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Influence of Cyanobacterial Biofertilizer on the Response of *Zea mays* Plant to Cadmium-stress

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CADMIUM (Cd) pollution is a thoughtful problem that alters crop yield and agricultural soils worldwide. Cyanobacteria are emerging candidates as eco-friendly biofertilizers. The present study evaluates the capability of super blue green biofertilizer (cyanobacteria biofertilizer (CB); *Nostoc* sp. and *Anabaena* sp.) to alleviate the Cd toxicity on 21 d-old *Zea mays* plants (applied as 2 and 10mM $CdSO_4$). Application of CB significantly improved maize growth and reduced Cd-accumulation. Cadmium-induced oxidative stress indicated by increment of malondialdehyde (MDA), H_2O_2 contents and electrolyte leakage (EL) was diminished upon application of CB. Cd-stress significantly induced L-phenylalanine ammonia-lyase (PAL) specific activity and phenolics accumulation in the leaves and roots of maize plants. The results revealed that Cd-stress markedly enhanced GSH/GSSG ratio, gltuthione reductase (GR), superoxide dismutase (SOD) and catalase (CAT) specific activities, while it decreased that of ascorbate peroxidase (APx) and guaiacol peroxidase (GPx) and insignificantly increased polyphenol oxidase (PPO). The isozyme pattern of peroxidase was changed upon Cd-exposure. CB application significantly reduced both of the Cd-induced accumulated phenolics content and reduced glutathione (GSH) while it increased total glutathione (TG). CB application significantly induced all tested antioxidant enzymes. The data revealed that CB application could reduce Cd toxicity in maize plants.

Keywords: Antioxidant enzymes, Biofertilizer, Cadmium, Glutathione, Oxidative stress, Phenolic.

Introduction

In the natural environment cadmium (Cd) exists at trace amounts, however it has become a primary heavy metal pollutant due to its increasing concentration in agricultural farm land (Goix et al., 2014). It is considered as an utmost pollutant due to its high toxicity and high solubility in water, besides it is easily absorbed from the soil by plant roots (Goix et al., 2014). Thus Cd is posing a severe hazard to biota, including plants, animals and humans. Unfortunately, it has been reported that crops in arable lands are the main source of Cd intake by humans (Gill & Tuteja, 2011). Numerous studies reported that excessive amount of Cd might interfere with several cellular functions with the toxicity manifestation ranging from decreased growth to plant destruction (Song et al., 2017), through stunting growth and chlorosis, interfering with various physiological processes such as photosynthesis, element assimilation (Lysenko et al., 2015), respiration (Bertoli et al., 2012), and cell division (Potters et al., 2007). Benavides et al. (2005) reported that these Cd-induced changes are reliant upon plant species, organ/tissue, metal concentration and duration of exposure.

Cadmium is not a redox metal, nevertheless, plants exposure to Cd results in the formation of reactive oxygen species (ROS) which prompted oxidative stress and produce several cellular damages namely increased lipid peroxidation, H_2O_2 build-up, modification in the expression of several genes and modulation of antioxidant system (Huybrechts et al., 2019). Nonetheless,

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plants utilize a diverse group of defense mechanisms to control these ROS such as redox reactions involving antioxidant enzymes as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) (Bhardwaj et al., 2009; Erdal & Turk, 2016; Biyani et al., 2019). More peroxidase enzymes like guaiacol peroxidase (GPOX), and polyphenol oxidase (PPO) are likewise known as stress mitigating enzymes as well as being involved in biosynthesis of lignin (Lee et al., 2007). Peroxidase isozymes have been highly expressed under heavy metals including Cd (Zheng et al., 2010; Soleiman et al., 2020).

One more defending reaction of plants to heavy metal toxicity is the expression of some non-enzymatic antioxidant like ascorbate (AsA), glutathione (GSH), carotenoids, tocopherols and phenolics (Gill & Tuteja, 2010). Plants experiencing heavy metal stress have been found to exude high levels of phenolic compounds. These Phenolics might control their modulation depending on the differences between the rate of their biosynthesis *via* phenylalanine ammonia-lyase activity (PAL) and their oxidation by metabolizing enzymes (*via* GPx and PPO) (Mongkhonsin et al., 2016). PAL is considered as a branching point enzyme between primary and secondary metabolism, besides being as one of the main lines for cell adaptation to various biotic and abiotic stresses comprising heavy metals (Rebey et al., 2017).

Glutathione has been reported as a thiol component that can directly reduces the generated ROS and acts as a metal chelator (Jozefczak et al., 2015). Generally, it is believed that managing a high reduced to oxidized glutathione (GSH/ GSSG) and reduced ascorbate to oxidized form (ASA/ DHASA) ratios are essential parameters for removing the inhibitory effect of generated ROS under various stresses (Ashraf et al., 2010).

In the last few years, a great concern has been raised about the problem of soil fertility deterioration and soil pollution with heavy metals due to extensive use of chemical fertilizers (Rashid et al., 2016). This problem can be reduced to a greater extent by using biofertilizers which help in maintaining the quality of the soil. Biofertilizers or plant growth promoting microorganisms (PGPM) are the products that contain cells of different microorganisms (bacteria, algae and fungi) which are agriculturally beneficial (Khanna et al., 2019). They support the soil environment with all types of micro- and macro-nutrients through fixation of nitrogen, phosphate and potassium solubilization or mineralization, excretion of plant growth regulating substances, manufacturing of antibiotics and biodegradation of organic matter in the soil (Khanna et al., 2019). Their application also exerts a defensive action during biotic and abiotic stresses encompassing heavy metals (Gouda et al., 2018). Furthermore, Gururani et al. (2013) suggested that utilization of plant growth promoting rhizobacteria (PGPR) with heavy metals-contaminated media might induce the activity of antioxidant enzymes *via* enhancing gene expression of these enzymes. Nowadays, application of PGPM such as cyanobacteria has been reported to enhance crop yield and soil fertility (Thajuddin & Subramanian, 2005) as well as increasing soil biomass after their death (Rodriguez et al., 2006) .Cyanobacteria are also involved in the enhancement of soil structure and recovery of saline and alkali soils by lowering pH and electrical conductivity, and by improving the hydraulic conductivity (Al-Sherif et al., 2015).

