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Studies to Reveal Importance of Fe for Cd-tolerance in *Brassica juncea*

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Abstract

A study was conducted to examine the role of iron (Fe) when *Brassica juncea* (Indian mustard) was exposed to cadmium (Cd). The mustard plants were grown in hydroponics divided into two sets one set of plant with normal Hoagland media and other set with Fe-deficient Hoagland media after fifteen days, plants were treated with $CdCl_2$ (150 μM) and studied at 48 and 72 hours after treatment (HAT). Fe-deficiency induced oxidative stress, the magnitude of stress was further higher in presence of Cd; however, oxidative stress was lesser during presence of Fe when thiobarbituric acid activity was studied. Activities of numerous enzymatic cellular antioxidants viz., superoxide dismutase, ascorbate peroxidase, glutathione reductase and catalase declined under Fe-deficiency and more severely during Cd exposure. However, Cd exposure in Fe-fed plants showed lesser degree of decline. Similar response was observed with contents of ascorbate, glutathione, soluble protein, chlorophylls and root-shoot ratio. Protein profile (SDS-PAGE) of *B. juncea* leaves showed differences among all treatments over control. The protein affected most fell between 6.5-14.5 kDa and 31-45 kDa which emanates under the protein category like HSP70 and chloroplast precursors/oxygen evolving protein nonetheless less affected under Fe-deficiency in absence of Cd. Study suggests essential requirement of Fe for providing tolerance to plant during Cd stress.

Additional keywords: *Brassica*, Antioxidants, Fe-deficiency, Cd-stress, SDS-PAGE

Abbreviations

TBARS (thiobarbituric acid), APX (ascorbate peroxidase), GR (glutathione

reductase), CAT (catalase), SOD (superoxide dismutase), HAT (hours after treatments), µM (micro-molar), SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), kDa (kilo Dalton), HSP (heat shock protein)

Acknowledgements
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Introduction
Metal ions such as iron, copper and zinc are indispensable micronutrient for all life and play important protagonists in numerous biochemical processes, including electron transfer reactions and plant antioxidant system (Qureshi et al., 2010), respiration, glycolysis and photosynthesis. Iron is used as co-factor and is present in heme-and iron-sulfur proteins (Van Hoewyk et al., 2007), which plays important roles in photosynthesis and N and S assimilation (Abdel-Ghany et al., 2005) in chloroplasts. Iron deficiency is a chief abiotic stress that disturbs many crop species, exclusively those grown in alkaline and calcareous soils (Lindsay and Schwab 1982). Whereas, these soils have abundant Fe but is not soluble thus often unavailable for plants obstructing growth rates and crop yield (Chen and Bark 1982).

Cadmium (Cd) is one of the most toxic metal pollutants in the soil surface layer (Sanita di and Gabrielli, 1999). Its accumulation in crops and soils is increasing concern to crop production and may produce oxidative stress (Qadir et al., 2004). Cd is well known for its phytotoxicity, which is associated with a number of morphological, physiological and biochemical events. Significant sources of Cd contamination are atmospheric decomposition derived from mining, smelting and fuel combustion, as well as the use of phosphate fertilizers and swedge sludge (Lungon-Moulin et al., 2004). Cd causes various phytotoxic symptoms including chlorosis, growth inhibition, water imbalance, phosphorus and nitrogen deficiency, reduced manganese transport and accelerated senescence. Cd can exert toxic effects through its high affinity for sulfhydryl groups in proteins and other biological molecules (Sanita di Toppi and Gabbrielli, 1999; Fagioni et al., 2009). Fe-deficiency during Cd exposure raises plants sensitivity and damages cells to greater level (Qureshi et al., 2010). Cd severely alters ascorbate-glutathione antioxidant system (Markovska et al., 2009) and metabolism of essential elements (Dong et al. 2006). The heavy metals induce oxidative stress because they interfere in electron transport system and activate several different types of ROS generating mechanisms (Qadir et al., 2004). A variety of proteins function as scavengers of ROS; these include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) and catalase (CAT) (Mittler, 2002; Asada, 1999; Bowler et al., 1992). Plants also respond to metal toxicity in different other ways too than ascorbate-glutathione pathway (Agehi et al., 2009). Such responses include immobilization, exclusion, chelation and compartmentalization of the metal ions, formation of peptide metal-binding ligand PCs (Phytochelatins) and the expression of more general stress-response mechanisms,

such as ethylene and stress proteins. A large number of stress proteins have been found to be induced by metal stress with a molecular mass of 10,000–70,000 kDa in plants.

Brassica juncea is an important oilseed crop which has been extensively used to demonstrate the response to Cd due its high tolerable threshold. *Brassica juncea* cv. Pusa Jai Kisan has been found to tolerate very high Cd levels.

The present study aims to examine the impact of iron deficiency (-Fe), Cd treatment under iron deficiency (-Fe+Cd) to reveal protective role of iron (+Cd+Fe) and comparison over control (+Fe) studied in terms of magnitude of oxidative stress (TBARS content), antioxidant response (enzymatic activities and non-enzymatic contents), photosynthetic pigments, root-shoot ratio and protein profile on SDS-PAGE.

Material and Methods

Plant material and treatments: Seeds of *Brassica juncea* cv. Pusa Jai kisan were collected from IARI (Indian Agricultural Research Institute), Pusa, New-Delhi, India.

