. Furthermore, increasing Cd level prompted oxidative stress measured in terms of malondialdehyde and H_2O_2 contents in maize plants. Application of N-Bio improved these attributes in Cd-stressed maize plants. Moreover, NO_3^- uptake and its assimilating enzymes (nitrate reductase, NR; glutamine synthase, GS; and, glutamate dehydrogenase GDH) were significantly increased in N-Bio-pretreated Cd-stressed plants than Cd-stressed ones and that was associated with a decrease of NH_4^+ content. N-Bio pretreatment also stimulated the accumulation of amino acids and markedly increased endogenous phytohormone content (IAA, GA_3) of Cd-stressed maize plants. These results revealed the potentiating effect of N-Bio pretreatment in regulating Cd-induced damages in maize plants. © 2021 Friends Science Publishers

Keywords: Biofertilizer; Cadmium stress; Root anatomy; Phytohormone; Nitrate reductase

Introduction

Plants are exposed to several abiotic and biotic stresses. In the present era, heavy metals have been widely distributed; being one of the major outstanding apprehensions for sustainable agriculture and human welfare (Edelstein and Ben-Hur 2018). Cadmium (Cd) is a non-essential heavy metal that occurs naturally in the environment in traces; however, its concentration is continuously increased due to extensive industrial processes, dispersal of sewage sludge and usage of phosphate fertilizers in agriculture (Liu *et al*. 2007). Furthermore, Cd uptake alters plant growth and development, and affects human health by its accumulation in the consumable parts of crop plants.

The processes related to Cd toxicity in plants are very complicated as it can affect several morphological and physiological processes even at low concentrations. Excessive amounts of Cd frequently elicits many stress symptoms in plants, such as decrease of carbon assimilation, generation of oxidative stress, inhibition of chlorophyll synthesis, reduction in nutrient uptake, impairment of photosynthesis and at last bringing about stunted growth, chlorosis, leaf epinasty, alterations in chloroplast ultrastructure, induction of lipid peroxidation, alterations in nitrogen (N) metabolism and interruption of antioxidant machinery (Shah *et al*. 2017; Farooq *et al*. 2020). It is well known that Cd is more easily absorbed by plants than any other heavy metals and more than 90% of the Cd is accumulated in roots (Hussain *et al*. 2021). Although most studies had focused on the impact of heavy metals on visible symptoms on aerial parts and root morphological characters, few studies had recorded toxic symptoms on the anatomical parameters of several plants grown under cadmium stress including maize (Gowayed and Almaghrabi 2013) and rice (Li *et al*. 2014). It is well known that stomata play an important role in adjustment of plant water balance and gas exchange. Alterations in stomatal structure and behavior have been observed due to different heavy metals toxicity as Cd (Mondal *et al*. 2013) and Pb (Divyajyothi and Sujatha 2019).

It has been shown that nitrate uptake and its assimilation by plants are differently affected under Cd treatment (Chaffei *et al*. 2004). Nikolić *et al*. (2017) reported that increasing Cd levels markedly decline NO_3^- uptake and

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its assimilation as indicated by suppressing nitrate reductase (NR) and glutamine synthetase (GS) activities. Singh and Prasad (2017) found a marked decline in glutamine synthetase–glutamate synthase (GS/GOGAT) activities in tomato plants imposed to Cd treatment. They suggested that the decline of both GS and GOGAT activities could result in the accumulation of NH_4^+ and a decrease of growth. On the other hand, other researchers (Chaffei *et al*. 2004; Skopelitis *et al*. 2006) have recorded an increase of glutamate dehydrogenase (NADH-GDH) activity under Cd stress.

The plants exposed to toxic elements tend to accumulate specific amino acid (AAs), which may have valuable functions and play various roles in plants (Xu *et al*. 2012; Zemanová *et al*. 2017). It is well recognized that long-term application of chemical fertilizers contributes to the pollution of soil and ground water with various heavy metals, soil degradation and destruction of soil microflora (Rashid *et al*. 2016). On the other hand, biofertilizers are different types of living population microorganisms, such as bacteria, fungi and cyanobacteria, live in plants root vicinity or rhizosphere and have the ability to stimulate plant growth through different modes of actions such as nitrogen fixation, degradation of organic materials and secretion of phytohormones (Sinha *et al*. 2014).

Recent studies have also demonstrated that application of various strains of plant growth promoting rhizobacteria (PGPR) can promote and improve several plants subjected to various environmental stresses (Naveed *et al*. 2014; Gouda *et al*. 2018). Bacteria of the genus *Azospirillum* and *Azotobacter* are among the best researched PGPR detected in the rhizosphere of many crop plants like wheat and tomato (Agami *et al*. 2017; Reddy *et al*. 2018). They exert their roles through N_2 fixation, secretion of several components as vitamins, plant growth regulators and several natural products as secondary metabolites in the rhizosphere (Vejan *et al*. 2016). Such capabilities accordingly result in enhanced growth of plants under various stresses like drought and heavy metal stress (Agami *et al*. 2017; Rezvi and Khan 2018). Previously, Pacwa-Płociniczak *et al*. (2011) stated that bacterial biosurfactants can bind toxic heavy metals, hence remove them from soil and increase plant tolerance.

Maize (*Zea mays* L.) is one of the most economically important cereal crops utilized for grain, silage, and biofuel goals (Tejada *et al*. 2016). An exponential increment in the world populace would request a higher crop production and hence more utilizations of chemical fertilizers. Therefore, the aim of the current investigation was to study the impact of Cd stress on *Zea mays* and evaluate the role of Nitrobien biofertilizer applied by seed inoculation in the response of maize plants to Cd stress. The modifications in some growth parameters, photosynthetic efficiency, stomatal behavior, and root anatomical structures were followed. In addition, nitrate, ammonia, free amino acids and hormonal content as well as nitrogen assimilating enzymes activity were evaluated.