Worldwide, maize is a very important agricultural crop, both for the population and for animal feed as well as an important candidate crop for ethanol production (Cakir, 2004). In Egypt, maize is considered the second most important crop and recently, high consumption of the nitrogen fertilizers has been significantly increased due to intensive farming which causes serious environmental problems (Abdel Monem et al., 2000). Consequently, it is of great importance nowadays to limit the use of chemical fertilizer in agricultural systems in order to maintain soil productivity for a sustainable agriculture mostly in developing countries. In this aspect, the current study was designed to assess the efficacy of the concurrent application of super blue green biofertilizer in reducing Cd toxicity by maize plants. The changes in the behavior of some antioxidant enzyme activities, Cd accumulation as well as changes in peroxidase isozymes were inspected.

Materials and Methods

Plant material, growth conditions and treatments

Maize (*Zea mays* L. cv. Nevertity) grains were obtained from the Agricultural Research Center, Giza, Egypt. Super blue green biofertilizer (cyanobacteria biofertilizer (CB); *Nostoc* sp. and *Anabaena* sp.) was 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 grains were rinsed with distilled water, soaked for 24hrs. at 25˚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 2mM and 10mM $CdSO_4$. The third group was irrigated with one tenth strength modified Hoagland solution supplemented with 100mL L^{-1} Bio (CB). The fourth group was irrigated with one tenth strength modified Hoagland solution supplemented with 100mL L^{-1} CB and 2 and 10mM $CdSO_4$. The pots were laid in an environmentally controlled growth chamber under the condition of 16-h photoperiod, 31°C/28°C ±2°C light/dark temperature and light intensity of about 23µmol $m^{-2}s^{-1}$ (cool white fluorescent tubes). The pots were 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 leaves and roots and quickly saved for estimation of the various growth parameters and chemical analyses. All chemical analyses were performed on roots and leaves.

Experimental methods

Growth parameters and cadmium content

The roots and shoots were separated and used for estimation of fresh (FM) and dry biomass (DM). Shoot height was measured to the nearest cm.

Cadmium content was measured following the method previously described by Chapman & Pratt (1961). Briefly, the leaves and roots tissues were collected, washed with distilled water, and dried at 70˚C for 48hrs. One g of dried roots and leaves was digested in 5mL HCl (2N). An aliquot of the digest was quantitatively analyzed for cadmium using an atomic absorption spectrophotometer (Perkin-Elmer, 2380).

Estimation of H_2O_2, lipid peroxidation, total phenolics and leaf electrolyte leakage

Hydrogen peroxide content was estimated following the method of Velikova & Loreto (2005). The tissue was homogenized in 0.1% (w/v) TCA, 0.5mL of the supernatant was mixed with 0.5mL of 10mM potassium phosphate buffer (pH 7.0) and 1mL of 1M KI, and the absorbance was read at 390nm. Lipid peroxidation was measured as the amount of malondialdehyde (MDA) produced by the thiobarbituric acid (TBA) reaction (Wang et al., 2009). One gram of fresh leaf samples was homogenized in 5mL 0.6% thiobarbituric acid in 10% trichloroacetic acid (TCA). The mixture was heated at 100°C for 15min. in a water bath. After cooling in ice, the mixtures were centrifuged at 5000*g* for 10 min. The absorbance of supernatants was read at 450, 532 and 600nm and MDA content was calculated using the following formula:

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

Total phenolics content was determined using the modified Folin–Ciocalteu spectrophotometric method (Demiray et al., 2009). The electrolyte leakage (EL%) of leaf was measured according to Deshmukh et al. (1991) where leaf samples were washed with deionized water to remove surface-adhered electrolytes, then placed in two sets of test tubes each containing 10mL distilled deionized water. One set of the tubes was incubated in a water bath at 40˚C for 30min. and the other set was incubated in boiling water bath at 100˚C for 15min and their respective electric conductivities L_1 and L_2 were measured by conductivity meter The electrolyte leakage is defined as:

$$EL\ (\%) = (L_1/L_2) \times 100.$$

Flourmetric estimation of cellular glutathione content

Reduced and oxidized glutathione (GSH and GSSG) contents were estimated fluorimetrically (Hissin & Hilf, 1976). The final assay mixture contained 100µL of dilute supernatant, 1.8mL phosphate EDTA buffer (pH 8.0) and 100µL of *o*-phthalaldehyde (OPT) containing 100µg of OPT. GSH content was calculated based on the fluorescence intensity at 425nm after excitation at 343nm. For GSSG assay, 0.5mL of the tissue supernatant was incubated with 200µL of 0.04 M *N*-ethylmaleimide (NEM) to interact with GHS. To the mixture, 4.3mL of 0.1 N NaOH was added, then 100µL of this mixture was taken for GSSG using the procedure outlined for GSH assay, except that 0.1N NaOH was used as the diluent rather than phosphate-EDTA buffer.