Experiments were conducted in 250 ml beakers containing Hoagland's nutrient solution of half strength in a culture room with 16 h photoperiod, a day/night temperature and relative humidity regimes of 25 ± 2^0C and 55–75%, respectively. Healthy seeds of uniform size were detergent-washed and surface sterilized with 4% sodium hypochlorite in double distilled water (DDW) for 5 min followed by 10 washings with DDW. Sterilized seeds were sown on wet paper towels in petriplates. Three-day-old seedlings were transferred to beakers containing Hoagland's solution (half strength) with two sets, one with Fe-deficiency and other with normal nutrient media (Fig 13 a, b). Hoagland's solution was changed every third day. Fifteen-days-old seedlings were subjected to 150 µM of $CdCl_2$ and studies 48 and 72 hours after treatment (HAT). The experimental design was taken as: +Fe –Cd (control), -Fe -Cd, -Fe +Cd and +Fe +Cd.

Thiobarbituric acid reactive substances: TBARS, considered as oxidative damage products, were determined in leaf samples by the method of Heath and Packer (1968). One gram of fresh tissue was ground in 10 cm^3 0.1% trichloroacetic acid (TCA) and centrifuged at 7826 g 5 min. The mixture of 1 cm^3 of supernatant with 4.0 cm^3 of 0.5% thiobarbituric acid (TBA) was heated at 95 ^{0}C for 30 min, cooled and centrifuged at 1957 g for 5 min. The absorbance of the supernatant was read at 532 nm and corrected for unspecific turbidity after subtraction from the value obtained at 600 nm. TBARS was then quantified using coefficient of absorbance of 155 mM^{-1} cm^{-1}.

Ascorbate (Asc), dehydroascorbate (DAsc) and total ascorbate (Asc + DAsc) were estimated by a modified method of Law et al. (1983). Fresh leaf tissue (0.5 g) was ground in 2 cm^3 of 0.1 M Na-phosphate buffer (pH 7.0) and 1 mM EDTA and centrifuged at 7826 g for 10 min. The supernatant was distributed in two separate sets for the assay of total ascorbate and ascorbate (Asc). To each sample (0.4 cm^3), 0.2 cm^3 of 10% TCA was added. After 5 min, 0.01 cm^3 of 5 M NaOH was added, mixed

and centrifuged for 2 min. To 0.2 cm^3 of the supernatant 0.2 cm^3 of Na-phosphate buffer (150 mM, pH 7.4) and 0.2 cm^3 of double distilled water (DDW) were added. For determination of Asc, another 0.2 cm^3 of supernatant was used with 0.2 cm^3 of Na-phosphate buffer and 0.1 cm^3 of 10 mM dithiothritol (DTT) and, after a thorough mixing, was left at room temperature for 15 min. 0.1 cm^3 of 0.5% N-ethylmaleimide was then added to each of the tubes and incubated at 24 °C for >30 s. Further, 0.4 cm^3 of 10% TCA, 0.4 cm^3 of 44% H_3PO_4, 0.4 cm^3 of 4% bipyridyl and 0.2 cm^3 of 3% $FeCl_3$ were added. After being vortex-mixed, samples were incubated at 37 ^{0}C for 60 min and absorbance was recorded at 525 nm.

Glutathione content: Reduced (GSH), oxidised (GSSG) and total glutathione (GSH + GSSG) were determined by the glutathione recycling method of Anderson (1985). Fresh leaf (0.5 g) was homogenized in 2 cm^3 of 5% sulphosalicylic acid at 4 ^{0}C. The homogenate was centrifuged at 7826 g for 10 min. To a 0.5 cm^3 of supernatant, 0.6 cm^3 of reaction buffer (0.1 M Na-phosphate, pH 7, 1 mM EDTA) and 0.04 cm^3 of 0.15% 5,5-dithiobis-2-nitrobenzoic acid (DTNB) were added and read at 412 nm after 2 min. To the same, 0.04 cm^3 of 0.4% NADPH and 0.002 cm^3 of glutathione reductase (GR; 0.5 enzyme unit) were added and reaction was run for 30 min at 25 ^{0}C. The samples were again read at 412 nm to determine the total glutathione.

Enzyme assays: The method of Dhindsa et al. (1981) was followed with slight modification for estimating SOD activity. Fresh leaf material (0.2 g) was homogenised in 2.0 cm^3 of extraction mixture containing 0.5 M Na-phosphate buffer, pH 7.3, 3 mM EDTA, 1% PVP, 1% Triton X 100 and centrifuged at 11 269 g at 4 ^{0}C. SOD activity in the supernatant was assayed by its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT). The assay mixture, consisting of 1.5 cm^3 reaction buffer containing 0.1 M Na-phosphate buffer, pH 7.5, 1% PVP, 0.2 cm^3 of L-methionine, 0.1 cm^3 enzyme extract with equal amount of 1 M $NaHCO_3$, 2.25 mM NBT solution, 3 mM EDTA, 60 μM riboflavin and 1.0 cm^3 of DDW was incubated under 15 W inflorescent lamp at 28 °C. 50% reduction of NTB was considered as one unit of enzyme activity.

APX activity was estimated by the method of Nakano and Asada (1981). Fresh leaf material (1 g), ground in 5 cm^3 of extraction buffer (0.1 M K-phosphate, pH 7, 3 mM EDTA, 1% PVP, 1% Triton X 100), was centrifuged at 7826 g for 10 min at 4 ^{0}C. APX activity was determined in supernatant by the decrease in absorbance of ascorbate at 290 nm, due to its enzymatic reakdown. 1 cm^3 of reaction buffer contained 0.5 mM ascorbate, 0.1 mM H_2O_2, 0.1 mM EDTA and 0.05 cm^3 of extract containing enzyme. The reaction was run for 5 min at 25 ^{0}C. APX activity was calculated by using coefficient of absorbance 2.8 mM^{-1} cm^{-1}. One unit of enzyme determines the amount necessary to decompose 1 μmol of ascorbate per min.