Materials and Methods

Plant material, growth conditions and treatments

Maize (*Zea mays* L. cv. Nevertity) seeds were obtained from the Agricultural Research Center, Giza, Egypt. Nitrobien biofertilizer (N-Bio) containing a combination of nitrogen-fixing bacteria; *Azotobacter* spp. and *Azospirillum* spp. were kindly supplied by biofertilizers Unit, General Organization of Agriculture Equalization Fund, Agriculture Research Centre, Ministry of Agriculture, Giza, Egypt. After surface-sterilization with 4% sodium hypochlorite for 10 min, the seeds were washed with distilled water several times, soaked for 24 h at 25^{o}C in aerated water and then transferred to weighed plastic pots filled with acid-washed quartz sand and clay (3:1). The pots were divided into four groups and each group consists of 3 replicates. The first group was left as a control without any treatment and irrigated with one tenth strength modified Hoagland solution (Epstein 1972). The second group was irrigated with one tenth strength modified Hoagland solution supplemented with 2 and 10 m*M* $CdSO_4$. In the third group the soaked seeds were inoculated with N-Bio; seed inoculation was performed by mixing maize seeds with the nitrobien using Arabic gum as adhesive material. The coated seeds were then air dried in shade for 30 min and the seeds were sown immediately in pots and irrigated with one tenth strength modified Hoagland solution. In the fourth group the seeds were inoculated with N-Bio and irrigated with one tenth strength modified Hoagland solution supplemented with 2 and 10 m*M* $CdSO_4$. The pots were placed in an environmentally controlled growth chamber under a 16-h photoperiod at an irradiance of about 23 μmol m^{-2} s^{-1} (cool white fluorescent tubes) and 31/28 ± 2°C light/dark temperature and irrigated with the treatment solutions every two-day interval throughout the whole experimental period. After 21 days, homologous plants were harvested, washed thoroughly from adhering soil particles, gently plotted, dissected to shoots and roots and quickly saved for estimation of the various growth parameters and chemical analyses. All chemical analyses were performed on roots and leaves.

Growth parameters

The roots and leaves were separated and taken for determination of fresh (FM) and dry biomass (DM). Shoot height was measured.

Light and scanning electron microscopy

The fragments of *Zea mays* L. (cv. Nevertity) roots from control and treated samples were fixed in a mixture of 2% formaldehyde and 2.5% glutaraldehyde in cacodylate buffer

at pH 7.4 for 2 h, thoroughly washed in the same buffer and then post fixed with 1.0% (w/v) osmium tetraoxide in the same buffer for 2 h at room temperature. Subsequently, the samples were transferred to re-distilled water and stained with a 0.5% aqueous solution of uranyl acetate. After passing through increasing concentrations of ethanol and embedded in Spurr's resin at 70°C (Spurr 1969). Semi-thin sections (1 μm) were observed with light microscope (Olympus, Japan) after staining with 2% uranyl acetate and lead acetate solutions (Venable and Coggeshall 1965). Samples preparation, visualization and photographing were carried out at the Electron Microscopic Unit, Faculty of Science, Alexandria University.

Scanning electron microscopy

Small pieces of fresh specimens of maize leaves from both control and treated samples were removed and fixed by immersing immediately in 4F1G in phosphate buffer solution (pH 7.2) at 4°C for 3 h. Specimens were then post fixed in 2% OSO_4 in the same buffer at 4°C for 2 h. Samples were washed in the buffer and dehydrated at 4°C through a graded series of ethanol. The samples were then dried by means of the critical point method, mounted using carbon paste on an Al-stub and coated with gold up to a thickness of 400A in a sputter-coating unit (JFC-1100 E). Observations of leaf morphology in the coded specimens were performed in a Jeol JSM-5300 scanning electron microscope operated between 15 and 20 KeV.

Estimation of photosynthetic pigments and quantum yield of PSII (Fv/Fm)

The photosynthetic pigments were determined according to methods described by Moran (1982) using N, N-dimethyl formamide (DMF). Absorbance was measured at two wavelengths of 646.8 and 663.8 nm using spectrophotometer (JENWAY, 6305, UV/Vis). Measurement of chlorophyll fluorescence was performed with OS-30P pulse modulated chlorophyll fluorimeter (Opti-sciences, Hudson, and USA) following the procedure described by Kooten and Snel (1990).

Estimation of lipid peroxidation and H_2O_2 content

Hydrogen peroxide content was determined according to the method of Velikova and Loreto (2005). The tissue was homogenized in 0.1% (w/v) TCA, 0.5 mL of the supernatant was mixed with 0.5 mL of 10 m*M* potassium phosphate buffer (pH 7.0) and 1 mL of 1 *M* KI, and the absorbance was read at 390 nm. Lipid peroxidation was monitored by spectrophotometric determination of malondialdehyde (MDA) using thiobarbituric acid (TBA) as described in Wang *et al*. (2009). The content of MDA was calculated on a fresh weight basis using the following formula:

$$\text{MDA } (\mu\text{mol g}^{-1}\text{ FM}) = [6.45(OD_{532}-OD_{600}) -0.56(OD_{450})\times 1000]/\text{wt.}$$

Estimation of total carbohydrate, total protein, nitrate and ammonia contents

The quantification of total available carbohydrate (TAC) was done following Murata *et al*. (1968). About 100 mg of finely powdered oven-dry plant material was hydrolyzed using 0.7 N HCl then assayed as glucose by phenol sulphoric acid method (Dubois *et al*. 1956). Absorbance was read at 490 nm using a UV-Vis Spectrophotometer (JENWAY, 6305, UV/Vis) with reference to known concentration of glucose.

Total protein (TP) content was determined according to the method of Hatree (1972) using Folin-phenol reagent. Absorbance was recorded spectrophotometrically at 650 nm using bovine serum albumin as a standard.

Nitrate contents were measured from an aqueous extraction of 0.2 g dried leaves or roots in 10 mL Millipore-filtered water. A 5-mL aliquot was dried in an air-drying oven at 60°C to complete dryness, after which 2 mL of the phenoldisulphonic acid reagent were added, the absorbance of the solution was measured with a spectrophotometer at 420 nm according to Johnson and Ulrich (1950)

Ammonium was extracted by homogenizing leaf and root segments in borate buffer (pH 8) containing 1.0% acetic acid. The homogenate was centrifuged for 10 min at 16 000 × g and the supernatant was used for determination of ammonium using phenol-hypochlorite method as described by Solorzano (1969) Absorbance was measured at 630 nm with reference to known concentrations of ammonium sulphate.

Nitrogen assimilating enzymes Assay

Nitrate reductase (NR, E.C. 1.6.6.1) was extracted and assayed following the method adopted by Saber *et al.* (1989). Fresh plant material (leaves or roots) was homogenized in 0.05 M Tris – HCl pH 7.5 followed by the addition of potassium nitrate and incubation at 30°C for 2 min. The reaction was started by addition of 0.6 μmol NADH.H and allowed to proceed for 15 min at 30ºC, then stopped by adding 0.1 M zinc sulphate solution and 95% ethanol. The NR activity was assayed according to the method adopted by Saber *et al.* (1989). The produced nitrite was estimated by measuring the absorbance at 290 nm and the NR specific activity was expressed as µmol NO_2^- produced mg^{-1} protein min^{-1}.