Enzymes assay

For estimation of antioxidant enzymes activity, frozen plant tissues were homogenized in ice-cold 0.1M potassium phosphate buffer (pH

6.8) containing 0.1mM EDTA. The homogenate was centrifuged at 15,000*g* for 20min. at 4°C. After centrifugation, the supernatant was used for assay of some antioxidant enzymes. Superoxide dismutase (SOD, EC 1.15.1.1) specific activity was measured by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) according to the method of Gong et al. (2005). The reaction mixture (3 ml) contained 50mM sodium phosphate buffer (pH 7.3), 13mM methionine, 75mM NBT, 0.1mM EDTA, 4mM riboflavin and enzyme extract. One unit of SOD was defined as the amount of enzyme that produced 50% inhibition of NBT reduction under the assay conditions. The SOD specific activity was expressed as Umg^{-1} protein min^{-1}. Catalase (CAT, EC 1.11.1.6) specific activity was determined as described by de Azevedo Neto et al. (2006) where the decomposition of H_2O_2 was followed at 240 nm and the enzyme activity was calculated using the extinction coefficient of 36 M^{-1} cm^{-1} and expressed as µmol $H_2O_2g^{-1}$ mg^{-1} protein min^{-1}. Ascorbate peroxidase (APX, EC 1.11.1.11) specific activity was determined following the method of Nakano & Asada (1981) where the H_2O_2-dependent oxidation of ascorbic acid (AsA) was followed by measuring the decrease in absorbance at 290nm, the enzyme activity was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1} and expressed as µmol H_2O_2 reduced mg^{-1} protein min^{-1}. Guaiacol peroxidase (GPX, EC 1.11.1.7) was assayed according to the method of Urbanek et al. (1991) where guaiacol oxidation to tetraguaiacol was followed for 5min. at 470nm. GPX activity was calculated using the extinction coefficient of 26.6 mM^{-1} cm^{-1} and was expressed as U mg^{-1} protein min^{1}. Polyphenol oxidase (PPO, EC 1.10.3.1) was assayed following the method described by Kumar & Khan (1982), where the formation of the purpurogallin was followed at 495 nm. PPO activity was expressed in U mg^{-1} protein min^{-1}. Glutathione reductase (GR, EC 1.6.4.2) was assayed following the oxidation of NADPH at 340 nm as described by Goldberg & Spooner (1983) and the enzyme activity was calculated using the extinction coefficient of 6.2mM^{-1} cm^{-1} and expressed as µmol $NADPH_2$ oxidized mg^{-1} protein min^{-1}.

L-phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) was assayed spectrophotometrically after the method of Jiang & Joyce (2003) by measuring the increase in OD_{290} nm due to the formation of trans-cinnamate. PAL specific activity was expressed as change in Δ_{290nm} mg^{-1} protein min^{-1}.

Native gel electrophoresis and staining of isoenzyme

PAGE for peroxidase (POD) isoenzyme was performed with a combination of agar- starch-Polyvinyl pyrrolidine (PVP) gel elechophoresis as described by Sabrah & El- Metainy (1985). POD isoenzymes were detected after Torres & Tisserat (1980) method. The gels were rinsed in water and stained in a 100mL of 0.01M sodium acetate – acetic acid buffer (pH 5.0) containing 0.1g benzidine and 0.5% hydrogen peroxide (H_2O_2).

Statistical analysis

Statistical analysis of the results was done using SPSS software package version 20.0 to obtain the mean, the standard error of each mean and for comparison between the different groups involved in this study, ANOVA was used to assess the significant difference among the control and treated groups. LSD was estimated $P \leq 0.05$.

Results

Growth parameters

The results of the present investigation clearly demonstrated that maize plants treated with CB showed better growth under both normal and Cd stress conditions. The maximum reduction in growth biomarkers (biomass accumulation, shoot height as well as water status) was observed at 10mM Cd (Tab. 1). However, CB treatment showed stimulatory effect on the growth at all levels of Cd treatment. At the end of the experimental period, the reduction in FM of leaves and roots of 10mM Cd-stressed plants was about 92% and 94%, respectively, compared to control. The corresponding values for CB treatment were 81% and 78%, respectively. The shoot height in 2mM Cd-stressed maize plants in presence of CB was 1.7-fold of 2mM Cd-stressed ones. The corresponding values in severely Cd-stressed plants were 2.7-fold. It is also noteworthy to observe that application of CB increased the water content from 65 and 33% in 10mM Cd-treated leaves and roots to 82% and 70%, respectively.

Cd accumulation

As shown in Table 1, the accumulation of Cd in the roots and leaves of maize plants was an aspect of Cd dosage. Nonetheless, upon CB supplementation, Cd accumulation was significantly decreased. Moreover, the accumulation of Cd in roots was greater than in leaves revealing the decrease of Cd allocation to the leaves. Seedlings grown in presence of 10mM Cd contained up to 86.5 and 36.9µg g^{-1} DM Cd in

roots and leaves, respectively. The corresponding values at CB-treatment -10mM Cd stress were 44.8 and 10.7µg g^{-1} DM, respectively.

Electrolyte leakage, lipid peroxidation, H_2O_2 and phenolics contents

There was a marked increase of EL in leaves of Cd-stressed maize plants grown in absence or presence of CB, but the attained values were greatly lower than those in absence of CB (Table 2). Increasing Cd concentrations in the nutrient medium up to 10 mM Cd resulted in a significant accumulation of H_2O_2 and MDA contents in the leaves and roots of maize plants, compared to untreated control plants (Table 2). The H_2O_2 and MDA contents in 10mM Cd-stressed leaves was 6.1-and 5.3-times, respectively, as in the untreated plants. The corresponding values in the roots were 6.4- and 6.3-fold respectively. CB application significantly declined H_2O_2 and MDA accumulation in Cd-treated plants in comparison to untreated Cd-stressed ones. After 21 d of experimental period, the decrease of H_2O_2 content in leaves and roots of CB- treated-10mM Cd stressed plants was 25% and 31%, respectively, compared to 10mM Cd-treated plants. Whereas, the decrease in MDA content in leaves and roots of CB-treated-10mM Cd-stressed plants was 26% and 29%, respectively compared to 10mM Cd-treated ones (Table 2). It was also seen that CB application insignificantly changed H_2O_2 and MDA contents in the leaves and roots under control conditions. Increasing Cd concentration resulted in a significant increase of phenolic compounds in the leaves and roots; the accumulation in the roots was greater than that in the leaves (Table 2). The application of CB mitigated the increase of phenolics in roots and leaves of CB- treated-10 mM Cd stressed plants by 28% and 22%, respectively in comparison to those without CB.