GR activity was determined by the method of Foyer and Halliwell (1976) modified by Rao 1992). Fresh leaf material (0.5 g), ground in 2 cm^3 of extraction buffer (0.1 M Na-phosphate, pH 7.0, 3 mM EDTA, 1% PVP, 1% Triton X 100) was centrifuged at 7826 g for 10 min. The supernatant was immediately assayed for GR activity through glutathione-dependent oxidation of NADPH at 340 nm. 1 cm^3

reaction mixture containing 0.2 mM NADPH, 0.5 mM GSSG and 0.05 cm^3 of enzyme extract was kept for 5 min at 25 ^{0}C. Corrections were made for any GSSG oxidation in the absence of NADPH. The activity was calculated using coefficient of absorbance of 6.2 mM^{-1} cm^{-1}. One unit of enzyme determines its amount necessary to decompose 1 μmol of NADPH per min.

CAT activity was determined by the method of Aebi (1984). Fresh leaf material (0.5 g), ground in 5 cm^3 of extraction buffer (0.5 M Na-phosphate, pH 7.3, 3 mM EDTA, 1% PVP, 1% Triton X 100) was centrifuged at 7826 g for 20 min at 4 ^{0}C. CAT activity in supernatant was determined by monitoring the disappearance of H_2O_2, measuring a decrease in absorbance at 240 nm. Reaction was run in a final volume of 2 cm^3 of reaction buffer (0.5 M Na-phosphate, pH 7.3) containing 0.1 cm^3 3 mM EDTA, 0.1 cm^3 of enzyme extract and 0.1 cm^3 of 3 mM H_2O_2 for 5 min. CAT activity was calculated by using coefficient of absorbance of 0.036 mM^{-1} cm^{-1}. One unit of enzyme determines the amount necessary to decompose 1 μmol of H_2O_2 per min.

Protein content: Protein was quantified with the help of Bradford (Bradford, 1976) reagent using bovine serum albumin as a standard. 0.5 g of fresh plant material was homogenized in 1 cm^3 phosphate buffer and was centrifuged at 7826 g from which 0.5 cm^3 supernatant was taken to which 0.5 cm^3 TCA was added. The sample was again centrifuged at 1370 g and the supernatant was discarded and the remaining was washed with NaOH to which 5 ml Bradford was added and the absorbance was taken at 595nm.

Photosynthetic pigments: Chlorophyll content was estimated by the method of Hiscox and Israelstam (1979). Fresh leaves were collected and kept in vials. 10 ml DMSO were added to it and were kept in an oven at 65 ^{0}C for 1 hour. OD was taken at 480, 645, 520 and 663 nm on the Beckman DU 640B spectrophotometer. The chlorophyll concentrations in mg fresh samples were calculated by the formula given by Arnon (1949).

Root-shoot ratio: The root-shoot length indicates length of plant arise from the root tip to upper most growing tip of the main axis. Plants were uprooted carefully and washed with cold DDW and were kept on moist filter papers to avoid desiccation. The lengths of root (root tip to root-stem junction) and shoot (root-stem junction to stem tip) were measured with the help of measuring scale in cm and recorded.

Protein profile: For protein profile on SDS-PAGE, leaves were frozen in liquid nitrogen and ground with the help of chilled pestle and mortar to a fine powder. This powder was extracted with 40 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.07% β-mercaptoethanol, 2% PVP and 1% Triton X100. The extract was centrifuged at 26 880 g for 60 min at 4 ^{0}C. The extract was mixed with 10% TCA (chilled) containing 0.07% β mercaptoethanol and 2 mM EDTA and left at -20 ^{0}C overnight. On second day the mixture was then centrifuged at 26 880 g for 15 min/4 ^{0}C and pellet was then washed twice with chilled acetone for 60 min. The pellet was then vacuum dried and

solubilized in soulibilization buffer containing Tris HCl (pH 7.5), 2mM EDTA, and 0.07 β-mercaptoethanol. Subsequently solubilized proteins were quantified by Bradford, using BSA as standard and 40 µg of protein were loaded in mini gel electrophoretic unit (Bio-Rad) on 12% acrylamide gel (laemmli, 1970). The gel was stained in Coomassie brilliant blue R250 for overnight and was then destained in destaining solution. The gel was scanned on gel documentation system (Bio-Rad, USA).

Statistical analysis: Statistical analyses of the data obtained were carried by the two-way ANOVA (Cochram and Cox 1957) to evaluate whether the values were significantly different.

Results

Thiobarbituric acid reactive substances (TBARS)

Fe-deficiency leads to 9.97% and 35% elevation in TBARS at 48 and 72 hours after treatment which was more intense in presence of Cd, 39% and 59% at 48 and 72 HAT, respectively. Presence of Fe proved very effective to control oxidative stress (TBARS) which was 10% and 20% higher as compared to control at 48 and 72 HAT, respectively (Fig 1, Table 1).

