Pak. J. Bot., 49(6): 2301-2308, 2017.

HEAVY METALS INDUCED LIPID PEROXIDATION IN SPINACH MEDIATED WITH MICROBES

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Abstract

Rapid growth of industrial sector and lack of proper disposal of industrial wastes heavily loaded our soil reservoirs with toxic metals which is a serious threat to all form of life. Among other mechanisms, lipid per-oxidation is a major threat to biological matrix. The aim of this research work was to evaluate the lipid peroxidation induced by heavy metals in spinach that is mediated by microbes (*Bacillus* spp. and *Coryne bacterium* spp.) isolated from contaminated soils irrigated with industrial effluents of Gadoon Industrial Estate, Swabi (GIES) and Hyatabad Industrial Estate, Peshawar (HIEP). The severity of the lipid peroxidation induced by heavy metals was determined by malondialdehyde (MDA) contents, glycine betaine (GB), proline (Pro), hydrogen per oxide (H_2O_2) contents, photosynthetic pigments (Chlorophyll "a", Chlorophyll "b" and "Carotenoids), total soluble sugar (TSS), total soluble proteins (TP), and cell viability (EC) estimation. Heavy metals contaminated soil significantly affected the photosynthetic system of the plant by lowering the content of Chlorophyll "a", Chlorophyll "b", carotenoids, total soluble sugar and total soluble proteins, while electrolyte contents, glycine betaine, proline, hydrogen peroxide and malondialdehyde contents in terms of lipid peroxidation were increased. Whereas the seeds inoculated with microbes showed significant increase in photosynthetic pigments, total soluble sugar and proteins contents with low cell leakage, glycine betaine, proline, hydrogen peroxide and malondialdehyde contents showing decrease in oxidative stress produced by heavy metals. Present results revealed that microbes inoculated plants showed low degree of lipid peroxidation which also confirmed the key role of microbes in bioremediation. Interestingly, *Coryne bacterium* spp., shows improved resistance to heavy metals contamination than *Bacillus* spp.

Key words: Heavy metals, Lipid peroxidation, Soil micro biota, MDA, Industrial effluents, Cell membrane stability.

Introduction

During growth, plants are exposed to the various stress factors in nature (Jahangir *et al.*, 2009). Among others, heavy metals are naturally present in soil, where rapid industrialization and agriculture activities boosts their concentration to toxic level (Bhatti *et al*., 2017), where it poses foremost threats to human health and ecosystem because of its high toxic nature, less solubility, mutagenic, carcinogenic, and non biodegradable. Furthermore, it plays key role in DNA damage, enzymes deformation and destruction of different membrane embedded structures of cells by producing toxic complexes which interfere with routine cellular function (Diels, 2002; Rajbanshi, 2008). The most drastic and commonly documented consequences of such heavy metal contamination are the creation of reactive oxygen species (ROS) that is formed as a byproduct of cellular respiration.

ROS level is increased during heavy metal stress which affects cellular comportments by damaging lipids, disintegration of lipo-membrane and inhibition of various enzyme (Khan *et al*., 2013a; Wu *et al*., 2012) that leads to the disruption of the natural balance between ROS production and cellular detoxifying system. Reactive oxygen species or oxidants are the peroxides, super oxides or singlet oxygen, which attack on carbon-carbon double bonds producing different cytotoxic compounds. Heavy metals cannot be degraded to harmless products but can only be transformed from one state to another and become less toxic (Lasat, 2002; Garbisu & Alkorta1997; Garbisu *et al*., 2003).

Lipids peroxidation is well known in plants, where these are exposed to different environmental abiotic stresses especially heavy metals stresses that disrupts important cellular organelles (Jahangir *et al.*, 2008) and resulting in low biomass, low germination rate, stunted growth and reduced crop yield (Alloway, 1990). Soil micro biota on other hand grow rapidly and utilized different soil substances for their nutritional purposes and provide plants fixed nitrogen, inhibit the effect of various environmental biotic and a biotic stresses by acquisition of nutrient resources (Glick, 2012). Microbes adopt different ways to cope with heavy metals in soil as by immobilization of heavy metals, by direct uptake of heavy metals or by using energy for this purpose which is available in the form of ATPs (Tebo *et al*., 1997; Nies & Silver, 1995). Due to positive role of microbes in the soil, heavy metals are either biotransformed or immobilized and are not available for plants and so level of lipid peroxidation is reduced in this case. Plant growth promoting bacteria can support their host plant (Jahangir *et al*., 2008) by releasing chelating substances and change the chemistry and mobility of heavy metals through the process of reduction, accumulation, immobilization and acidification to overcome the heavy metal induced stress and so improves plant growth (Glick, 2010; Yousuf *et al*., 2010). Microorganisms resistant to higher metals concentration can be used for cycling of metals, bioremediations and reclamation of polluted sites (Jiang *et al*., 2008). Present study was proposed to investigate lipid peroxidation induced by heavy metals in spinach grown in contaminated soils and the mediated potential of microbes isolated from same soil.

Material and Methods

Soil, seed and bacterial strains collection: Heavy metals contaminated soil was obtained from industrial estate of Gadoon, Swabi and Hyatabad industrial estate, Peshawar, Khyber Pakhtunkhwa. The soil was analyzed for heavy metals contamination according to the approved methods of United States Environmental protection Agency (Anon., 3050b, 1996). Spinach (*Spinacia oleracea* L.) seeds were obtained from National Agricultural Research Center (NARC) Islamabad, Pakistan while Heavy metals resistant bacterial strains were obtained from Plant Stress Molecular Biology Lab, Kohat University of Science and Technology, Kohat isolated from heavy metals contaminated soil.

Seeds Sterilization and microbial inoculation: Seeds were surface sterilized with 3.5% sodium hypochlorite solution and were inoculated with heavy metal resistant strains having cell suspension of 10^7–10^9 CFU/ml for 10 hours (Chanway & Nelson, 1990). Seeds were grown in pots having 1.5 kg control and heavy metal contaminated soil. The soil was air dried and passed through 2mm steel mesh. The treatment included a control soil (soil without heavy metals), heavy metals contaminated soil, and heavy metals contaminated soil with seeds inoculated with microbes. Pots were irrigated with tap water as required and harvested after 60 days. After harvesting, plants (cut 2 cm above the ground) were analyzed for lipid peroxidation by different methods.

Lipid peroxidation (TBARS) estimation: MDA contents were estimated by the method described by Velikova *et al*. (2000). Reaction mixture of 25 mg Trichloroacetic acid and 2.5 mg of Thiobarbituric acid was prepared in double de-ionized water. Similarly second reaction mixture was prepared by adding 1.5 ml of enzyme extract and 2.5 ml of first reaction mixture and incubated at 100ºC for 10 min in hot water bath. The reaction was terminated by placing the reaction mixture at ultra low temperature for 10 min. To achieve proper homogenization, mixture was gently vortexed and then centrifuged at 1300 rpm for 15 min, to obtain clear crude enzyme extract. Absorbance was noted at 532 nm while non specific absorption at 600 nm to estimate the amount of MDA by an extinction coefficient of $155 nM^{-1}\ cm^{-1}$.