Glutathione content

Results in Table 3 indicated that both GSH and TG contents were markedly increased in the leaves and roots of maize plants under Cd stress in presence or absence of CB, whereas GSSG content markedly decreased compared to control. At the end of experimental period, the increase of TG and GSH contents in leaves of 10 mM Cd-stressed plants was 13% and 46%, respectively, compared to control. The corresponding values after application of CB treatment were 13% and 24%, respectively, compared to the 10 mM Cd-stressed ones. It is clearly demonstrated that the decline of GSH in roots and leaves of maize plants subjected to Cd stress in absence or presence of CB treatment was associated with a marked increase of glutathione-redox potential (GSH/GSSG). At the end of experimental period, the GSH/GSSG fractions in roots and leaves of 10mM Cd-stressed plants increased from 1.22 and 1.37 to 1.67 and 1.74 in presence of CB-treatment, respectively.

Enzymes activities

The specific activity of PAL was significantly increased by increasing Cd level in the leaves and roots of maize plants, particularly the roots (Table 2). Application of CB treatment markedly increased PAL specific activity. The PAL specific activity of 10 mM Cd-stressed roots and leaves in presence of CB was 1.5- and 3- fold of 10mM Cd-stressed plants.

TABLE 1. Changes in fresh and dry biomasses (FM and DM), shoot height, water content % and Cd accumulation in 21-day-old maize plants in response to Cd-stress and biofertilizer (Bio) application

Treatment	Cd conc. (mM)	FM (mg $plant^{-1}$)		DM (mg $plant^{-1}$)		Shoot Height (cm)	Water content (%)		Cd accumulation (µg g^{-1} DM)	
		Roots	Leaves	Roots	Leaves		Roots	Leaves	Roots	Leaves
-Bio	0	4.9±0.44^{b}	18.4±1.11^{b}	0.8±0.04^{b}	2.1±0.31^{bc}	^{c}9.1±1.01	89±5.69	90±4.98	n.d.	n.d.
	2	3.1±0.25^{c}	11.9±1.43^{c}	0.5±0.02^{b}	1.9±0.13^{cd}	5.7±0.41^{de}	81±6.31	84±7.11	22.7±2.28^{c}	10.1±1.34^{a}
	10	0.3±0.08^{e}	1.5±0.30^{e}	0.2±0.02^{c}	0.3±0.07^{e}	1.4±0.22^{f}	33±2.99	65±7.42	86.5±10.72^{a}	36.9±1.69^{a}
+ Bio	0	9.3±0.77^{a}	27.1±2.47^{a}	0.8±0.09^{a}	2.7±0.22^{a}	12.1±1.21^{ab}	91±7.00	92±7.67	n.d.	n.d.
	2	7.2±0.65^{b}	21.9±2.44^{b}	0.7±0.08^{a}	1.9±0.15^{b}	10.8±0.98^{bc}	90±9.00	91±10.11	13.8±1.38^{d}	5.9±0.54^{c}
	10	1.1±0.08^{d}	5.2±0.52^{d}	0.3±0.03^{c}	0.9±0.08^{d}	4.6±0.42^{e}	70±7.78	82±6.83	44.8±4.98^{b}	10.7±0.97^{b}

- Values are means of 3 independent replicates ±SE.

- Means followed by different letters are significantly different at P≤ 0.05 according to the least significant difference (LSD).

TABLE 2. Changes in electrolyte leakage EL (%), hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and total phenolics content as well as PAL activity in roots and leaves of 21-day-old maize plants in response to Cd-stress and biofertilizer (Bio) application

Treatment	Cd conc. (mM)	EL (%)	H_2O_2 content (µmol H_2O_2 g^{-1} FM)		MDA content (µmol g^{-1} FM)		Phenolics content µmol eq gallic acid g^{-1}) (DM		PAL Δ_{290nm} mg^{-1} protein min^{-1}	
			Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves
-Bio	0	5.7±0.78	10.1±2.25^{d}	15.0±3.89^{e}	9.0±2.01^{d}	9.0±3.33^{d}	104±11.56^{d}	114±8.77^{e}	0.03±0.002^{e}	0.03±0.001^{d}
	2	16.0±1.62	32.2±4.37^{c}	39.5±2.56^{c}	19.6±2.58^{c}	15.4±4.17^{c}	321±29.18^{d}	202±16.83^{c}	0.07±0.01^{d}	0.04±0.001^{d}
	10	40.9±3.80	64.9±7.32^{a}	91.0±6.14^{a}	56.7±6.66^{a}	47.9±4.98^{a}	1039±94.45^{a}	901±69.31^{a}	0.16±0.01^{b}	0.11±0.01^{b}
+ Bio	0	7.9±0.66	11.9±1.09^{d}	16.3±1.48^{e}	8.8±0.74^{d}	10.2±0.93^{d}	95±8.64^{d}	103±10.30^{e}	0.03±0.001^{e}	0.04±0.001^{d}
	2	12.9±1.43	13.1±1.19^{d}	24.1±2.99^{d}	12.1±1.34^{cd}	13.5±1.12^{c}	352±39.11^{c}	191±17.36^{c}	0.08±0.01^{c}	0.17±0.01^{c}
	10	33.5±3.35	47.7±4.34^{b}	72.2±6.56^{b}	44.5±4.05^{b}	36.3±3.03^{b}	747±83.00^{b}	705±54.23^{b}	0.24±0.02^{a}	0.34±0.03^{a}