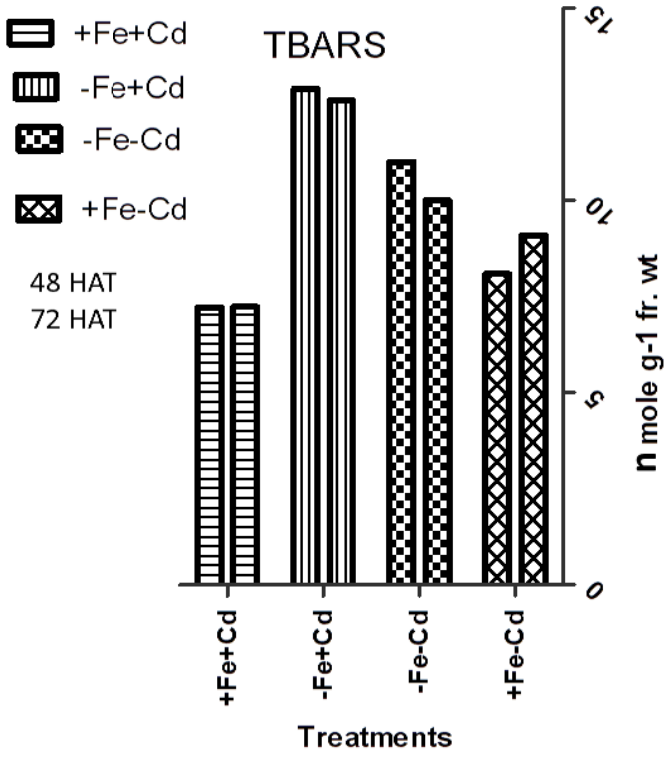

Figure 1: Impact of Cd on magnitude of oxidative stress in terms of TBARS under Fe-deficient and Fe-fed conditions.

Table 1: Influence of Fe-deficiency and Cd treatment on cellular antioxidants, soluble proteins, in leaves. Values are Mean±SE of five replicates. Parentheses include per cent variation from control.

	Hours after treatment (HAT)							
	48				72			
Parameter/Treatment	+Fe -Cd	-Fe -Cd	-Fe +Cd	+Fe +Cd	+Fe -Cd	-Fe -Cd	-Fe +Cd	+Fe +Cd
TBARS	9.096±0.120 (00)	10.003±0.125 (9.97%)	12.66±0.0762 (39.18%)	7.23±0.224 (20.5%)	8.096±0.216 (00)	11.0.±0.061 (35.86%)	12.9±0.525 (59.3%)	7.22±0.278 (10.8%)
SOD	9.58±0.340 (00)	8.5±0.125 (11.27%)	7.2±0.234 (24.8%)	10.22±0.042 (6.688%)	9.68±0.340 (00)	8.6±0.012 (11.15%)	7.1±0.026 (26.65%)	10.23±0.347 (5.68%)
APX	13.12±0.201 (00)	8.68±0.0123 (33.84%)	7.35±0.024 (43.97%)	12.9±0.231 (1.6%)	12.9±0.210 (00)	8.69±0.201 (32.2%)	7.4±0.401 (41.66%)	13.12±0.120 (1.7%)
CAT	54.3±0.001 (00)	25.8±0.031 (52.4%)	14.24±0.201 (73.77%)	58.3±0.002 (7.3%)	54.32±0.091 (00)	24.1±0.021 (55.63%)	14.25±0.021 (73.77%)	60.4±0.041 (11.19%)
GR	23.19±0.012 (00)	20.21±0.0134 (12.85%)	19.34±0.0478 (16.6%)	25.16±0.0378 (8.3%)	23.18±0.034 (00)	20.22±0.081 (12.76%)	19.32±0.0109 (16.65%)	29.8±0.0129 (28.5%)
Ascorbate (Total)	32.23±0.034 (00)	30.21±0.020 (6.26%)	29.6±0.010 (8.16%)	35.12±0.102 (8.96%)	32.22±0.291 (00)	30.22±0.094 (6.20%)	29.2±0.008 (9.37%)	35.16±0.071 (9.12%)
Glutathione (Total)	25.16±0.0130 (00)	22.19±0.0120 (11.88%)	20.21±0.032 (19.67%)	28.19±0.0102 (12.04%)	25.12±0.012 (00)	22.18±0.012 (11.77%)	20.21±0.0109 (19.67%)	29.2±0.019 (12.04%)
Soluble protein content	18.356±64.40 (00)	18.2±34.89 (0.84%)	16.29±43.56 (11.25%)	20.5±49.01 (11.68%)	18.34±62.2 (00)	18.3±31.8 (0.84%)	16.2±50.019 (11.25%)	21.6±50.01 (11.65%)

Cellular antioxidants

Activity of several enzymatic antioxidants and levels of non-enzymatic antioxidants were studied in *Brassica juncea*. Iron deficiency lead to decrease in cellular antioxidants viz; SOD (superoxide dismutase) was 11% decreased, APX (ascorbate peroxidase) 33.84% and 32.2% GR (glutathione reductase) 12.85% and 12.76% and CAT (catalase) 52% and 55%at 48 and 72 hours after treatment and the contents of

non-enzymatic antioxidants viz., ascorbate was 6.26% and 6.20% and glutathione 11.88% and 11.77% decreased. However, in presence of iron they were (SOD 6% and 5%, APX 1.67% and 1.70%, CAT 7.36% and 11.19%, GR 8.49% and 28.5%, ascorbate 8.96% and 9.12%, glutathione 12%) increased at 48 and 72 HAT correspondingly (Fig 2-7).