Cell viability in term of electrical conductivity: Cell viability was determined as described by Jamil *et al*. (2011). Equal number of small strips of 1 cm were cut and placed in 20 ml double distilled water in closed tubes. The tubes along with plant strips were incubated for 24h at 10ºC and then at 25ºC to measure electrical conductivity (Cv1). After that, samples were autoclaved for 40 min and electrical conductivity (Cv2) was noted by using conductivity meter (BMS EC Meter EC-4001).

Determination of photosynthetic pigments: Photosynthetic pigments were determined by taking equal amounts of dried plant material and MgO (25 mg) along with 5 ml methanol in autoclaved closed caped tubes, to prevent evaporation of methanol. The mixture was completely homogenized by vortex and placed at room temperature for 15 min and then placed on shaker for overnight at room temperature. Samples were then centrifuged at 4000 rpm for 5 mins at 25ºC. Supernatant was removed and absorbance were noted at three different wavelengths 666 nm, 653 nm, and 470 nm on UV- visible spectrophotometer using methanol as standard. Total pigments like carotenoids, chlorophyll "a" and chlorophyll "b" were determined by using the formulae proposed by Lichtentaler & Wellburn (1985).

$$C_a = 15.65\ A_{666} - 7.340\ A_{653}$$
$$C_b = 27.05\ A_{653} - 11.21\ A_{666}$$
$$C_{x+c} = 1000\ A_{470} - 2.860\ C_a - 129.2\ C_b / 245$$

Proline estimation: Proline contents of plant samples were estimated by using protocol of Bates *et al*. (1973) with slight modifications as described by Jamil *et al*. (2012). Plant material (100 mg) was completely homogenized with the help of homogenizer with 5 ml of 3% sulfo-salicylic acid following centrifugation at 4000 rpm for 30 minutes at 25ºC. Equal volume of supernatant (1000 ul) and acid ninhydrin solution (1.25 g ninhydrine in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid) were thoroughly mixed, along with 1 ml glacial acetic acid and was placed in oven for an hour at 100ºC. Reaction was stopped by placing the samples in ultra low temperature for 10 min. After these reaction mixtures were completely homogenized with 2 ml toluene and placed at 25ºC until two layers were formed. Supernatant was separated from the mixture and absorbance was measured by spectrophotometer at 520 nm by using toluene as blank. Total Proline content was determined according to formula:

$$(\mu g\ proline/ml \times 2/115.5) / (0.1/5)$$

Glycine betaine estimation: Methodology of Grieve & Grattan (1983) was used to calculate glycine betaine contents. Plant materials of 1000 µg were homogenized in 10 ml de ionized water, followed by complete vortex for 10 min and then filtration. Crude extract of 1000 µl was mixed with 1000 µl of 2M HCl. After that 500 µl mixture and 200 µl potassium tri-iodide solutions was mixed in glass tube, again vortex and cool down the reaction with occasional shaking for 2 hours. Mixture was supplemented with 2000 µl distilled water and 20 ml pre cooled 1-2 dichloromethane. Two clear layers were formed in the tube placed on ice bath. Optical density of the organic layer was measured at 365 nm. The concentrations of the Glycine betaine were calculated with the help of standard curve.

Hydrogen peroxide contents: Hydrogen per oxide contents were determined by using methodology of Nankano & Asada (1980). Reaction mixture of 4000 µl was prepared with 1000 µl Potassium phosphate buffer (PBS), and 2000 µl Potassium Iodide (KI) and 1000 µl of enzyme extract. Absorbance of the mixture was measured at 390 nm.

Determination of soluble proteins: Fresh spinach leaves were grinded in liquid nitrogen and completely homogenized with the help of electrical homogenizer by adding 10 ml phosphate buffer having pH 7.8 and centrifuged at 14000 rpm at 4ºC for 20 minutes. Supernatant was removed and stored at 4ºC. Soluble

protein was measured using Bradford assay (Bradford, 1976). Reaction mixture of 2.02 ml was prepared having composition of 500 µl Bradford reagent and 2000 µl distilled water and 20 µl extracted protein. The mixture was shaken vigorously and kept at room temperature for 5 minutes. The absorbance was noted at 595 nm having distilled water as a blank.

Determination of soluble sugar: Soluble sugar was quantified by the method of Dey (1990) with slight modification. Fresh spinach leaves (50 mg) were grinded into fine powder in 3 ml pre warmed solution of 90% ethanol. The sample was incubated at 80ºC for 1 hour and the top aqueous layer was transferred to another sterile tube. The debris were grinded again in 3 ml pre-warmed 90% ethanol solution and incubated again at 80ºC for 1 hr and its supernatant was collected, again. Now both the supernatants were mixed and diluted to 15 ml with distilled water. Plant extract of 1 ml was taken and mixed with 1 ml solution of 5% phenol. The mixture was added slowly with 5 ml solution of concentrated H_2SO_4 and then diluted up to 10 ml with distilled water. The mixture was then incubated for 30 minutes and stirred vertically with glass rod .The absorbance was measured at 485 nm by using filtered distilled water as blank.

Statistical analysis: Analysis of variance (ANOVA) was performed by using statistics 9 software; verticals bar represent standard deviation (SD) while alphabets represents significant differences between treatments.

Results

Thiobarbituric acid reactive substances – TBARS: Malondialdehyde (MDA) contents were high in plants grown in contaminated soil of GIES and HIEP, by showing MDA contents as 16.72 and 13.95 µmol in contaminated soil of both industrial estates as compared with control i.e. 12.49 µmol (Fig. 1). On other hand, plants raised from microbes inoculated seeds showed low amount of MDA with both microbes "a" (*Bacillus* spp.) and "b" (*Coryne bacterium* spp.) as 12.19 and 11.52 µmol in GIES while 8.9 and 10.92 µmol in HIEP respectively (Fig. 1).

Glycine betaine: High level of Glycine betaine (GB) i.e. 3.18 and 2.62 mg/g was determined in plants grown in GIES and HIEP soils, while seeds inoculated with microbes showed low level of GB as compared to non inoculated seeds plants (Fig. 2A). Both microbes (a and b) alleviate the drastic effect of heavy metals by lowering the concentration of GB in plants as 1.98 and 1.48 mg/g in plants grown from seeds inoculated with microbe (a) and 1.62 and 0.96 mg/g in plants grown from seeds inoculated with microbe (b) in both GIES and HIEP soils as shown in Fig. 2A.