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

TABLE 3. Changes in the content of oxidized glutathione (GSSG), reduced glutathione (GSH) and total glutathione (TG) in roots and leaves of 21-day-old maize plants in response to Cd-stress and biofertilizer (Bio) application

Treatment	Cd conc. (mM)	Glutathione content (µg 100 g^{-1} DM)							
		Roots				Leaves			
		GSSG	GSH	TG	GSH / GSSG	GSSG	GSH	TG	GSH / GSSG
-Bio	0	78.62±6.55^{a}	55.38±4.62^{d}	134.00±11.17^{c}	0.70±0.064^{e}	101.19±8.4^{bc}	81.59±6.80^{e}	182.78±15.23^{e}	0.81±0.090^{d}
	2	70.14±7.01^{b}	67.38±6.74^{c}	137.52±13.75^{c}	0.96±0.087^{d}	98.65±9.87^{c}	88.71±8.87^{e}	187.36±18.74^{e}	0.89±0.081^{cd}
	10	64.70±5.88^{c}	78.64±7.15^{b}	143.34±13.03^{b}	1.22±0.128^{b}	87.29±7.94^{d}	119.45±10.8^{b}	206.74±18.79^{bc}	1.37±0.144^{b}
+ Bio	0	71.30±5.94^{b}	66.65±5.55^{c}	137.95±10.61^{c}	0.93±0.078^{d}	110.43±9.2^{a}	91.13±7.59^{d}	201.56±15.50^{c}	0.83±0.069^{d}
	2	69.59±6.96^{b}	72.25±7.23^{bc}	141.84±14.18^{bc}	1.04±0.095^{c}	108.38±10.^{a}	106.55±10.6^{c}	214.93±21.49^{b}	0.98±0.098^{c}
	10	62.85±5.71^{c}	111.46±10.13^{a}	174.31±15.85^{a}	1.67±0.176^{a}	85.45±7.77^{d}	148.40±13.4^{a}	233.85±21.26^{a}	1.74±0.183^{a}

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

As far as antioxidant enzymes activity is concerned, the increase of Cd-stress in presence or absence of CB-treatment showed various responses. Cd-stress significantly increased SOD, CAT and GR specific activity in roots and leaves of maize plants in comparison to their controls (Fig. 1). After 21 d experimental period, SOD, CAT and GR specific activities in leaves of 10 mM Cd-exposed maize plants was 4.8-, 3.6 and 3.1-fold of untreated controls, respectively. CB-treatment resulted in an induction of SOD and GR specific activity in the Cd-treated roots and leaves, in comparison to those non-treated ones. The increase of SOD and GR specific activity in CB -treated 10mM Cd-stressed leaves was 27% and 113%, respectively compared to 10mM Cd-treated plants. The corresponding values for roots were 21% and 177%, respectively. It is noteworthy that CAT specific activity in CB -treated maize plants was significantly increased in presence of Cd compared to control, but the attained values were lower than those plants treated only with Cd.

APx specific activity was significantly inhibited with increasing Cd concentrations either in presence or absence of CB but the attained values were mostly higher than those in CB-non-treated plants. The APx specific activity in roots and leaves of 10mM Cd-stressed plants in presence of CB- was 1.3- and 1.6- fold, respectively, of the Cd-stressed non-inoculated plants.