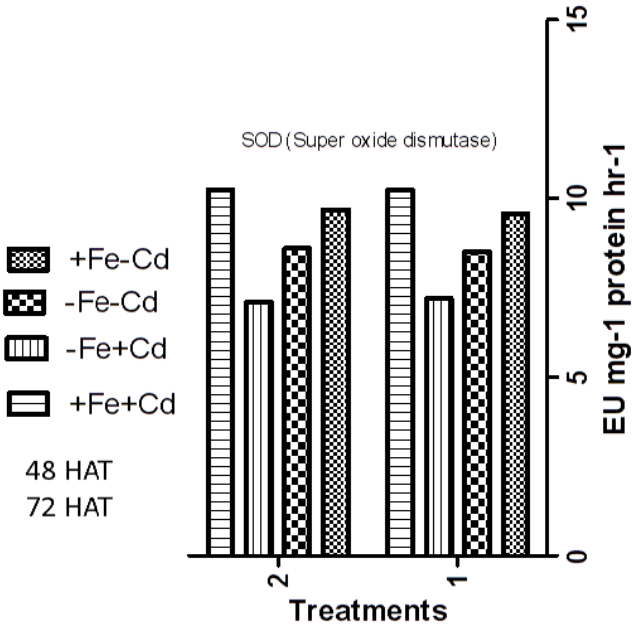

Figure 2: Impact of Cd on SOD (superoxide dismutase) activity under Fe-deficient and Fe-fed conditions

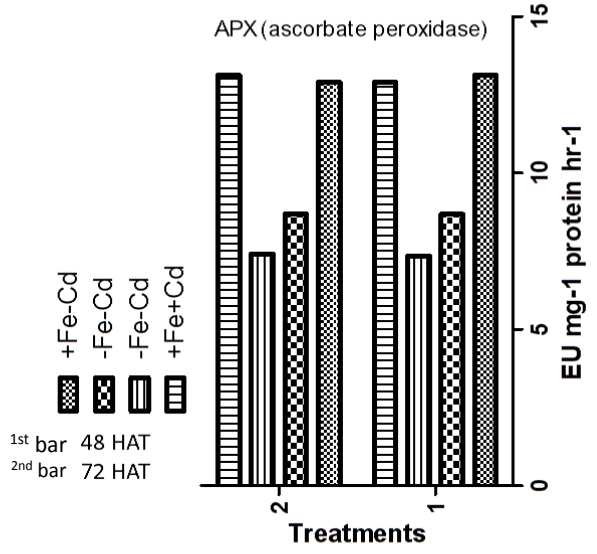

Fig 3: Impact of Cd on APX (ascorbate peroxidase) activity under Fe-deficient and Fe-fed conditions.

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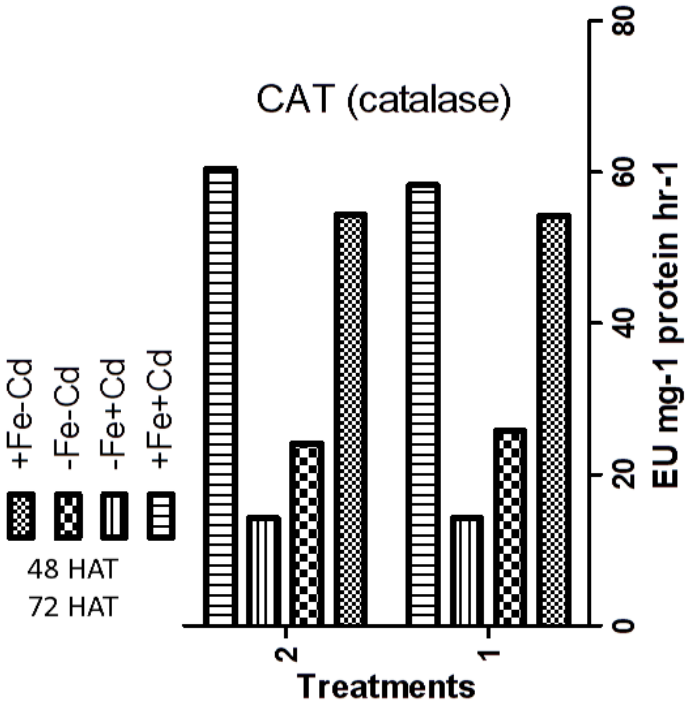

Figure 4: Impact of Cd on CAT (catalase) activity under Fe-deficient and Fe-fed conditions.

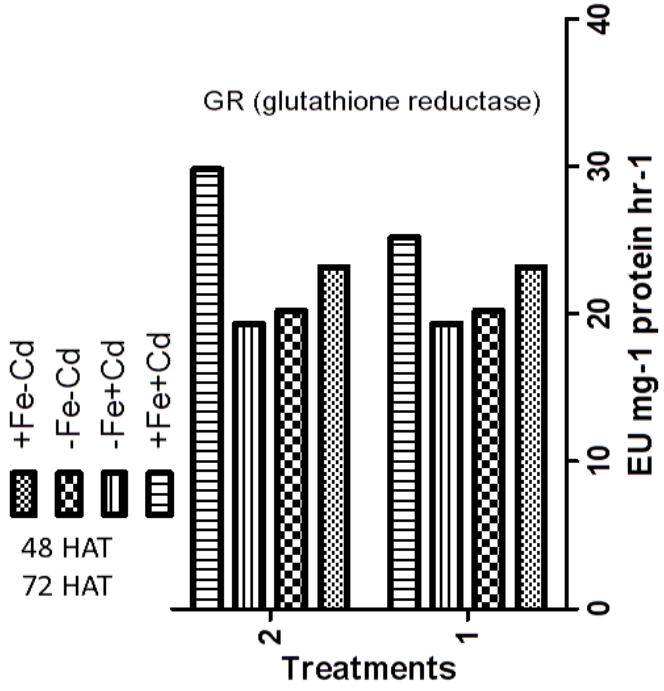

Figure 5: Impact of Cd on GR (glutathione reductase) activity under Fe-deficient and Fe-fed conditions.