Glutamine synthetase (GS, EC. 6.3.1.2): was extracted as described by O'Neal and Joy (1973). Plant material was homogenized in 25 m*M* tris-HCl buffer (pH 7.8), 1 m*M* $MgCl_2$, 14 m*M* β-mercaptoethanol and 1% (w/v) polyvinyl pyrrolidone (PVP). GS specific activity was determined using hydroxylamine as substrate and the formation of γ-glutamylhydroxamate (γ-GHM) was determined colorimetrically at 540 nm after complexion with acidified ferric chloride (Canovas *et al*. (1991). The GS specific activity was expressed in μmol glutamyl hydroxamate mg^{-1} protein min^{-1}.

The NADH-dependent glutamate dehydrogenase (NADH-GDH, EC.1.4.1.2) was extracted according to Turano *et al.* (1996). Frozen samples were homogenized using a cold mortar and pestle with grinding medium consisting of 100 m*M* Tris–HCl (pH 7.5), 14 m*M* β-mercaptoethanol and 1% (w/v) PVP. NADH-GDH specific activity assays were carried out according to Groat and Vance (1981). The oxidation of NADH was measured by a UV-vis Spectrophotometer (TU-1901, Purkinje General, Beijing, China) at 340 nm for 7–10 min and the specific activity of GDH in units of μmol of NADH oxidized mg^{-1} protein min^{-1} was calculated using an extinction coefficient for NADH at 340 nm.

High-performance liquid chromatography (HPLC) analysis

Plant phytohormons: Endogenous phythormones, namely auxins (as Indole-3-butyric acid), abscisic acid (ABA) and gibberellic acid (as GA_3) were estimated by HPLC. The plant roots and leaves (1 g) were thoroughly extracted in 80% methanol containing 0.1% butylhydroxytoluene (Kettner and Dorffling 1995). The extract was centrifuged at 5000 *g* for 5 min at 4°C and the supernatant was reduced to aqueous phase using rotary evaporator. The pH of aqueous phase was adjusted to 2.5–3.0 and extracted four times with half volume of ethyl acetate. The ethyl acetate was dried completely using rotary evaporator and the dried sample was re-dissolved in 1 mL of methanol (100%). 50 μL of methanol extract was analyzed using HPLC system (Agilent technologies 1200 series and UV/VIS detector 200 LC, U.S.A.) equipped with a 5-μm column (Exclipse XDB-18; 4.6 X 150 mm; Brownlee). The solvent used was methanol-2% acetic acid and H_2O (40:20:20) as the mobile phase, run isocratically at flow rate of 1 mL min^{-1}. The detector was set at 254 nm for the integration of peak areas after calibration with the external standard.

Free amino acids: For estimation of free amino acids, samples were homogenized in 1:10 (w/v) glass distilled water and the homogenate was centrifuged at 5000 rpm for 15 min at 4°C. The supernatant was treated with methanol 1:1 (v/v), centrifuged at 10,000 rpm for 5 min and collected for analysis using the previously mentioned HPLC system. Free amino acids were determined as their stable OPA derivative (Williams 1986). The mobile phase consisted of Solvent A (sodium acetate buffer pH 6.8) and solvent B (glacial acetic acid and methanol), run isocratically at flow rate of 1 mL min^{-1}. The detector was set at excitation 230 and emission 450 nm for the integration of peak areas after calibration with the external standards.

Statistical analysis

Statistical analysis of the results was carried out according to Duncan's multiple range tests using SPSS-20. Data were subjected to one-way ANOVA following the method of Sokal and Rohlf (1995). Differences between treatment-means were considered statistically significant at $P \leq 0.05$

Results

Growth parameters

The N-Bio pretreatment enhanced the biomass accumulation of maize seedlings as well as shoot height. Increasing Cd concentration in the nutrient solution resulted in a significant decline of FM, DM and shoots height of N-Bio pretreated and untreated maize plants compared to the control; but the attained values of the former were greater than the latter (Table 1). The same trend was observed for water status of leaves and roots. At the end of the experimental period, the reduction in FM of leaves and roots of 10 m*M* Cd-stressed plants was 88 and 92% respectively, compared to control. The corresponding values for N-Bio pretreatment were 68 and 78%, respectively. In 10 m*M* Cd-treated roots, the water content raised from 39 to 82% upon application of N-Bio. The shoot height in 10 m*M* Cd-stressed maize plants in presence of N-Bio was 3.4-fold the value of 10 m*M* Cd-stressed ones.

Light microscope of root cross sections

Cd stress had a strong negative impact on the anatomical structure of roots in comparison to control plants (Fig. 1). The epidermal and cortical cells were severely ruptured with shrinkage and disturbance of pith parenchyma cells as well as a decrease in the diameter and number of metaxylem elements. In addition, there was a marked disorganization and crimple structures of xylem and phloem elements. Obviously, N-Bio pretreatment of maize seeds mostly improved the adverse effect of Cd on the root anatomical structure.

Scanning electron microscope

A clear stomatal structure in control and N-Bio-pretreated plants were observed, whereas marked variations in stomatal opening and guard cell shape were noticed in response to Cd stress and N-Bio application (Fig. 2). The stomata of the 10 m*M* Cd treated maize seedlings were highly defective with their completely collapsed, irregularly thickened guard cells and their stomatal opening almost remained closed. On the other hand, N-Bio- pretreatment of Cd-stressed leaves increased the stomatal opening compared to Cd- stressed ones which might reveal the role of N-Bio in enhancing the CO_2 diffusion.

Photosynthetic pigments and photosynthetic efficiency

Contamination of nutrient solution with various Cd levels significantly decreased Chl *a*, *b* and total photosynthetic pigments content as well as Fv/Fm values. The carotenoid

Table 1: Effect of biofertilizer application on fresh and dry biomasses, shoot height and water content in the leaves of maize seedlings grown at 2- and 10-m Cd for 21 d

Treatments	Cd conc. (mM)	FM (mg plant^{-1})		DM (mg plant^{-1})		Shoot Height (cm)	Water contents (%)	
		Roots	Leaves	Roots	Leaves		Roots	Leaves
-Bio	0	5.64 ± 0.63^{c}	19.83 ± 1.65^{c}	0.71 ± 0.06^{bc}	1.75 ± 0.13^{cd}	8.8 ± 0.73^{b}	87 ± 6.69^{a}	91 ± 7.00^{a}
	2	3.95 ± 0.40^{d}	14.01 ± 1.56^{d}	0.64 ± 0.05^{cd}	1.68 ± 0.15^{de}	6.5 ± 0.54^{c}	84 ± 9.33^{a}	88 ± 8.00^{a}
	10	0.44 ± 0.03^{e}	2.35 ± 0.24^{e}	0.27 ± 0.02^{e}	0.71 ± 0.06^{f}	1.7 ± 0.14^{d}	39 ± 3.90^{c}	70 ± 7.00^{b}
+ Bio	0	12.94 ± 1.18^{a}	36.57 ± 3.32^{a}	1.12 ± 0.11^{a}	3.71 ± 0.37^{a}	15.4 ± 1.71^{a}	91 ± 8.27^{a}	93 ± 8.45^{a}
	2	9.66 ± 0.97^{b}	33.10 ± 3.01^{a}	0.91 ± 0.08^{ab}	2.48 ± 0.21^{b}	14.2 ± 1.29^{b}	91 ± 8.27^{a}	93 ± 10.33^{a}
	10	2.75 ± 0.23^{d}	11.52 ± 0.96^{b}	0.49 ± 0.03^{d}	1.53 ± 0.14e	6.8 ± 0.62^{c}	82 ± 9.11^{b}	87 ± 9.67^{b}
	P = 0.05	0.003*	0.0087*	0.005*	0.003*	0.017*	0.039*	0.04