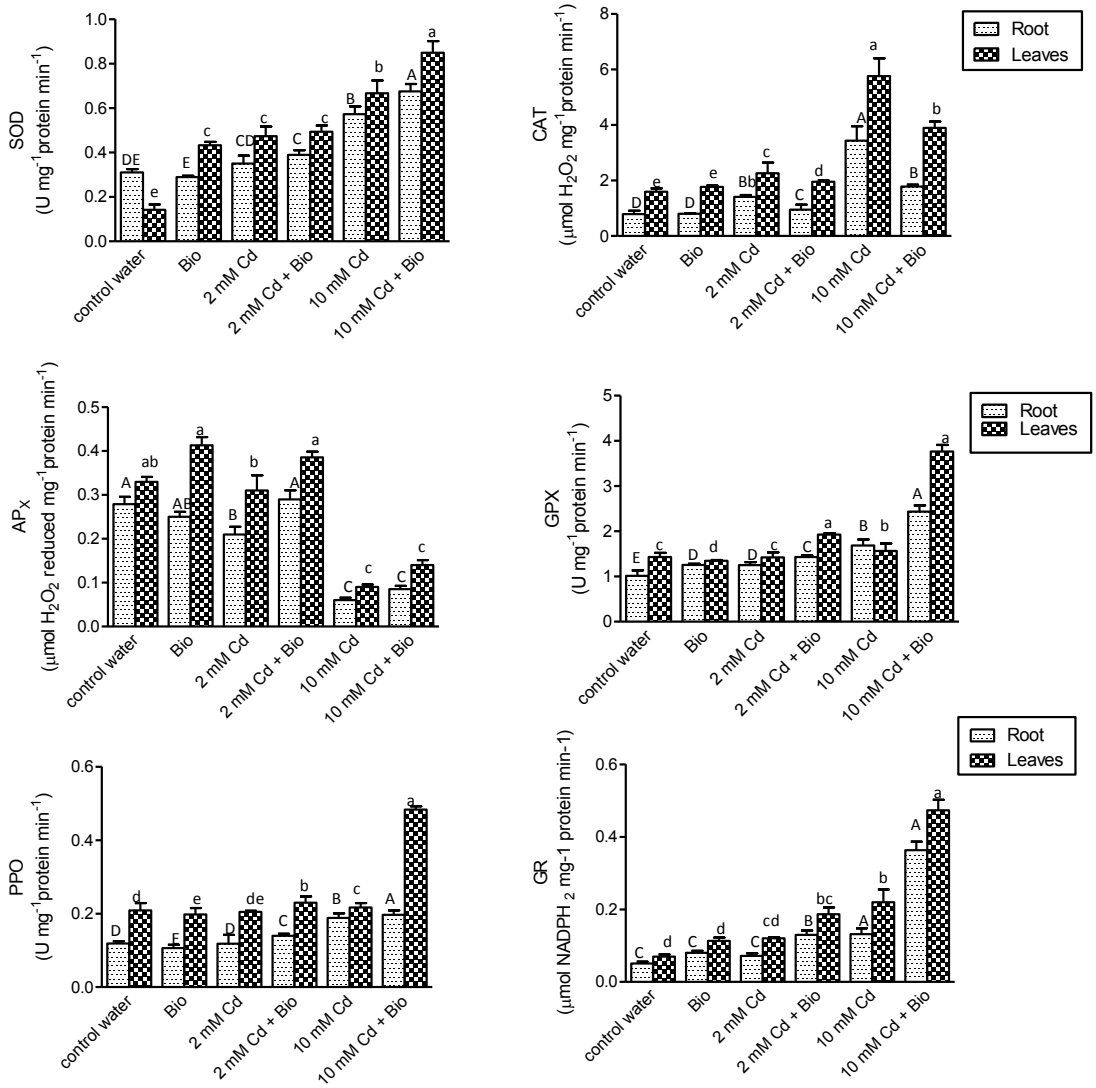

Fig. 1. Changes in the specific activities of superoxide dismutase (SOD),catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), guaiacol peroxidase (GPx) and polyphenol oxidase (PPO) in roots and leaves of of 21-day-old maize plants in response to Cd-stress and biofertilizer (Bio) application [Values are the means of 3 independent replicates ± SE; Means followed by different letters are significantly different at P ≤ 0.05 according to the least significant difference (LSD)].

As shown in Fig. 1, Cd stress insignificantly affected both GPx and PPO specific activities in leaves, whereas it significantly induced GPX and PPO in the roots of maize plants compared to the unstressed plants. CB treatment significantly enhanced their activities in the stressed plants, compared to the untreated plants. In presence of CB-treatment, the GPX and PPO specific activities in leaves of 10 mM Cd-treated plants were 2.4-and 2.2-fold respectively of those Cd-stressed ones. The corresponding values for roots were 1.4-and 1.0-fold respectively

Peroxidase isozyme

There was a marked variation in band numbers and intensities of peroxidase isozyme patterns depending upon the treatments. As shown in Fig. 2, the anodic band (A1) was expressed in all treatments as a heterogeneous band with high activity level. Whereas the second anodic band (A2) was expressed as homozygous bands in all treatment except for Cd one; their activities were varying ranging from low in control treatment to moderate in CB-treatment and CB+Cd-treatment. On the other hand two heterogeneous bands were detected in 10mM Cd-treated leaves.

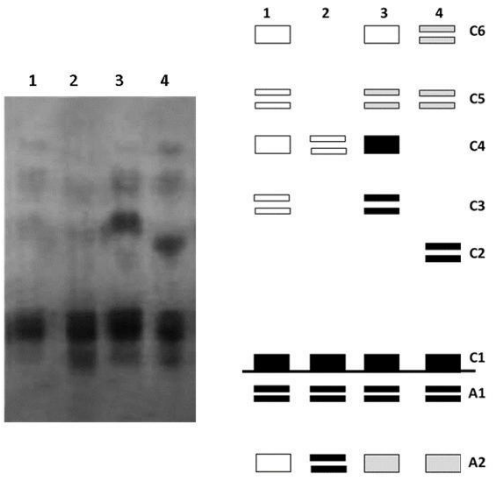

Fig. 2. Effect of Cd-stress and biofertilizers (Bio) application on peroxidase isozyme patterns in leaves of 21-day-old maize plants; (1): Control, (2):10mM Cd, (3): Bio, (4): Bio + 10mM Cd. [A: Anodic, C: Cathodic].

The Zymogram demonstrated five cathodic (C) peroxidase isozyme bands varying in their activity in control. The first cathodic band (C1) was expressed as homologous locus with high activity level in all treatments. In control treatment, 5 cathodic bands were expressed with low activity; the C4 and C6, are homogeneous while C3 and C5 are heterogeneous. In 10mM Cd- stressed maize leaves C3, C5 and C6 cathodic bands disappeared while C4 band was expressed as heterogeneous locus with low activity level. Treatment with CB resulted in five peroxidases cathodic isozymes with high or moderate activity. Treatment of maize plants with CB in presence of 10 mM Cd resulted in an expression of heterogeneous C2 with high activity and C5 and C6 with moderate activity and that was accompanied with a disappearance of C3 and C4 in comparison to control.

Discussion

Exposure of maize plants to different Cd concentrations brought about a significant decline in FM, DM as well as water content along with an increment in the EL% and Cd accumulation. Similar observations were reported for various plants growing under Cd-stress such as *Triticum aestivum* (Liu et al., 2007) and *Pisum sativum* (Głowacka et al., 2019). It has been described that imposing heavy metals stress induces the formation of ROS and that enhances the oxidative stress on different cell components such as proteins, lipids, chlorophylls and DNA resulting in disturbance of cellular activities and inhibition of growth (Singh et al., 2018). In this connection, the present results showed that exposure of maize plants to different Cd concentrations caused a significant increase of H_2O_2 and MDA accumulation in the leaves and roots. Furthermore, these findings were clearly associated with a decrease of water content and increase of EL% revealing the serious role of generated H_2O_2 in the peroxidation of polyunsaturated fatty acids of plasma membranes, and hence lost their integrities. In accordance with these views, several studies have concluded that Cd stress resulted in an increase of H_2O_2 content and lipoxgenation of plasma membranes in several plant species including wheat (Wang et al., 2017) and tomato (Singh et al., 2018). It is well known that H_2O_2 is generated in various cytoplasmic organelles including chloroplasts, mitochondria and peroxisomes during photosynthesis and respiration. Thus the drastic decline in maize growth under Cd stress might be in part associated with enhancing ROS generation and the destructive effect of generated H_2O_2 on plasma membranes and several cellular components leading to suppression of growth.