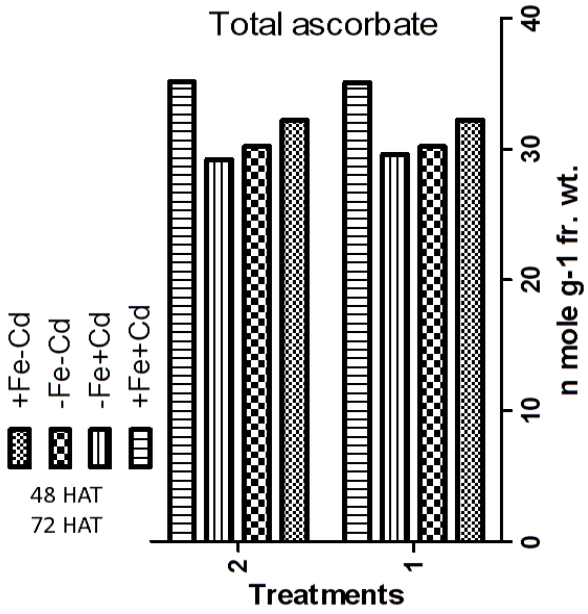

Figure 6: Impact of Cd on total ascorbate under Fe-deficient and Fe-fed conditions.

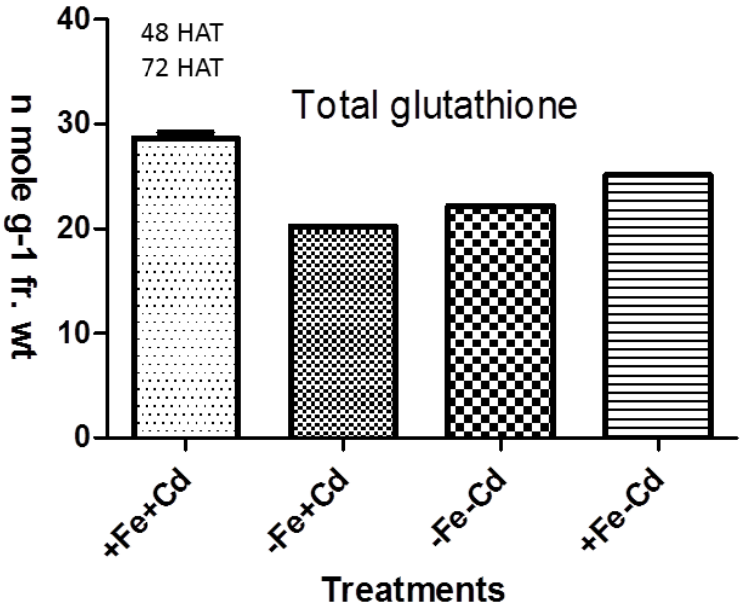

Figure 7: Impact of Cd on total glutathione under Fe-deficient and Fe-fed conditions.

Soluble protein content

Soluble protein content estimated under Fe-deficiency and Cd stress was 11.22% and 11.66% decreased which was 11% less intense in presence iron under Cd stress at 48 and 72 HAT respectively. Presence of iron proved to be competent to control loss of protein content in presence of Cd (Fig 8).

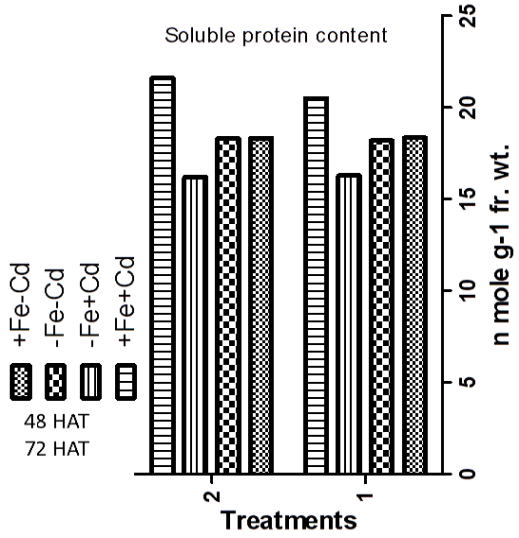

Figure 8: Impact of Cd soluble protein content under fe-deficient and Fe-fed conditons.

Photosynthetic pigments

The photosynthetic pigments such as chlorophyll *a*, in absence of iron were 0.84% decreased which was 19.35% and 15.34% more intense in presence of Cd content at 48 and 72 hours after treatment respectively. Consequently Chlorophyll *b* and carotenoid was7.89%, 18.48%, 11.76% and 10.48% decreased in absence of iron both at 48 and 72 hours after treatment correspondingly. The effect was more extreme (Chl *a* 19.35% and 15.34%, Chl *b* 30.66% and 34.21%, carotenoid 27.59% and 27.55% at 48 and 72 HAT respectively) in presence of Cd (Fig 9).