Proline: Increase level of proline was noted in non inoculated plants of GIES and HIEP as 0.76 and 0.96 µmol/g respectively, while plants raised from seed inoculated with microbe's showed reduced amount of proline indicating positive role of microbes in alleviating heavy metals stress (Fig. 2B). Microbes inoculated plants showed low level of proline accumulation as 0.76 and 0.33 µmol/g in GIES soil with microbe a and b, while similar results was observed in HIEP soil as 0.44 and 0.6 µmol/g as shown in Fig. 2B.

Hydrogen Peroxide contents: High level of Hydrogen per oxide content i.e., 1.02 and 1.05 µmol was found in plants grown in GIES and HIEP soils, while seeds inoculated with microbes showed low level of Hydrogen peroxide as compared to non inoculated seeds plants (Fig. 3). Both microbes (a and b) alleviate the harsh effect of heavy metals by lowering the concentration of Hydrogen per oxide in plants as 0.83 and 0.76 µmol in plants grown from seeds inoculated with microbe a and 0.57 and 0.78 µ mol in plants grown from seeds inoculated with microbe b in both GIES and HIEP soils (Fig. 3).

Total soluble sugar: Total soluble sugar content was decreased in plants grown in heavy metals contaminated soil of GIES and HIEP i.e., 20.48 and 21.56 mg/g as compared to control (25.26 mg/g) as shown in Fig. 4. Microbes inoculated plants showed high level of total soluble sugar as 23.36 and 24.7 mg/g inoculated with microbe (a and b) in GIES soil, while similar results were noted in HIEP soil as 24.6 mg/g and 23.8 mg/g (Fig. 4).

Total soluble proteins: A decrease was noted in TSP contents of the plants grown in heavy metals contaminated soil of GIES and HIEP i.e., 22.4 and 18.53 mg/g as compared to control i.e. 31.36 mg/g (Fig. 4). On other side, plants grown from seed inoculated with microbes lowers the drastic effect of heavy metals by increasing the level of TSP (27.2 and 22.2 mg/g) in GIES and HIEP soil (22.5 and 21.2 mg/g (Fig. 4).

Cell viability: Heavy metals concentration in contaminated soil caused increase in cell membrane injury. Seeds inoculated with microbes showed low level of conductivity as compared to non inoculated seeds (Fig. 5). High electrolyte leakage (86.93 and 80.7 µs/cm) was found in plants grown in GIES and HIEP soils respectively as compared to control (61.43 µs/cm) while microbes inoculated plants showed low level of electrolyte leakage i.e., 55.46 and 65.9 µs/cm in GIES soil inoculated with microbe (a) while electrolyte leakages were 58.1 and 69.9 µs in HIEP soil inoculated with microbe (b) as shown in Fig. 5.

Photosynthetic pigments: Photosynthetic pigments (chlorophyll "a" chlorophyll "b" and carotenes) were significantly affected by heavy metals present in contaminated soil of GIES and HIEP as shown in Table 1. Chlorophyll "a" contents in plants grown in control soil were 11.7 mg/g while these values were lower in plants grown in GIES and HIEP i.e. 8.1 and 6.1 mg/g. Similarly Chlorophyll "b" and Carotenoids values were 4.9 and 7.2 mg/g in control while in plants grown in GIES and HIEP soils, these values were 4.2 and 4.2 mg/g, and 3 and 4 mg/g respectively (Table 1). On other side significant increase was noted in plants grown from microbes inoculated seeds i.e. chlorophyll a, b and Carotenoids (11.8, 5.6 and 6.3mg/g) in control soil, while in contaminated soil of GIES these values were 9.8, 4, and 6mg/g with microbe (a) and 12.4, 5.1 and 6.3mg/g with microbe (b) respectively. Same pattern of results was noted in HIEP soil, where chlorophyll a, b and Carotenoids contents with microbe (a) were 8, 3.5 and 5.2 mg/g and 10.3, 4.6 and 5.3 mg/g with microbe (b) (Table 1).

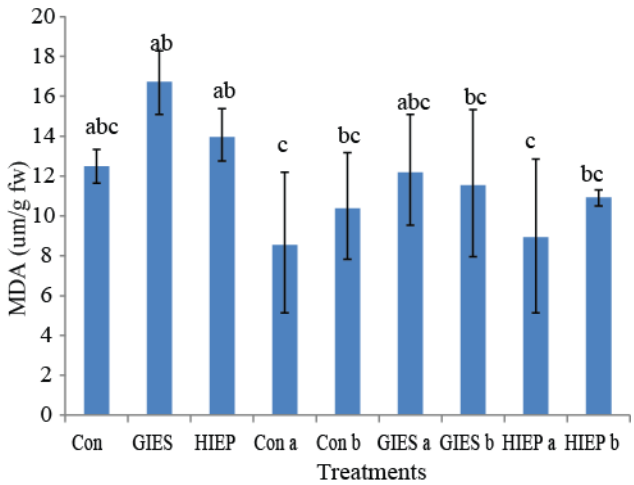

Fig. 1. Effect of control and heavy metals contaminated soil on MDA content of spinach plant mediated with microbes. **Con**: Control; **GIES**: Gadoon Industrial estate; **HIEP**: Hyatabad Industrial estate; **Con a**: Control + *Bacillus* spp.; **Con b**: Control + *Coryne bacterium* spp.; **GIES a**: Gadoon Industrial estate + *Bacillus* spp; **GIES b**: Gadoon Industrial estate + *Coryne bacterium* spp.; **HIEP a**: Hyatabad Industrial estate + *Bacillus* spp.; **HIEP b**: Hyatabad Industrial estate + *Coryne bacterium* spp.