Values are means of 3 independent replicates ±SE. means followed by different letters are significantly different at $P \leq 0.05$ according to the least significant difference (LSD)

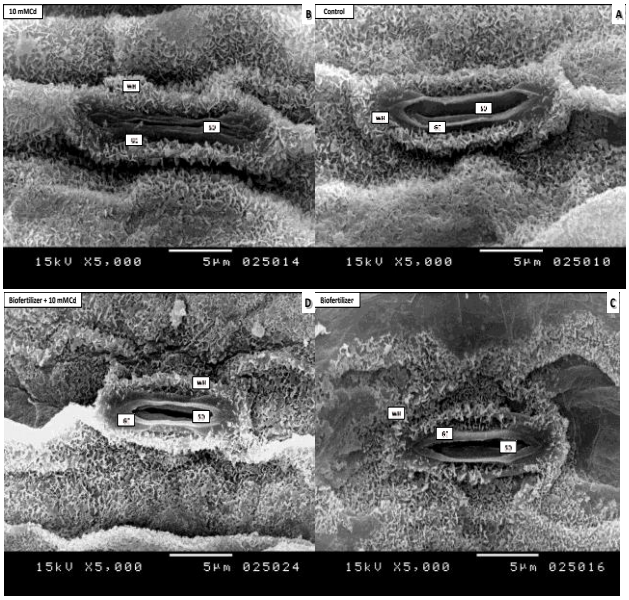

Fig. 1: Scanning electron microscopy (SEM) of abaxial leaf surface from 21-day-old maize plants in response to Cd-stress and biofertilizer application. (**A**): untreated control, (**B**): 10 m*M* Cd, (**C**): biofertilizer and (**D**): biofertilizer +10 m*M* Cd

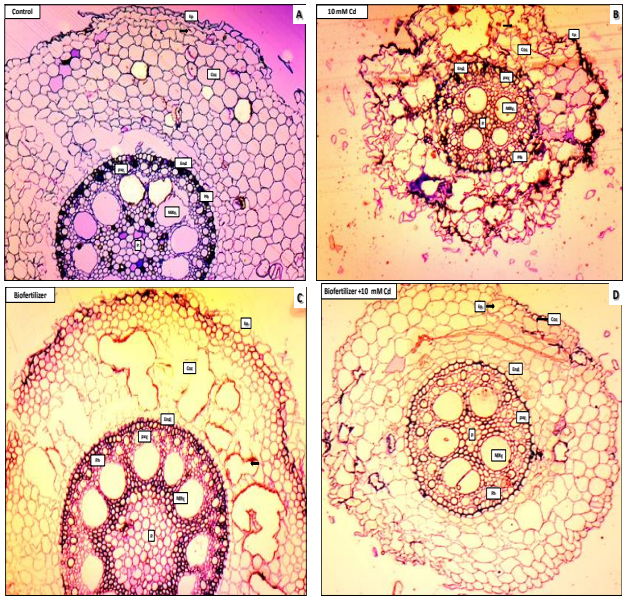

Fig. 2: Light microscope photography showing transverse sections of 21-day-old maize roots in response to Cd-stress and biofertilizer application (**A**): untreated control, (**B**): 10 m*M* Cd, (**C**): biofertilizer and (**D**): biofertilizer +10 m*M* Cd. (Ep) Epidermis, (End) Endodermis, (Cor) Cortex, (Ph) Phloem, (Mxy) Metaxylem, (Pxy) Protoxylem and (P) Pith

contents were insignificantly changed compared to control (Table 2). Inoculation of maize seeds with N-Bio significantly increased total pigments content and Fv/Fm values in Cd-stressed leaves versus those of non-inoculated ones. It is interesting to demonstrate that in absence of Cd, inoculation of N-Bio significantly increased the photosynthetic pigments content compared to non-inoculated control.

H_2O_2 content and Lipid peroxidation

After prolonged exposure of maize plants to Cd stress, there was a significant increment in H_2O_2 content in a concentration dependent aspect; it was about 5.4- and 4.6-folds the value of control in response to 10 m*M* Cd in the leaves and roots, respectively (Fig. 3). Inoculation with N-Bio resulted in a significant depression in H_2O_2 accumulation compared to non-inoculated treatment. At N-Bio-10 m*M* Cd- treatment, the decrease in H_2O_2 content was 41 and 55% in the leaves and roots, respectively compared to the value at 10 m*M* Cd alone.

In parallel with changes in H_2O_2, Cd treatment significantly increased MDA content, indicting lipid peroxidation in the leaves and roots of maize plants compared to control. However, inoculation of maize seeds with N-Bio reduced the Cd-induced MDA accumulation (Fig. 3).

Carbohydrate, protein, nitrate and ammonia contents

Increasing Cd- levels significantly reduced TAC and TP in the leaves and roots of maize seedlings (Table 3). At 10 m*M* Cd treatment, the TAC content of leaves and roots were 49 and 38% of control, respectively. The corresponding values for TP were 36 and 30%, respectively. The interactive effect of N-BiO and Cd displayed a significant

Table 2: Effect of biofertilizer application on photosynthetic pigments (mg g^{-1} FM) and quantum yield of PSII (Fv/Fm) in the leaves of maize seedlings grown at 2- and 10-m Cd for 21 d.