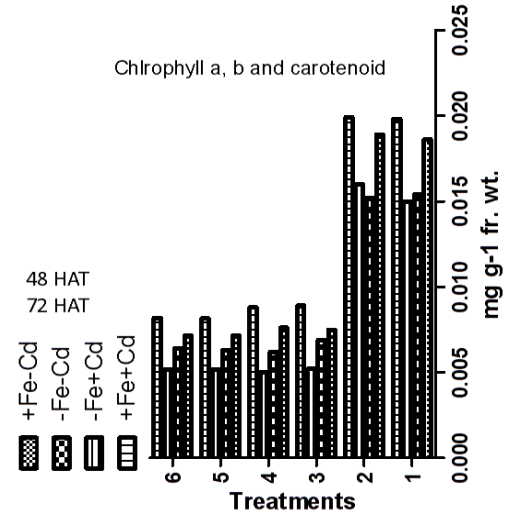

Figure 9: Impact of Cd on chlorophyll a, b and carotenoid activity under Fe-deficient and Fe-fed conditions.

Root-Shoot ratio

The length of root and shoot was 16% decreased in absence of Fe, however, it was 36% more intense in presence of Cd and Cd-stress at 48 and 72 HAT as compared to that of control as shown in figure 10. Presence of iron abolishes the effect of cadmium exposure.

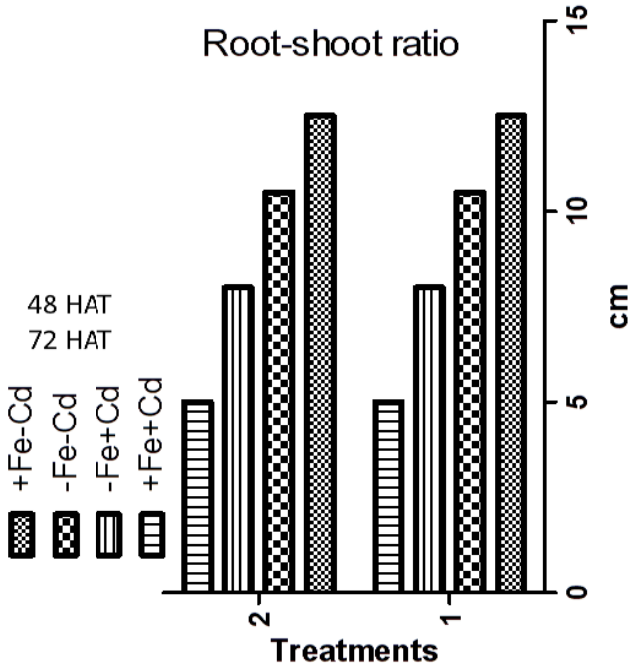

Figure 10: Impact of Cd on root-shoot ratio activity under Fe-deficiend and Fe-fed conditions.

Protein profile (SDS-PAGE)

1D profile of leaf protein of *Brassica juncea* showed enormous number of bands studied at 72 hours after treatment. In absence of iron the proteins were found to be down regulated as compared to that of control which was more severe in presence of Cd as compared to that of control having molecular weight between 31-45 kDa and 14.4-21.5 kDa. However, the bands were found more intense in presence of cadmium, some protein bands were found to up-regulated as compared to that of control in presence of Cd (Fig 11). These proteins were analyzed with reference to certain literatures and were found that these proteins could be certain stress responsive and photosynthetic proteins like DNA heat shock protein, oxygen evolving enhancer protein and RuBisCO which keeps the plant overwhelmed from stress and helps from noxious effects of toxic metals in order to maintain the normal physiological functions. From our study it showed that presence of iron helps the plants to overcome the stress effects of toxic metals.

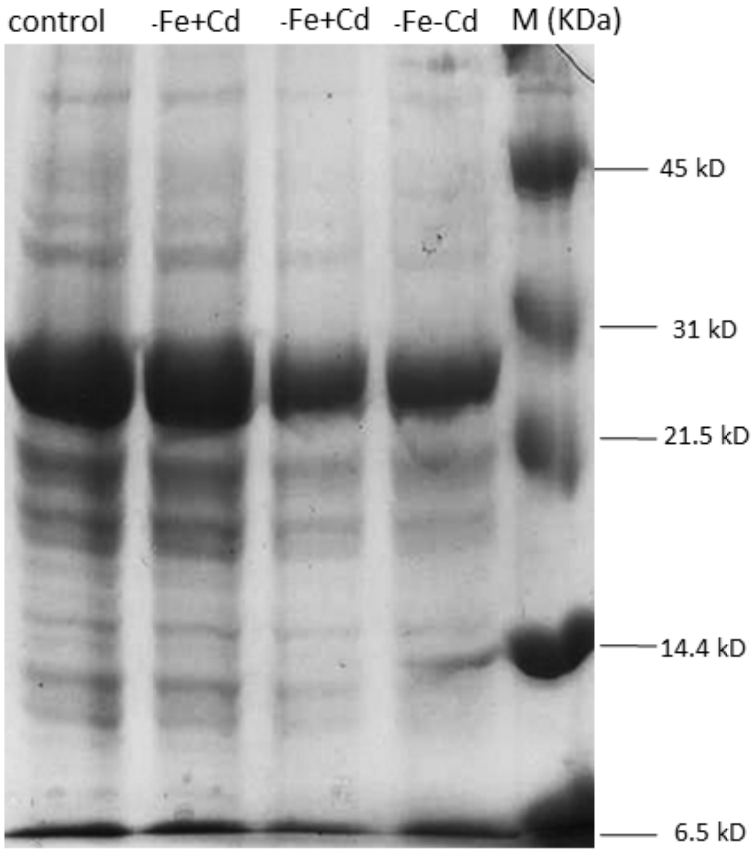

Figure 11: Brassicajuncea leaf protein profile (SDS-PAGE) in response to Fe-deficiend and Cd-Stress.