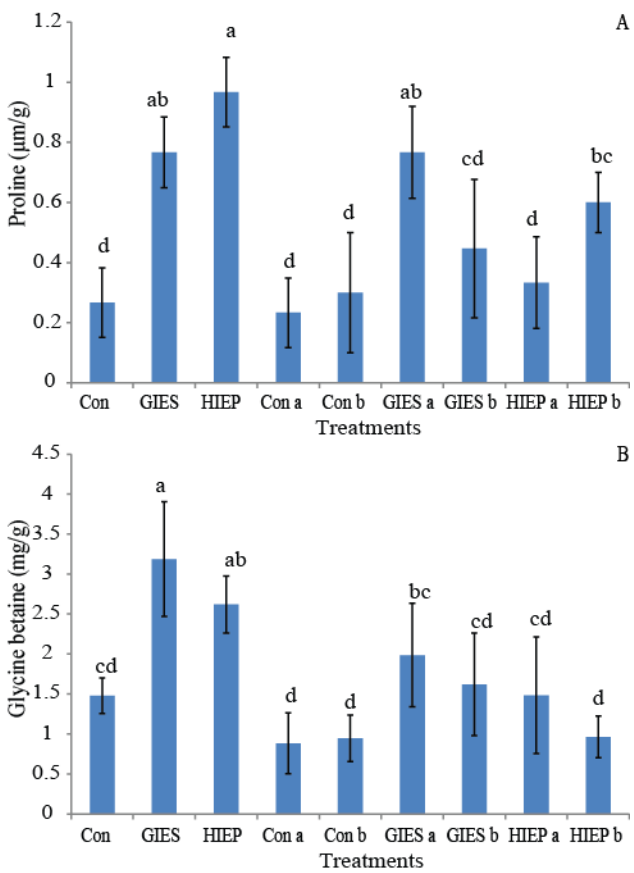

Fig. 2. Effect of control and heavy metals on contaminated soil on Glycine betaine (GB) (A) and Proline contents (Pro) (B) of spinach plant mediated with microbes. **Con**: Control; **GIES**: Gadoon Industrial estate; **HIEP**: Hyatabad Industrial estate; **Con a**: Control + *Bacillus* spp.; **Con b**: Control + *Coryne bacterium* spp.; **GIES a**: Gadoon Industrial estate + *Bacillus* spp.; **GIES b**: Gadoon Industrial estate + *Coryne bacterium* spp.; **HIEP a**: Hyatabad Industrial estate + *Bacillus* spp.; **HIEP b**: Hyatabad Industrial estate + *Coryne bacterium* spp.

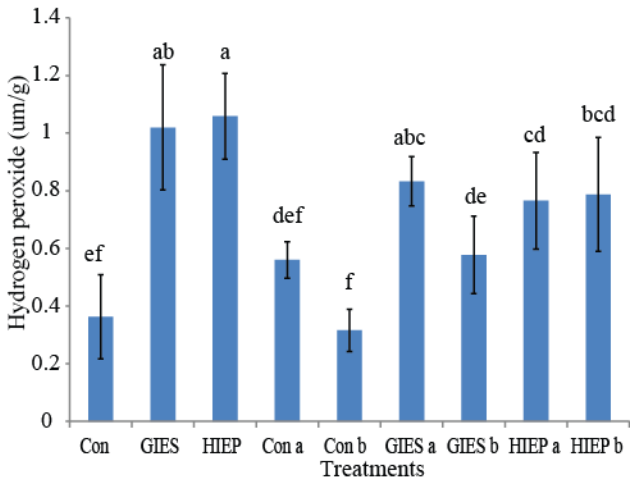

Fig. 3. Effect of control and heavy metals contaminated soil on Hydrogen peroxide (H_2O_2) of spinach plant mediated with microbes. **Con**: Control; **GIES**: Gadoon Industrial estate; **HIEP**: Hyatabad Industrial estate; **Con a**: Control + *Bacillus* spp.; **Con b**: Control + *Coryne bacterium* spp.; **GIES a**: Gadoon Industrial estate + *Bacillus* spp.; **GIES b**: Gadoon Industrial estate + *Coryne bacterium* spp.; **HIEP a**: Hyatabad Industrial estate + *Bacillus* spp.; **HIEP b**: Hyatabad Industrial estate + *Coryne bacterium* spp.

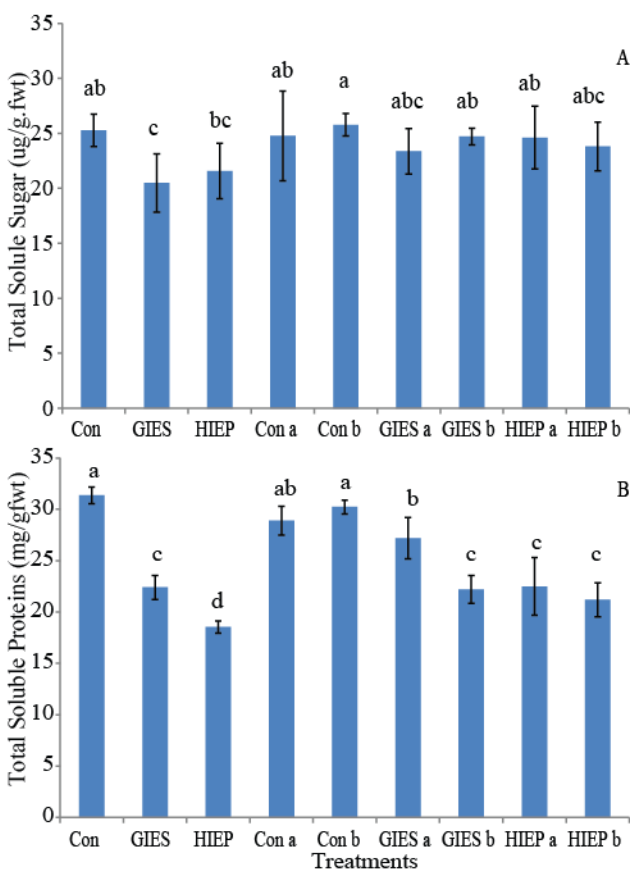

Fig. 4. Effect of control and heavy metals contaminated soil on Total soluble sugar (TSS) and Total Soluble proteins (TSP) contents of spinach plant mediated with microbes. **Con**: Control; **GIES**: Gadoon Industrial estate; **HIEP**: Hyatabad Industrial estate; **Con a**: Control + *Bacillus* spp.; **Con b**: Control + *Coryne bacterium* spp.; **GIES a**: Gadoon Industrial estate + *Bacillus* spp.; **GIES b**: Gadoon Industrial estate + *Coryne bacterium* spp.; **HIEP a**: Hyatabad Industrial estate + *Bacillus* spp.; **HIEP b**: Hyatabad Industrial estate + *Coryne bacterium* spp.

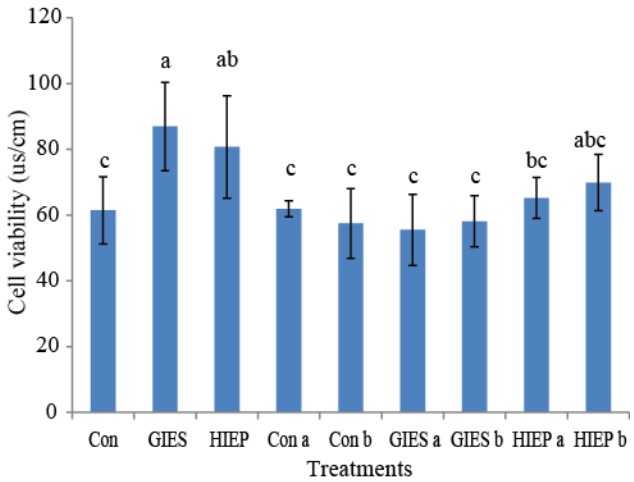

Fig. 5. Effect of control and heavy metals contaminated soil on Electrical conductivity of spinach plant mediated with microbes. **Con**: Control; **GIES**: Gadoon Industrial estate; **HIEP**: Hyatabad Industrial estate; **Con a**: Control + *Bacillus* spp.; **Con b**: Control + *Coryne bacterium* spp.; **GIES a**: Gadoon Industrial estate + *Bacillus* spp.; **GIES b**: Gadoon Industrial estate + *Coryne bacterium* spp.; **HIEP a**: Hyatabad Industrial estate + *Bacillus* spp.; **HIEP b**: Hyatabad Industrial estate + *Coryne bacterium* spp.