Treatments	Cd conc. (m)	Pigment content (mg g^{-1} FM)				Quantum yield of PSII (F_V/F_M)
		Chl. *a*	Chl. *b*	Carot.	Total	
-Bio	0	22.86 ± 2.54^{b}	12.08 ± 1.10^{ca}	5.75 ± 0.52^{d}	40.68 ± 3.39^{a}	0.807 ± 0.09^{a}
	2	14.94 ± 1.15^{c}	7.54 ± 0.66^{d}	6.40 ± 0.71^{d}	28.88 ± 2.72^{c}	0.730 ± 0.08^{bc}
	10	5.12 ± 0.47^{d}	2.38 ± 0.20^{d}	7.46 ± 0.42^{cd}	14.96 ± 1.08^{d}	0.562 ± 0.04^{d}
+ Bio	0	34.92 ± 2.49^{a}	10.03 ± 0.63^{b}	9.56 ± 0.87^{bc}	54.51 ± 6.06^{a}	0.815 ± 0.07^{a}
	2	28.78 ± 3.20^{ab}	14.22 ± 1.19^{ac}	10.79 ± 0.98^{bc}	53.79 ± 4.48^{a}	0.780 ± 0.07^{b}
	10	12.63 ± 1.64^{c}	6.60 ± 0.96^{d}	17.54 ± 1.75^{a}	36.77 ± 4.25^{b}	0.721 ± 0.07^{c}
	$P = 0.05$	0.019*	0.009*	0.035*	0.028*	0.007*

Values are means of 3 independent replicates ±SE. means followed by different letters are significantly different at $P \leq 0.05$ according to the least significant difference (LSD)

Table 3: Effect of biofertilizer application on nitrate (mg g^{-1} DM), ammonium (µg g^{-1} DM), total available carbohydrates (mg g^{-1} DM) and total protein contents (mg g^{-1} DM) in the leaves and roots of maize seedlings grown at 2- and 10-m*M* Cd for 21 d.

Treatments	Cd conc. (m*M*)	NO_3^- content (mg g^{-1} DM)		NH_4^+ content (μg g^{-1} DM)		TAC (mg g^{-1} DM)		TP (mg g^{-1} DM)	
		Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves
-Bio	0	1.50 ± 0.045^{a}	2.53 ± 0.281^{c}	3.38 ± 0.307^{d}	10.74 ± 1.193^{dc}	66.79 ± 6.07^{b}	91.32 ± 10.15^{b}	19.43 ± 1.49^{b}	42.51 ± 4.72^{bc}
	2	0.82 ± 0.047^{c}	1.74 ± 0.158^{d}	12.64 ± 1.149^{c}	21.16 ± 1.924^{c}	62.69 ± 5.70^{b}	81.08 ± 9.01^{c}	16.91 ± 1.30^{c}	37.88 ± 3.16^{cd}
	10	0.38 ± 0.072^{e}	0.48 ± 0.051^{f}	54.06 ± 5.691^{a}	77.22 ± 8.128^{a}	25.47 ± 1.96^{d}	45.04 ± 4.50^{d}	5.76 ± 0.58^{d}	15.16 ± 1.26^{e}
+ Bio	0	1.48 ± 0.053^{a}	4.59 ± 0.399^{a}	4.21 ± 0.468^{d}	6.08 ± 0.676^{f}	74.65 ± 6.22^{a}	101.65 ± 8.47^{a}	24.64 ± 2.46^{a}	51.11 ± 4.26^{a}
	2	1.09 ± 0.041^{b}	3.06 ± 0.269^{b}	6.99 ± 0.583^{cd}	8.83 ± 0.803^{ef}	73.52 ± 7.35^{a}	94.57 ± 7.88^{b}	23.26 ± 2.11^{a}	48.38 ± 4.03^{a}b
	10	0.55 ± 0.058^{d}	1.06 ± 0.112^{c}	25.36 ± 2.669^{b}	35.56 ± 3.743^{b}	42.52 ± 3.54^{c}	78.32 ± 6.02^{c}	12.53 ± 1.14^{c}	32.27 ± 3.23^{d}
	$P = 0.05$	0.001*	0.003*	0.010*	0.009*	0.017*	0.011*	0.025*	0.009*

Values are means of 3 independent replicates ±SE. means followed by different letters are significantly different at $P \leq 0.05$ according to the least significant difference (LSD)

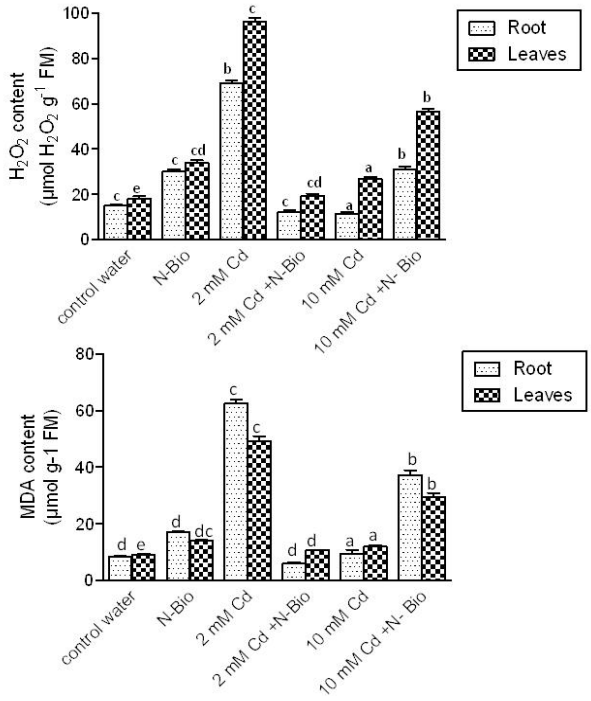

Fig. 3: Effect of N-biofertilizer application on hydrogen peroxide (H_2O_2) and malondialdehyde (MDA**)** in the roots and leaves of maize seedlings grown at 2- and 10-m*M* Cd for 21 d

Values are means of 3 independent replicates ±SE. means followed by different letters are significantly different at $P \leq 0.05$ according to the least significant difference (LSD)

increment in TAC and TP contents compared to those of Cd treated-plants. The TAC and TP contents in N-Bio inoculated leaves in presence of 10 m*M* Cd were 1.7- and 2.1-folds, respectively compared to those of non-inoculated ones. The corresponding values in roots were 1.9- and 2.5-folds, respectively.

Supplementing the nutrient solution with various Cd levels significantly suppressed NO_3^- content in leaves and roots of maize plants either pretreated or untreated with N-Bio, compared to their controls; but attained values of the latter were markedly higher than the former (Table 3). The increase in the NO_3^- content in the leaves and roots of N-Bio inoculated plants grown at 10 m*M* Cd was 121 and 45% compared to those of non inoculated ones.

Conversely to the NO_3^- trend, there was a marked accumulation of NH_4^+ content in leaves and roots of maize plants with increasing Cd concentrations in growth media in absence or presence of the N-Bio, but the attained values in the latter were lower than those in the former. On the other hand, N-Bio- pretreatment of Cd-stressed plants decreased the NH_4^+ accumulation under Cd stress. The NH_4^+ content in leaves and roots of N-Bio-10 m*M* Cd-treated plants decreased by 54 and 53%, respectively compared to N-Bio-pretreated plants alone.