Application of CB significantly improved the growth of Cd-stressed maize plants compared to untreated plants as evident by the increase in all measured growth indices. These findings were in accordance with numerous other reports for different plant species such as maize (Thajuddin & Subramanian, 2005) and rice (Prasanna et al., 2012). The enhancement of maize growth may be related to the ability of CB to supply the plants by several nutrients, in addition to lowering soil pH that may promote soil nutrient availability (Zaki et al., 2019). Khalil & El-Noemani (2015) suggested that CB improves soil chemical properties and strengthen biological and physical characters which represent supportive factors for root development. Furthermore, it is well documented that CB have enormous potential as a source of various bioactive compounds, phytohormones, vitamins and amino acids in addition to their ability of N_2 fixation and increasing soil biomass after their death and decay (Saadatnia & Riahi, 2005). The results in this study revealed that CB significantly decreased Cd uptake and its accumulation in the leaves and roots of maize plants compared to those of untreated ones which was associated with a decrease of EL% as well as H_2O_2 and MDA contents. Therefore, the prevailing investigation explores also the role of CB for immobilization of Cd in soil to maize roots and enhancement of the antioxidative systems.

The decrease of Cd accumulation in CB-treated maize plants might be attributed to precipitation of Cd as insoluble Cd-phosphate in soil (Gonzalez-Chavez et al., 2004), elimination of Cd from soil *via* adsorption on microbial wall components or/ and absorption of Cd by microbial cells (Rajkumar et al., 2010). Furthermore, under Cd- stress, the Cd accumulation in roots was greater than that in leaves; this could be explained by inducing the Cd-binding capacity in cell walls and xylem elements of roots as well as phosphate of plasma membranes (Lux et al., 2011). Hence, the decrease of uptake and accumulation of Cd in roots might be considered as one feature of defense mechanisms to protect the phytostability of CB-treated maize plants.

Gururani et al. (2013) reported that application of plant growth promoting microorganisms (PGPMs) might result in enhancing gene expression of antioxidant enzymes under heavy metals stress. Whereas, Islam et al. (2014) reported that the increase of non-enzymatic contents and enzymatic antioxidants activity induced by PGPMs could be introduced in ameliorating the oxidative damage in plants subjected to heavy metals stress. In agreement with these views, application of CB, as noted later, markedly induced non-enzymatic and enzymatic antioxidants in maize plants under Cd stress, revealing the role of tested CB in alleviating the detrimental consequences of generated ROS on the biological activities.

Zouari et al. (2016) suggested that plants can incorporate phenolic compounds as a defensive mechanism against metal stress to bypass their destructive effects at a cellular level, such as membrane damage, which may disturb the electron transport due to an enhancement of oxidative stress or eventually cell death. Furthermore, the synthesis of phenolics is depending upon the difference between increasing PAL activity and phenolic metabolizing enzymes (eg. GPx and PPO). In the present study, application of various Cd concentrations with the growth media resulted in a significant induction of PAL specific activity in leaves and roots of maize plants. In addition, there was an increase of total phenolics content in leaves and roots of Cd-stress maize plants and that accompanied with an insignificant activity of GPx and PPO compared to untreated control. These observations could be explained by enhancing the phenolics biosynthesis, *via* increasing PAL activity, and lowering their oxidation as H-donors for GPx and PPO. Moreover, the accumulation of phenolics content might be considered as toxic agent for plant cell and /or induce IAA-oxidase causing the decline in the growth hormone content (Taffouo et al., 2009) and eventually reduce mize growth. Increased PAL activity was reported in several plants treated with Cd such as soybean and lupine (Pawlak-Sprada et al., 2011). Głowacka et al. (2019) reported that Cd treatment induced the PAL activity and lowered GPx activity in the root tissues of pea plants.

On the other hand, CB treatments resulted in a significant suppression of total phenolics content, increased specific activity of PAL, GPx and PPO and decline of H_2O_2 content indicating the reduction of generated ROS among GPx and PPO using phenolics as H-donors. These findings might be explained by a marked increase of external IAA uptake, secreted by CB, and decline the IAA-oxidase activity due to consumption of phenolics as H-donors for GPx and PPO and/ or participation in lignin biosynthesis (Ali et al., 2006).Therefore, one role of CB in the induction of a defense mechanisms-under this study-might be related to enhancement of the lignin biosynthesis and improvement of the anatomical structure of Cd-stressed maize roots (data not shown), hence alleviate the negative effect of Cd on maize growth.

Gill & Tuteja (2010) proposed that increasing GR activity and glutathione pool together with AsA and APx activity play a role in scavenging the generated H_2O_2 *via* AsA-GSH cycle under stress conditions. In the current investigation, however there was a significant increment of GR activity and GSH/GSSG ratio in roots and leaves of Cd-stressed maize plants, while the APx activity was significantly decreased. Parallel to these observations, there was a significant accumulation of H_2O_2 revealing the insufficient elimination of generated H_2O_2 *via* AsA-GSH cycle. Noctor et al. (2002) stated that GSH is consumed as a substrate for induction AsA-GSH cycle and/ or as reductants for glutathione peroxidase and glutathione-S-transferase. Moreover, Jozefczak et al. (2015) concluded that GSH is considered as metal chelator due to the great affinity of metal to its thiol group and as a precursor of phytochelatins biosynthesis. Therefore, the increase of GSH content in maize plants imposed to Cd stress might be introduced as a defense mechanism, in scavenging generated H_2O_2 directly as H-donor for glutathione peroxidase and glutathione-S-transferase and as chelating agents for Cd and/or indirectly as enhancing phytochelatins synthesis.