Leaf number and size: It was found that plant raised from seed inoculated with microbes grown in contaminated soil of both industrial estates was more in leaf number and size. Plant raised from seeds inoculated with microbe grown in control soil had 5 leaves by comparing with non inoculated control plant (4 leaves). Similarly microbes inoculated plants grown in GIES and HIEP contaminated soil had better growth and leaf number i.e. 6 and 5 leaves respectively, by comparing with non inoculated plants i.e., 5 leaves as shown in Fig. 6 A & B. Similarly plant raised from seeds inoculated with microbe was larger in size as compared to plant non inoculated with microbe (Fig. 6A). Negative correlation was observed between MDA and Chlorophyll "a" (-0.462), chlorophyll "b" (-0.367) and Carotenoids contents (-0.455), while regression values of chlorophyll "a" "b" and Carotenoids with MDA were 9.43-0.29, 7.00-0.19 and 15.98- 0.48 respectively as shown in Fig. 7.

Table 1. Effect of control and heavy metals contaminated soil on Chlorophyll "a", Chlorophyll "b", Total Chlorophyll (a+b) and Carotenoids contents of spinach plant mediated with microbes.

Treatments	Chlorophyll (a) (mg/g)		Chlorophyll (b) (mg/g)		Total Pigments (a+b) (mg/g)		Carotenoids (mg/g)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cont	11.7	0.850 *bc*	4.9	0.608 *bcd*	16.6	4.855 *bc*	7.2	0.550 *b*
GIES	8.1	1.078 *de*	4.2	1.001 *bcde*	12.4	2.734 *de*	4.2	0.950 *ef*
HIEP	6.1	0.5 ***e***	3.0	0.568 *e*	9.1	2.144 *f*	4.0	0.723 *f*
Cont (A)	11.8	0.550 *bc*	5.6	1.201 *b*	17.5	4.384 *bc*	6.3	0.665 *bcd*
Cont (B)	14.8	1.385 *a*	7.5	0.550 *a*	22.3	5.114 *a*	8.2	1.050 *a*
GIES (A)	9.8	1.517 *cd*	4.0	0.556 *cde*	13.8	4.148 *d*	6.0	0.781 *cd*
GIES (B)	12.4	0.173 *b*	5.1	0.981 *bc*	17.5	5.114 *b*	6.3	0.680 *bc*
HIEP (A)	8.0	1.357 *de*	3.5	1.137 *de*	11.6	3.181 *e*	5.2	0.702 *de*
HIEP (B)	10.3	2.193 *bc*	4.6	0.577 *bcd*	14.9	4.030 *c*	5.3	0 *cde*

Con: Control; **GIES**: Gadoon Industrial estate; **HIEP**: Hyatabad Industrial estate; **Con a**: Control + *Bacillus* spp.; **Con b**: Control + *Coryne bacterium* spp.; **GIES a**: Gadoon Industrial estate + *Bacillus* spp.; **GIES b**: Gadoon Industrial estate + *Coryne bacterium* spp.; **HIEP a**: Hyatabad Industrial estate + *Bacillus* spp.; **HIEP b**: Hyatabad Industrial estate + *Coryne bacterium* spp.

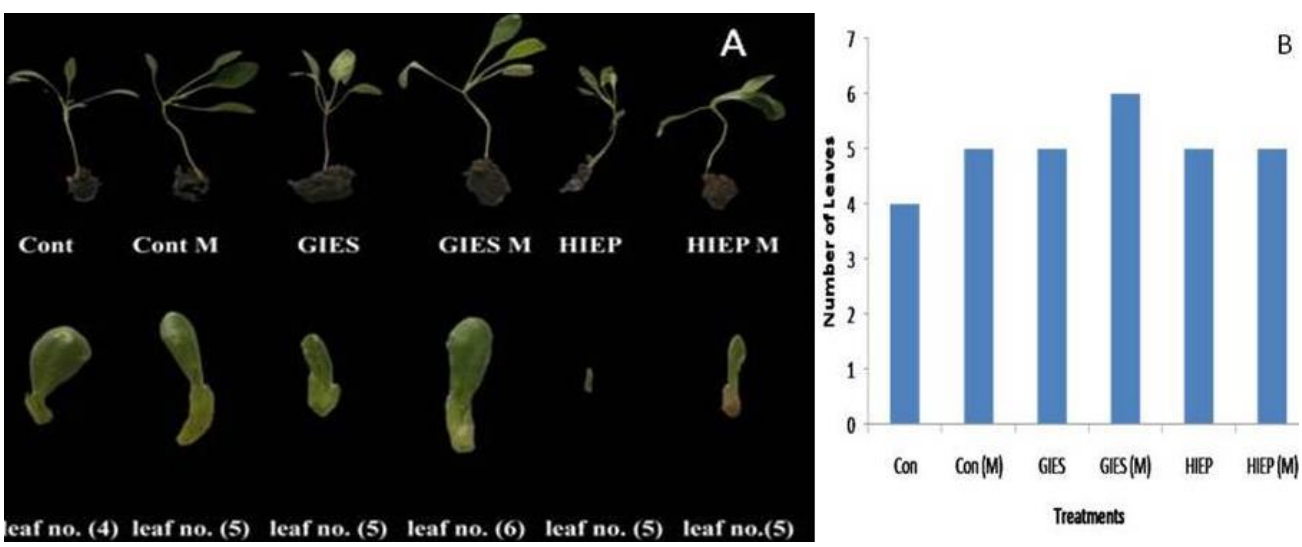

Fig. 6 (A&B). (A) Effect of microbes on leaf sizes of spinach plant associated with microbes grown in control and heavy metals contaminated soil. (B) Effect of microbes inoculation on number of leaves of spinach plant associated with microbes. **Con**: Control; **GIES**: Gadoon Industrial estate; **HIEP**: Hyatabad Industrial estate; **GIES M**: Gadoon Industrial estate + Microbes; **HIEP M**: Hyatabad Industrial estate + Microbes.

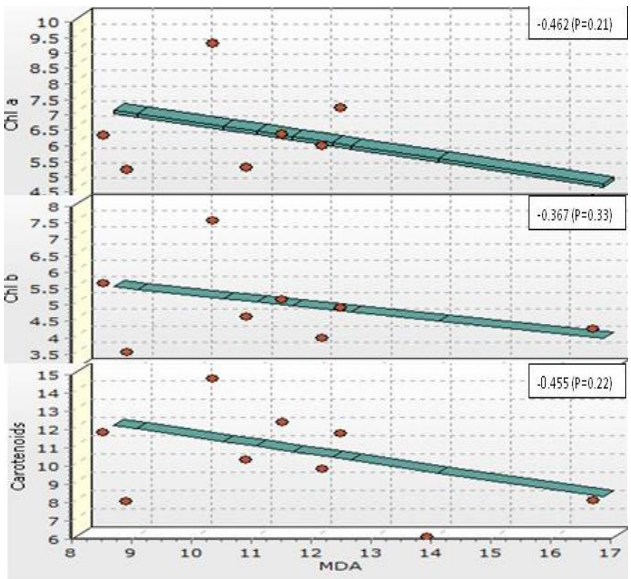

Fig. 7. Coefficient of linear correlation "R" and Regression line between MDA and Photosynthetic pigments i.e. chlorophyll "a" "b" and Carotenoids.