Changes in phytohormones and free individual amino acids

The 10 m*M* Cd stress brought about significant decline of IAA and GA_3 contents in leaves and roots of N-Bio inoculated and non-inoculated maize plants, whereas ABA significantly increased in comparison to controls (Table 4). The decrease of IAA and GA_3 contents in leaves of Cd-stressed plants were 49 and 66% respectively compared to the control. The corresponding values for roots were 66 and 44% respectively. Contrarily, the increase of ABA in leaves

Table 4: Effect of biofertilizer application on plant phytohormons (ng g^{-1} FM) in the leaves and roots of maize seedlings grown at 2- and 10-mM Cd for 21 d. (ABA): Abscisic acid; (IAA): indole acetic acid and (GA_3): gibberellic acid.)

Treatment	Cd conc. (mM)	Hormone Content (ng g^{-1} FM)					
		IAA		GA_3		ABA	
		Roots	Leaves	Roots	Leaves	Roots	Leaves
-Bio	0	18.14 ± 1.64^{b}	22.05 ± 2.20^{c}	36.15 ± 3.27^{c}	48.11 ± 4.80^{c}	9.09 ± 0.75^{c}	10.08 ± 0.83^{c}
	10	6.22 ± 0.46^{c}	11.30 ± 0.92^{d}	20.10 ± 2.00^{d}	16.21 ± 1.33^{d}	23.06 ± 1.92^{a}	35.10 ± 3.89^{a}
+ Bio	0	31.11 ± 2.38^{a}	46.09 ± 4.18^{a}	51.14 ± 5.67^{a}	106.11 ± 10.60^{a}	9.09 ± 0.75^{c}	11.11 ± 1.00^{b}
	10	17.09 ± 1.31^{b}	32.12 ± 2.91^{b}	44.12 ± 4.00^{b}	70.10 ± 7.78^{b}	16.08 ± 1.60^{b}	16.10 ± 1.78^{b}
	P	0.021*	0.029*	0.012*	0.009*	0.015*	0.011*

Values are means of 3 independent replicates ±SE. means followed by different letters are significantly different at $P \leq 0.05$ according to the least significant difference (LSD)

Table 5: Effect of biofertilizer application on free individual amino acid content (mg 100 g^{-1} DM) in the roots and leaves of maize seedlings grown at 2- and 10-m*M* Cd for 21 d**.**

Amino acids	Treatment			
(mg 100 g^{-1} DM)	Control	Bio	10 m*M* Cd	10 m*M* + Bio
Aspartic acid	1.49	2.07	2.46	3.00
Glutamic acid	1.98	5.40	0.61	3.43
Glutamine	1.16	0.88	0.07	0.19
Arginine	1.35	1.17	2.31	1.02
Lysine	1.19	0.98	0.38	0.33
Alanine	0.34	0.95	0.16	1.29
Glycine	0.34	0.72	0.04	0.34
Isoleucine	0.23	0.47	0.12	0.17
Leucine	1.45	1.06	0.57	0.87
Serine	0.19	0.92	0.10	0.13
Theronine	1.39	4.51	0.23	2.63
Tyrosine	0.58	0.18	0.34	0.46
Phenylalanine	0.51	1.20	1.40	0.33
Methionine	0.41	0.37	0.15	1.20
Cysteine	0.34	0.44	0.09	0.32
Tryptophan	1.44	3.46	0.17	0.98
Total	14.39	24.78	9.02	16.69

and roots of 10 m*M* Cd-stressed maize plants was 248 and 154% compared to control, respectively. N-Bio pretreatment alone or in combination with Cd stress had a stimulatory effect on the endogenous IAA and GA_3 while it decreased the ABA content, compared to their respective controls.

Among 16 detected free amino acids, 10 m*M* Cd resulted in a marked increase in arginine (Arg, 71%), aspartic acid (Asp, 65%) and phenylalanine (Phe, 176%) compared to control (Table 5). Other amino acids were markedly decreased such as glutamic acid (Glut, 69%), glycine (Gly, 88%) and cysteine (Cys, 74%). Application of N-Bio in presence of 10 m*M*-Cd mainly led to suppression in the level of 3 amino acids namely Arg, Lyc and Phe while the other detected amino acids were markedly increased compared to non-inoculated Cd-stressed leaves.

Enzyme activities

Generally, prolonged exposure to Cd-stress in presence or absence of N-Bio significantly decreased both NR and GS specific activity in leaves and roots of maize plants in comparison to their controls (Fig. 4). The decrease of NR and GS specific activity in severely Cd-stressed leaves was 79 and 75%, respectively compared

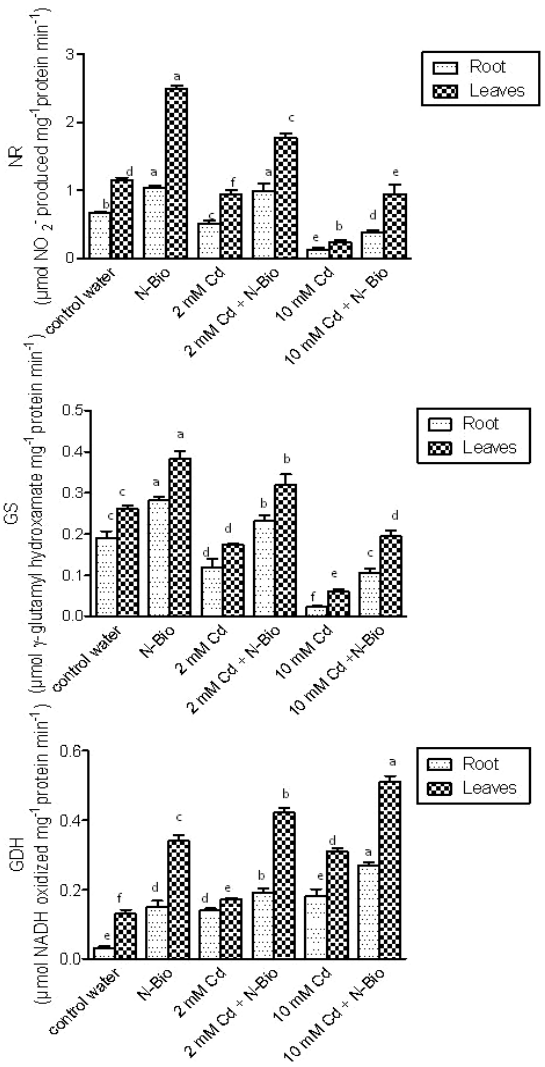

Fig. 4: Effect of N-biofertilizer application on NR, GS and GDH specific activities in the roots and leaves of maize seedlings grown at 2- and 10-m*M* Cd for 21 d

Values are means of 3 independent replicates ±SE. means followed by different letters are significantly different at $P \leq 0.05$ according to the least significant difference (LSD)

to control. The corresponding values for roots were 81 and 88%, respectively. The same trend was observed upon N-Bio pretreatment but the attained values for both NR and

GS specific activities were much less than in Cd-treated plants.