However, the suppressed growth of maize plants due to Cd exposure pointed out to the inability of these defense mechanisms to shift off the destructive effect of Cd. On the other hand, application of CB showed a significant increase of TG and GSH contents, an increase of GR and APx activities and decrease of H_2O_2 content. These results might indicate that CB could induce GR activity in expense $NADPH_2$ for reduction of GSSG, instead of plasma membrane-associated NADPH oxidase which use O_2 as H-acceptor, and maintain the ascorbate in the reduced form which enhances AsA-GSH cycle and APx activity for eliminating the generated H_2O_2.

To detoxify excess ROS and avert cellular damage, the activities of various scavenging enzymes must be modulated (Singh et al., 2009). Cd-stress significantly increased the specific activities of SOD and CAT enzymes in the leaves and roots of maize plants, while that of APX decreased and that of GPx were insignificantly increased compared to untreated control. These observations were accompanied with a significant increase of H_2O_2 accumulation revealing that both APx and GPx have low ability to eliminate the generated H_2O_2. In addition, the decline of APx and GPx activities might be attributed to the inhibitory effect on peroxidase isozyme forms, as indicated by the marked decrease of number and activity of peroxidase isoforms in leaves of Cd-stressed maize plants compared to control. Induction or inhibition of POD isoenzymes under Cd stress in cell culture of barley (Huttova et al., 2006) and licorice (Zheng et al., 2010) has been reported earlier. The increased activities of CAT and SOD under Cd stress are in accordance with those recorded for several plant species (Mobin & Khan, 2007; El-Amier et al., 2019). Conversely, Bhardwaj et al. (2009) showed a significant decline of CAT activity in Cd-stressed french bean plants. Głowacka et al. (2019) reported that Cd caused a reduction in CAT, GPx and APx activities in the root tissues of pea plants.

It is interesting to demonstrate that application of CB enhanced the specific activities of SOD, APx, GPx, and PPO in leaves and roots of Cd stressed plants in comparison to non-treated plants indicating the ameliorating effect of CB of the inhibitory effect and the oxidative stress caused by Cd-stress on maize plants. These observations are partially in concomitant with that of Prasanna et al. (2012) who reported that inoculating a set of hybrids of maize plant with different cyanobacterial formulations resulted in an increase in PAL, PPO and GPx activities.

Conclusion

The results of the prevailing study propose that application of CB (*Nostoc* sp. and *Anabaena* sp.) enhanced the growth of 21 day-old Cd-stressed maize plants. The effect of CB might be attributed to both reduced Cd metal accumulation in the seedlings as well as suppression of Cd-induced oxidative damage of plasma membranes. CB induced the non-enzymatic and enzymatic antioxidants compared to the uninoculated- Cd stressed plants. CB application significantly reduced accumulated phenolics content and increased TG and GSH. Additionally, CB application significantly induces all tested antioxidant enzymes (CAT, SOD, GR, GPx, PPO and APx).

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Author contribution: 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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تأثير المخصب الحيوي من البكتيريا الخضراء المزرقه علي استجابه نبات الذرة تحت تأثير اجهاد الكادميوم

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يعتبر تلوث الكادميوم من المشاكل التي تؤثر على انتاجيه المحاصيل الزراعية والتربة الزراعية في جميع أنحاء العالم. وتعد البكتيريا الخضراء المزرقه من المخصبات الحيوية الناشئه حديثا كمخصبات صديقة للبيئة. تهدف هذه الدراسة إلى تقييم قدرة المخصب الحيوي من البكتيريا الخضراء المزرقه (*Nostoc* sp. and *Anabaena* sp) علي التخفيف من سمية الكادميوم في نبات الذرة (مطبقة على شكل 2 و 10 ملي مول كبريتات الكادميوم). وقد وجد ان المعالجة باالمخصب الحيوي من البكتيريا الخضراء المزرقه استطاعت أن تحسن نمو الذرة بشكل ملحوظ وساعدت على انخفاض تراكم الكادميوم. كما ادى استخدام هذا المخصب الحيوي إلى تقليل الأضرار التأكسدية الناجمة عن الكادميوم كما هو مبين من انخفاض محتوي فوق اكسيد الهيدروجين، واكسدة الليبيدات، وتسرب الأيونات. تسببت زيادة الكادميوم في زيادة معنويه للفينولات الكلية وكذلك نشاط انزيم الفينيل الانين امونيا ليز في أوراق وجذور نباتات الذرة. وبالتزامن ادى اجهاد الكادميوم إلى حدوث تحسن في نسبة الجلوتاثيون المختزل إلى المؤكسد وكذلك نشاط انزيمات الجلوتاثيون ريدكتيز، سوبراوكسيد ديسميوتيز، انزيم الكاتليز بينما انخفض نشاط انزيم أسكوربات البيروكسيديز، في حين ان كل من انزيم الجواياكول بيروكسيديز والبوليفينول اوكسيديز لم يظهرا اى زيادة معنوية. كذلك كان هناك تغييرفي نمط المشابهات من البيروكسيديز عند التعرض لإجهاد الكادميوم. ولقد وجد أن استخدام المخصب الحيوي من البكتيريا الخضراء المزرقه استطاع أن يقلل من تراكم الفينولات الكلية، وادى إلى زياده الجلوتاثيون الكلي والمختزل. وعلاوة على ذلك فقد تم تسجيل زياده معنويه في نشاط كل انزيمات مضادات الأكسدة المختبرة عند استخدام المخصب الحيوي. خلصت الدراسة إلى أن استخدام المخصب الحيوي من البكتيريا الخضراء المزرقه يمكن أن يقلل من سمية الكادميوم في نبات الذرة.