Discussion

Lipids peroxidation is the oxidative degradation of lipids caused by reactive oxygen species (ROS) due to oxidative stresses. In plants, reactive oxygen species (ROS) are routinely generated during respiration process in mitochondria or in endoplasmic reticulum and play important role in cell signaling (Sharma *et al*., 2012), but upon exposure to stress conditions the production of ROS become high enough to damage plants tissues, cell viability, root growth and chloroplast development due to peroxidation of lipids of various membrane embedded structures of the cell. Oxidative stresses are induced by various types of environmental factors like heavy metals, drought, and salt (Jahangir *et al.*, 2009). Climatic changes and anthropogenic activates boost the concentration of heavy metals which affect plant growth and development (Alloway, 1990).

Plant-microbes interaction on other hands shows positive effect on plant physiology and productivity by providing plants fixed nitrogen, reduced the negative effect of various environmental biotic and a biotic stresses (Glick *et al*., 2007), enhanced nodules formation in host plants by entophytic bacteria (Wang *et al*., 2007), minimizing the deleterious effect of plant pathogens and by facilitating plant to obtain acquisition of nutrient resources (Glick, 2012; Yasmin *et al.*, 2013). Malondialdehyde (MDA) is one of the important indicators of lipid peroxidation, in our study we found high values of MDA content in the plants grown in soil of HIEP and GIES, while these values were reduced when plants grown from seeds primed with microbes indicating low level of lipid peroxidation. Microbes adopt different ways to cope with heavy metals in soil and immobilize the heavy metals entry into plants (Tebo *et al*., 1997).

Microbes uptake metals from contaminated medium by chemiosmotic gradient of lipomembrane or by using energy molecules in the form of ATPs, while some microbes selectively uptake metals from soil leaving soil free from metals (Nies & Silver, 1995). *Bacillus* spp are actively involved in the biotransformation process of metals, oxidation of manganese, arsenic, chromium, selenium are often used for energy production and electron acceptor for anaerobic respiration (Santini *et al*., 2000; Quitantana *et al*., 2001). It is clear from the literature that microbes immobilized and biotransformed the metals from very toxic form to less toxic form and reduced their bioavailability to the plant lowering the oxidative damage with low level of lipid peroxidation and MDA content (Barkay & Schaefer, 2001). Similarly (Islam *et al*., 2016) reported that seed inoculated with *P. vermicola* showed resistance by restricting excess to heavy metals uptake and decreased occurs in MDA and H_2O_2 contents under cupper stress.

Osmolytes like proline and glycine betaine have protective role in plants commonly used as indicators of environmental stresses (Girija *et al*., 2002; Pandit *et al*., 2011). According to our results the amount of proline and glycine betaine were increased in the contaminated soil while in microbes inoculated seed raised plant, these amount was reduced indicating that microbes increased plant resistance to heavy metals (Dimkpa *et al*., 2009). According to Barkay & Schaefer, (2001), microbes either bio-transformed or immobilized the metals by reducing their bioavailability to plant system minimizing the stress effect ultimately lowering the concentration of proline and glycine betaine. Cell membrane is the first line of defense against heavy metals in medium, and heavy metals affect cell membrane by increase leakage from the membrane (Hall, 2002).

Heavy metals induce different types of oxidative stresses and disrupt the function of mitochondrial respiration (Shanker *et al*., 2005). Soluble sugar such as glucose, sucrose and fructose are important metabolic sources and play key role in cell signaling during different types of a biotic stresses including heavy metals, by reducing the amount of soluble sugar which ultimately affect the seed germination and seedling growth (Rosa *et al*., 2004). Reduction in total soluble sugar content of plants grown in heavy metals contaminated soil may due to inhibition of photosynthetic activity leads to low production of glucose in grana of chloroplast, so low availability of glucose in cytosol for glycolysis, resulting decrease in sugar content in the cell. Secondly stimulated metabolic path ways for energy production also utilized the remaining glucose resulting lowering the total sugar content of the cell (John *et al*., 2008; Bhardwaj *et al*., 2009; Rosa *et al*., 2009). Similar results were also noted by (Nayek *et al*., 2010) in spinach.

It is observed that heavy metals stress reduced the amount of total soluble sugar and this may be due to destruction of the photosynthetic machinery, and high need of energy due to oxidative stress and toxicity responses. In case of total soluble proteins, the same pattern was noted in plants grown in heavy metals contaminated soil. This might be due to reduced protein synthesis, catalytic activity of heavy metals and protein hydrolysis (John *et al*., 2008; Bhattacharya & Choudhuri, 1997; Ericson & Alfinto, 1984; Costa & Spitz, 1997; Mohan & Hosetti, 1997; Davis *et al*., 1987). On other side microbes alleviated the drastic effect of heavy metals and reduced the toxicity of heavy metals by increasing level of total soluble proteins and sugar in the plant (Fig. 4). The increased level of soluble sugar and protein in the plants in contaminated soil may be due to less excess of the plant to heavy metals as microbes are proven to uptake and immobilized the metals present in the soil by

chemiosmotic gradient of lipomembrane, direct uptake of heavy metals, biotransformation of heavy metals from high toxic form to low toxic form and by utilizing the heavy metals for energy production (Barkay & Schaefer, 2001; Santini *et al*., 2000; Nies & Silver, 1995). Secondly heavy metals in a medium enhance the production of ethylene which reduce or inhibit plant growth (Jackson, 1991; Goldstain, 1986) on the other hand bacteria in soil protect plant from negative effect of heavy metals and shows positive effect on plants growth by reducing the level of ethylene by hydrolyzing with ACC deaminase activity (Glick *et al*., 1998).

A reduction in photosynthetic pigments was noted in plants grown in the contaminated soil of both industrial estates (Table 1). Reduction in the chlorophyll contents may be due to the replacement of Mg and Fe ions of chlorophyll by toxic heavy metals (Pourraut *et al*., 2011), inhibition of the respective enzymes system essential for plant photosynthesis like Rubisco, and carbonic anhydrase (Mobin & Khan, 2007), inhibition of the Calvin cycle (Ali *et al*., 2015), lowering CO_2 fixation, decrease accumulation of protein pigment complexes. Heavy metals toxicity also damage carotenoids which serve as antioxidants against ROS and minimize different photochemical damage (Sengar *et al*., 2008). On other side microbes tolerant to heavy metals minimize the drastic affect of metals by reducing their uptake from the medium and by utilizing these metals for their nutritional purposes (Glick, 2010; Rajkumar *et al*., 2012). Furthermore, photosynthetic pigments were negatively correlated with metal exposures and MDA contents, indicating oxidative stress cause by metals which disrupt membrane bounded chloroplast lowering the photosynthetic ability of the plant (Leblebici *et al*., 2015).