In contrast to NR and GS trends, contaminating the nutrient media with various Cd levels significantly increased GDH specific activity in leaves and roots of N-Bio-untreated or treated maize plants (Fig. 4). At 10 m*M* Cd stress, the GDH specific activity in leaves and roots was 2.3- and 6-folds respectively, compared to untreated control. The corresponding values in N-Bio-pretreated leaves and roots were 1.5- and 1.8-folds, respectively.

Discussion

Cd-stress negatively influenced several growth parameters of maize plants. Several studies have shown that growth biomarkers have been depressed due to Cd-stress in a number of plants including *Solanum melongena* L. (Singh and Prasad 2014) and *Zea mays* L. (Liu *et al*. 2007). It has been reported that increasing Cd accumulation in plant organs could enhance the generation of ROS which caused the damage of plasma membranes, photosynthetic pigments, and various cellular components, leading to reduction of growth (Liu *et al*. 2007). In accordance with these views, the current study demonstrated that Cd stress induced a significant accumulation of H_2O_2 and MDA in leaves and roots of maize plants and disturbed the root anatomical structure causing immature xylogenesis and dysfunctions of phloem which reflect the decrease of water and photosynthates allocation. Moreover, the disturbance of the anatomical feature of maize roots was accompanied with a marked accumulation of Cd in roots (Data not shown) and a significant decline of IAA and GA_3 contents indicating the inhibitory effect of Cd on auxins biosynthesis and disordering the mitotic divisions leading to suppression of cell divisions and elongation and hence the growth. Soudeh and Zarinkamar (2012) reported that inhibition of root growth may be the result of decreased cell division and or disorderliness in the activity and contents of phytohormones like auxins in response to heavy metals stress. Cd-stress seems to provoke a series of structural alterations with possible functional implications in the maize plant such as shrinkage of root diameter (Li *et al*. 2014) and reduction in the metaxylem vessels diameter (Gowayed and Almaghrabi (2013) which is considered as important factor affecting root capacity as translocation conduits.

Comstock (2002) reported that the regulation of guard cell has become a crucial model system for explaining the regulatory signals that control stomatal behavior. Cd exposure profoundly alters the behavior of stomata in maize leaves (Fig. 1). Similar observations were reported for many plant species grown under different heavy metals stress (Mondal *et al*. 2013; Divyajyothi and Sujatha 2019). The defective stomata probably might have lost a functional closing mechanism, and therefore were unable to regulate the exchange of water vapor and CO_2, which decreases both transpiration and photosynthesis (Fatemy *et al*. 1985). Furthermore, this adverse stomatal closure may be due to the loss of turgor of the guard cells and the damage to the guard subsidiary cells (Priyadarshini and Sujatha 2011).

In the present study, Cd stress triggered a significant reduction in the photosynthetic pigments content and the quantum yield of PSII (F_v/F_m). These observations are in accordance with those reported for coriander (Haneef *et al*. 2013) and tomato (Singh and prasad 2017). The suppression in photosynthetic pigments content has been reported to be related to the inhibitory effect of Cd on specific enzymes responsible for their synthesis and induction of some degradative enzymes such as chlorophyllase as well as destruction of the photosynthetic machinery and reduction in the chlorophyll proteins content (Singh and Prasad 2017). In addition, Singh and Prasad (2014) suggested that the decrease of Fv/Fm values in plants under Cd stress could be related to the decline in the active reaction centers and inability of PSII to reduce the primary acceptor (QA) resulting in a decrease of electron transport and photosynthetic activity. Therefore, the decline of chlorophyll contents in Cd-stressed maize plants could be related to the enhancement of lipid peroxidation of thylakoids and chloroplast membranes as indicated by increasing MDA content in the leaves. Moreover, the decrease of TP content, due to increase of oxidative stress by generated ROS (H_2O_2) might inhibit the biosynthesis and content of pigment-protein complexes of photosystems, hence reduce the photosynthetic activity and growth.

The results of this study apparently demonstrated that N-Bio pretreatment significantly increased the growth of Cd-stressed maize plants. Fukami *et al*. (2017) reported that spraying cells or metabolites of *Azosperillum brasilense* Ab-V5 and Ab-V6 enhanced the growth of maize plants. Gothandapani *et al*. (2017) have reported that application of various plant growth promoting microorganisms (PGPMs) induces the growth biomarkers in several plants. Earlier**,** Sokhangoy *et al*. (2012) concluded that biofertilizers might enhance the nutrient availability to plants causing an increase of growth. Whereas, Etesami (2018) suggested that secretion of plant hormones (*e.g*., IAA) by biofertilizers might induce the plant growth**.** Therefore, the increase of growth biomarkers in N-Bio-pretreated maize plants under Cd stress could be attributed to increasing the absorption of plant hormones (IAA) and nutrients including NO_3^- from the rhizophere. Data recoded in this study (Table 3 and 4) pointed out that, there was a marked increase of NO_3^- content and plant growth regulators (IAA and GA3) in leaves and roots of N-Bio-pretreated maize plants under Cd stress in comparison to untreated ones. These observations were accompanied with a marked increase of TAC and TP contents, revealing the stimulation of growth. Several studies in *Eruca sativa* (Kamran *et al*. 2015) and *Zea mays* (Roychowdhury *et al*. 2017) grown under Cd stress showed a marked reduction in growth in response to Cd stress, while PGPR application could contribute to improved growth indices.

Furthermore, N-Bio treatment markedly increased the photosynthetic pigment contents and Fv/Fm ratio of Cd-treated maize leaves compared to non-treated plants. Similarly, Khanna *et al*. (2019) found that plant growth promoting microorganism's inoculation resulted in an increase of photosynthesis and growth of Cd-stressed tomato plants. The findings in this study might be explained by decreasing the generation of ROS and oxidative damage of chloroplasts and thylakoids membranes and enzymatic proteins as well as increasing the availability of essential elements introducing in chlorophyll biosynthesis (as indicated by the increase of NO_3 content). Moreover, inoculation of maize seeds with N-Bio significantly declined ABA content that was associated with an increase of stomatal opening. These observations might reflect the increase of CO_2 diffusion and hence increase photosynthesis. Haneef *et al*. (2013) reported that PGPMs are capable of shifting off the toxic effect of heavy metals through enhancing the mobilization of elements such as N, K, P, Mg and Fe.

Nitrogen (N) is one of the essential nutrients involved in biosynthesis of various cell components such as amino acids, protein and chlorophylls that reflect its essential role in sustaining the growth of plants. Decreased TP content in the maize plants grown on excess Cd was in accordance with earlier observations in several plants such as chamomile plants (Kovacik and Backor 2017). It has been reported that the decrease of protein content under Cd stress may be related to enhancement of protein hydrolysis and/or decrease of protein synthesis in addition to the suppression in amino acids biosynthesis (Xu *et al*. 2012). It can be suggested that the decrease of TP content in Cd-stressed maize plants, in this study, might be attributed to increasing ROS generation, as indicated by increase of H_2O_2 accumulation, which cause oxidative damage for protein. Moreover, the decrees of C-skeleton, due to decline of TAC and inhibition of NO_3^- uptake and its assimilation, *via* NR and GS (Fig. 4), could result in a marked decrease of amino acids biosynthesis and hence protein synthesis.

It is well documented that plants exposed to different abiotic stresses modulate total and amino composition (Zemanová *et al*. 2017). The findings in this study indicated an increase of some amino acids while others were decreased. These variations might reveal the participation of various amino acids in the biosynthesis of secondary metabolites involved in strategy mechanism (Chaffei *et al*. 2004; Xu *et al*. 2012). The decrease of arginine and phenylalanine could be involved in the biosynthesis of polyamine and phenolic compounds, while the decrease of methionine, cysteine, glutamic and glycine content might be introduced in the synthesis of phytochelatine and thiol compounds such as GSH and thiol-non proteins. On the other hand, the decrease of tryptophan content might reflect the suppression of IAA concentration in Cd-stressed maize plants. Costa and Spitz (1997) reported that increasing of asparagine accumulation in Cd-treated *in vitro* lupin tissues culture could participate in the synthesis of chelate peptide for detoxification of the Cd toxicity. It is interesting to demonstrate that methionine content decreased markedly in 10 m*M* Cd-stressed plants while it increased upon application of N-Bio compared to stressed non-inoculated plants. These findings might reflect the role of N-Bio in the protection of maize growth from the inhibitory effect of ethylene via secreting of 1-amino cyclopropane 1-carboxylate (ACC). Ahmad *et al*. (2013) reported that N-Bio can produce several protective enzymes such as ACC deaminase. Kang *et al*. (2010) postulated that ACC, the precursor of ethylene is synthesized from methionine *via* ACC synthase activity, and then produced ethylene could inhibit the growth. Thus, the decreased methionine content in Cd-treated leaves, in this study, was accompanied with a marked reduction of growth, and that might be explained by enhancing ethylene production under Cd stress causing a marked inhibitory effect on maize growth.

It has been firmly established that NO_3^- is taken up *via* specific channel systems and ATP-dependent plasma membranes-associated carriers (Forde 2000). The suppression of NO_3^- content and NR activity in leaves and roots of maize plants imposed to Cd stress might be correlated with Cd-induced disturbance of plasma membranes that might disordered the specific NO_3^- channels and plasma membrane-associated NO_3^- carriers. Moreover, the disturbance of cortical cells and vascular system of Cd-stressed roots could lead to suppression of NO_3^- transportation through xylem elements from roots to leaves. Furthermore, the decline of TAC, the requisite C-skeleton and source of H-donors for nitrogen assimilation might indirectly inhibit NR activity. Besides, the reduction of NR activity in Cd-treated maize plants could be related to the oxidative damage of NR enzyme and NO_3^- carrier proteins, by generated ROS and interaction of functional SH groups of the enzyme with Cd as well as suppression of NR gene expression (Erdal and Turk 2016).

It is noteworthy that parallel to the suppression of NO_3^- content and NR activity in leaves and roots of maize plants imposed to Cd stress, there was a significant increase of $NH4^+$content and induction of NADH-GDH. Similarly, several studies have demonstrated a notable increment of NADH-GDH activity under Cd stress (Chaffei *et al*. 2004; Skopelitis *et al*. 2006). Singh and Prasad (2017) reported that the suppressed GS and GOGAT activities in Cd-treated tomato seedlings could resulted in a marked disturbance in NH_4^+ assimilation process, and that leads to an increase of NH_4^+ accumulation and a decrease of protein content. Britto and Kronzucker (2002) suggested that NAD(P)H-GDH activity (an alternative enzyme for NH_4^+ assimilation) might be enhanced when GS/GOGAT cycle is inhibited to protect the cell damage caused by accumulated NH_4^+. Thus, it is possible that the increase of NADH-GDH activity was to compensate the decrease of GS activity to sustain NH_4^+ assimilation resulting in a marked accumulation of NH_4^+. Application of N-Bio partially shifted off the inhibitory

consequences of Cd on both processes as indicated by the significant increase of NR, GS and GDH activities as well as NO_3^- content in N-Bio-pretreated Cd-stressed plants compared to those of Cd-stressed ones. The increase of NO_3^- uptake and NO_3^- transportation and its assimilating enzymes in N-Bio-pretreated maize plants might be attributed to decreased Cd-bioavailability to root system *via* increase of Cd adsorption on organic matter (Pacwa-Płociniczak *et al*. 2011) and reduction of oxidative damage, hence controlling plasma membrane integrity.

Conclusion

This study pointed out that N-Bio treatment (*Azotobacter* spp. and *Azospirillum* spp.) was able to mitigate Cd toxicity in maize plants causing an increase in growth biomarkers. However, N-Bio pretreatment may have restored the growth of maize plants *via* increasing of NO_3^- uptake (content) and modulation of its assimilating enzyme activities (NR, GS, and GDH) as well as suppression of oxidative damage of plasma membranes and improvement of Cd-induced alteration in root anatomical structure. Moreover, inoculation with N-Bio may have enhanced the uptake of N-Bio secreted phytohormones (IAA, GA_3) which was accompanied with a decrease of ABA content leading to an increase of stomatal opening, CO_2 diffusion and finally increasing the photosynthetic efficiency.

Author Contributions

The authors confirm contribution to the paper as follows: study conception and design: Saber N.E., Abou-Zeid H.M., Ismail G.S.M., following lab experiments: Abdelrahim B.I., Saber N.E., Abou-Zeid H.M., Ismail G.S.M. Data analysis and interpretation: Saber N.E., Abou-Zeid H.M., Ismail G.S.M., Abdelrahim, B.I., draft manuscript preparation: Ismail G.S.M., Abou-Zeid H.M., critical revision of the article and final approval of the version to be published: Saber N.E., Abou-Zeid H.M., Ismail G.S.M.

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