Conclusion

Different markers were used to analyze lipid peroxidation in spinach grown in contaminated soil. Present study confirmed that heavy metals induced lipid peroxidation by increasing proline, glycine betaine contents, hydrogen peroxide and electrical conductivity. Whereas decreasing total soluble sugar and total soluble proteins along with lowering photosynthetic ability of plants. Microbes have the potential to alleviate the drastic affect of heavy metals in a medium by lowering lipid per oxidation of important membrane bounded organelles.

Acknowledgment

Financial support for this study was provided by HEC through research grant no. 4372, Pakistan.

References

Ali, B., R.A. Gill, S. Yang, M.B. Gill, M.A. Farooq, D. Liu, M.K. Daud, S. Ali and W. Zhou. 2015. Regulation of cadmium-induced proteomic and metabolic changes by 5-aminolevulinic acid in leaves of *Brassica napus* L. *PLoS ONE*, 10: 123328.

Alloway, B.J. 1990. Heavy metals in soils. *John Wiley and Sons, Inc, NY*. 370

Anonymous. 1996. Method 3050B: Acid Digestion of Sediments, Sludges, and Soils. *Revision* 2.

Barkay, T and J. Schaefer. 2001. Metal and radionuclide bioremediation: issues, considerations and potentials. *Curr. Opin. Microbiol*., 4: 318-323.

Bates, L.S., R.P. Waldren and J.D. Teare. 1973. Rapid determination of free proline for water stress studies. *Plan Soil*, 39: 205-207.

Bhatti, M.F., M.I. Ashraf, Z. Masoom, S.U. Malik. 2017. Impact of industrial effluents on growth of dominant tree species of Islamabad, Pakistan. *Pak. J. Bot*., 49: 1833-1839.

Bhardwaj, S.K., M. Baharnoori, B. Sharif-Askari, A. Kamath, S. Williams and L.K. Srivastava .2009. Behavioral characterization of dysbindin-1 deficient sandy mice. *Behav. Brain Res*., 197: 435-441.

Bhattacharyya and M.A. Choudhuri. 1997. Effect of Pb and Cd on the biochemical changes in the leaves of terrestrial (Vigna) and aquatic (Hydrilla) plants under solution culture. *Indian J. Plant Physiol*., 32: 99-103.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Ann. Rev. Biochem.*, 72: 248-254.

Chanway, C.P and L.M. Nelson. 1990. Field and Laboratory Studies of *Triticum Aestivum* L. inoculated with Co-Existent Growth promoting Bacillus strains. *Cell Biol*., 22: 789-795.

Costa, G and E. Spitz .1997. Influence of cadmium on soluble carbohydrates, free amino acids, protein content of *In vitro* cultured *Lupinus albus*. *Plant Sci*., 128: 131-140.

Davis, C.E., V.D. Jolley, G.D. Mooso, L.R. Robinson and R.D. Horrocks. 1987. Quality of stockpiled Bigalta limpograss forage at varying fertility levels. *Agron. J*., 79: 229-35.

Dey, P.M. 1990. Oligosaccharides. *Meth plan Bioch*. Academic press, London.

Diels, L., N. Van der Lelie and L. Bastiaens. 2002. New developments in treatment of heavy metal contaminated soils. *Rev. Environ. Sci. and Biotech.*, 1: 75-82.

Dimkpa, C.O., D. Merten, A. Svatos, G, Büchel and E. Kothe. 2009. Siderophores mediate reduced and increased uptake of cadmium by *Streptomyces tendae* F4 and sunflower (*Helianthus annuus*), respectively. *J. Appl. Microbiol*., 107:1687-1696.

Ericson, M.C. and A.E. Alfinito. 1984. Proteins produced during salt stress in tobacco cell cultures. *Plant Physiol*., 74: 506-509.

Garbisu, C. and I. Alkorta. 1997. Bioremediation: principles and future. *J Clean Technol. Environ. Toxicol. Occup*., 6: 1-16.

Garbisu, C. and I. Alkorta. 2003. Basic concepts on heavy metal soil bioremediation. *The Eur. J. Min. Pro. Environ. Prot*., 3: 66.

Girija, C., B.N. Smith and S.M. Swamy. 2002. Interactive effects of sodium chloride and calcium chloride on the accumulation of proline and glycinebetaine in peanut (*Arachis hypogaea* L.). *Environ. Exp. Bot*., 47: 1-10.

Glick, B.R. 2010. Using soil bacteria to facilitate phytoremediation. *Biotech Adv*., 367: 374.

Glick, B.R. 2012. Plant growth-promoting bacteria: mechanisms and applications. *Scientifica*, 963-401.

Glick, B.R., D.M. Penrose and J. Li. 1998. A model for lowering of plant ethylene concentration by plant growth promoting bacteria. *J. Theor. Biol.*, 190: 63-8.

Glick, B.R., Z. Cheng, J. Czarny and J. Duan. 2007. Promotion of plant growth by ACC deaminase-containing soil bacteria. *Eur. J. Plant Pathol.*, 119: 329-39.

Goldstein, H. 1986. Multilevel mixed linear model analysis using iterative generalised least squares. *Biometrika*, 73: 43-56.

Greive, C.M. and S.R. Grattan. 1983. Rapid assay for determination of water-soluble quaternary amino compounds. *Plant Soil*, 70: 303-307.

Hall, J.L. 2002. Cellular mechanisms for heavy metal detoxification and tolerance. *J. Exp. Bot*., 53: 1-11.

Islam, M.S., M.K. Ahmed, M. Raknuzzaman, M. Habibullah-Al-Mamun and M.K. Islam. 2016. Heavy metal pollution in surface water and sediment: A preliminary assessment of an urban river in a developing country. *Ecol. Indic.*, 48: 282-291.

Jackson, M.B. 1991. Ethylene in root growth and development. *Plant Hor. Ethy.*, 159-181.

Jahangir, M., H.K. Kim, Y.H. Choi and R. Verpoorte. 2008. Metabolomic response of *Brassica rapa* submitted to pre-harvest bacterial contamination. *Food Chem*., 107(1): 362-368.

Jahangir, M., I.B. Abdel-Farid, H.K. Kim, Y.H. Choi and R. Verpoorte. 2009. Healthy and unhealthy plants: The effect of stress on the metabolism of Brassicaceae. *Env. Exp. Bot*., 67(1): 23-33.

Jahangir, M., I.B. Abdel-Farid, Y.H. Choi and R. Verpoorte. 2008. Metal ion-inducing metabolite accumulation in *Brassica rapa*. *J. Plant Physiol*., 165(14): 1429-1437.

Jamil, A., S. Riaz, M. Ashraf and M.R. Foolad. 2011. Gene expression profiling of plants under salt stress. *Crit. Rev. Plant Sci*., 30: 435-458.

Jamil, M., S. Bashir, S. Anwar, S. Bibi, A. Bangash, F. Ullah and E.S. Rha. 2012. Effect of salinity on physiological and biochemical characteristics of different varieties of rice. *Pak J. Bot.*, 44: 7-13.

Jiang, C.Y., X.F. Sheng, M. Qian and Q.Y. Wang. 2008. Isolation and characterization of a heavy metal-resistant *Burkholderia* sp. From heavy metal-contaminated paddy field soil and it's potential in promoting plant growth and heavy metal accumulation in metal-polluted soil. *Chemosphere*, 72: 164.

John, R., P. Ahmad, K. Gadgil and S. Sharama. 2008. Effect of cadmium and lead on growth, biochemical parameters and uptake in *Lemna polyrrhiza* L. *Plant Soil and Env.*, 54: 262-270.

Khan, M.U., R.N. Malik and S. Muhammad. 2013. Human health risk from heavy metals via food crops consumption with wastewater irrigation practices in Pakistan. *Chemosophere*, 93: 2230-2238.

Lagos-Quintana, M., R. Rauhut, W. Lendeckel and T. Tuschl. 2001. Identification of novel genes coding for small expressed RNAs. *Science*, 294: 853-858.

Lasat, H.A. 2002. Phytoextraction of toxic metals: A review of biological mechanisms. *J. Environ. Qual*., 31: 120.

Leblebici, Z., A. Ahmet, D. Aksoy and D. Fatih. 2015. Influence of nutrient addition on growth and accumulation of cadmium and copper in (*Lemna gibba*). *Chem Speci & Bioavail*., 22: 157-164.

Lichtenthaler, H. and A. Wellburn. 1985. Determination of total carotenoids and chlorophylls A and B of leaf in different solvents. *Biochem. Soc. Trans.*, 11: 591-592.

Mobin, M. and N.A. Khan. 2007. Photosynthetic activity, pigment composition and antioxidative response of two mustard (*Brassica juncea*) cultivars differing in photosynthetic capacity subjected to cadmium stress. *J. Plant Physiol*., 164: 601-610.

Mohan, B.S. and B.B. hosetti. 1997. Potential phytotoxity of lead and cadmium to Lemna minor grown in sewage stabilization ponds. *Environ. Pollut*., 98: 233-238.

Nankano, Y. and K. Asada. 1980. Hydrogen-peroxide is scavenged by ascorbate-specific peroxidase in spinach-chloroplasts. *Plan Cell Physiol*., 22: 867.

Nayek, S., S. Gupta and R.N. Saha. 2010. Metal accumulation and its effects in relation to biochemical response of vegetables irrigated with metal contaminated water and wastewater. *J. Hazard. Mat*., 178: 588-595.

Nies, D.H. and S. Silver. 1995. Ion efflux systems involved in bacterial metal resistances. *J. Ind. Micro.*, 14: 186-199.

Pandit, S., A. Sengupta, S. Kale and D. Das. 2011. Performance of electron acceptors in catholyte of a two-chambered microbial fuel cell using anion exchange membrane. *Bioresour Technol.*, 102: 2736-2744.

Pourraut, B., M. Shahid, C. Dumat, P. Winterton and E. Pinelli. 2011. Lead uptake, toxicity and detoxification in plants. *Rev. Environ. Conta. Toxicol.*, 213: 113-136.

Rajbanshi, A. 2008. Study on heavy metal resistant bacteria in Guheswori sewage treatment plant. *Our Nature*, 6: 52-57.

Rajkumar, M., S. Sandhya and M.N.V. Freitas. 2012. Perspectives of plant associated microbes in heavy metals Phytoremedeation. *Biotechnol. Adv.*, 30: 1562-74.

Rosa, M., M. Hilal, J.A. González and F.E. Prado. 2004. Changes in soluble carbohydrates and related enzymes induced by low temperature during early developmental stages of quinoa (*Chenopodium quinoa*) seedlings. *J. Plant Physiol*., 161: 683-9.

Rosa, M., M. Hilal, J.A. González and F.E. Prado. 2009. Low-temperature effect on enzyme activities involved in sucrose-starch partitioning in salt-stressed and salt-acclimated cotyledons of quinoa (*Chenopodium quinoa* Willd.) seedlings. *Plant Physiol.* Biochem., 47: 300-7.

Santini, J.T., A.C. Richards, R. Scheidt, M.J. Cima and R. Langer. 2000. Microchips as controlled drug-delivery devices. Ang. Chemie *Intern. Edi.*, 39: 2396-2407.

Sengar, R.S., S. Gupta, M. Gautam, A. Sharma and K. Sengar. 2008. Occurrence, uptake, accumulation and physiological responses of nickel in plants and its effects on Environment. *Res. J. Phytochem*., 2: 44-60.

Shanker, A.K., C. Cervantes, H. Loza-Tavera and S. Avudainayagam. 2005. Chromium toxicity in plants. *Environ Intern.*, 31: 739-53.

Sharma, A.B., R.S. Dubey and M. Pessarakli. 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *J. Bot*., 1-26.

Tebo, B.M., W.C. Ghiorse, L.G. Waasbergen, P.L. Siering and R. Caspi. 1997. Bacterially mediated mineral formation: Insights into manganese (II) oxidation from molecular genetic and biochemical studies. *Rev. in Miner*., 35: 225-266.

Velikova, V., I. Yordanov and A. Edreva. 2000. Oxidative stress and some antioxidant systems in acid rain treated bean plants: Protective role of exogenous polyamines. *Plant Sci*., 151: 59-66.

Wang, Z.Y., M.Y. Bai, E. Oh and J.Y. Zhu. 2007. Brassino steroid signaling network and regulation of photomorpho genesis. *Ann. Rev. of Gen*., 46: 701-724.

Wu, L., Z. Li, C. Han, L. Liu, Y. Teng, X. Sun and P. Christie. 2012. Phytoremediation of soil contaminated with cadmium, copper and polychlorinated biphenyls. *Int. J. Phytoremed.*, 14: 570-584.

Yasmin, S., F.Y. Hafiz, M. schmid and A. Hartmann. 2013. Plant – beneficial rizobacteria for sustainable increased yield of cotton with reduced level of chemical fertilizers. *Pak J. Bot*., 45: 655- 662.

Yousaf, S., K. Ripka, T. Reichenauer, V. Andria, M. Afzal and A. Sessitsch. 2010. Hydrocarbon degradation and plant colonization by selected bacterial strains isolated from Italian ryegrass and birds foot trefoil. *J. App. Micro*., 109: 1401.

(Received for publication 17 November 2016)