Figure 12: a. Plants grown under normal Hoagland nutrient media, b. Plants grown under Fe-deficient Hoagland nutrient media.

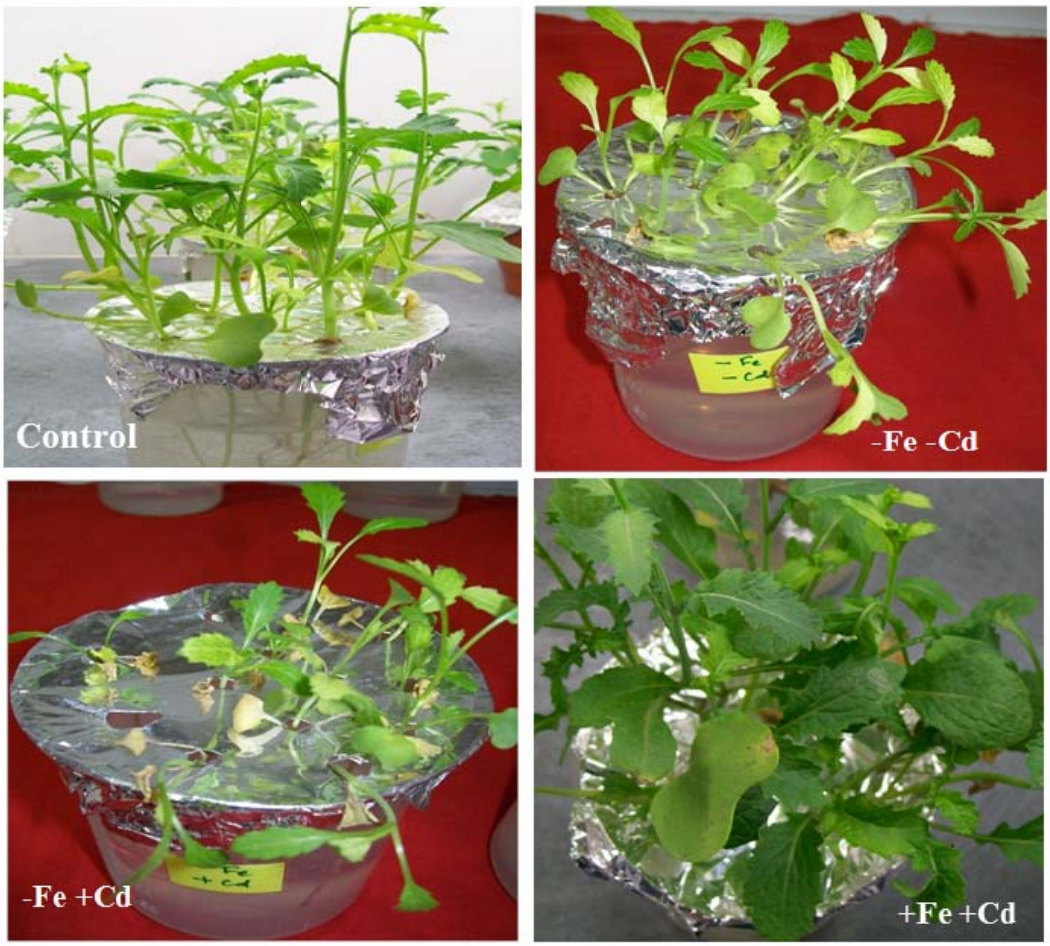

Figure 13: Brassica grown under separate and cumulative influnce of Fe-deficiency and Cd-stress along with control.

Discussion

Fe deficiency is reported worldwide in a number of agriculturally important crops, with deficiency usually occurring in alkaline soils and being attributed to the lowered availability of iron at high pH in calcareous soils (Romheld and Marschnner 1986). In our present work, we studied role of iron under Cd stress in order to reveal the importance of Fe for Cd tolerance. The Cd-stress was found to adversely affect plant growth and metabolism in *B. juncea*. TBARS formation is considered to be the general indicator of oxidative damage such as lipid peroxidation. Peroxidation reaction in photosynthetic tissues and plastids are initiated by either excited chloroplasts or oxygen species derived from the superoxide anion radical. Metal ions block the electron flow in PSII, which leads to the formation of excited chlorophyll, which in turn causes production of free radicals (Kato and Simizu, 1985). Our study revealed that under Fe-fed conditions magnitude of oxidative stress was low which was severe with Cd stress and Fe-deficiency. This may be due to the formation of

ROS in chloroplast and peroxisomes which leads to exclusion of hydrogen ions from unsaturated fatty acids (Delledonne, 2005; Qureshi et al., 2010).

To mitigate and repair the damage caused by ROS, plants have evolved complex antioxidant both enzymatic and non-enzymatic systems. During the present investigation Fe tend to decrease the enzymatic activities which were more intense when plants were exposed to Cd. It was suggested that the decrease in magnitude enzymes activity could be the consequence of de novo synthesis of enzymatic protein (Allen et al., 1997). This is for these enzymes are the iron comprehending and their ensue oxidative stress in chloroplast, peroxisomes and mitochondria which pointers to break down of certain iron enclosing genes thus, leads to inactivation of these stress responsive proteins to overcome the oxidative stress (Qureshi et al., 2005). ROS occasionally amend and restrain certain genes when contemporary only in roots but when the concentration goes more it unquestionably lead to demodulation of several metabolic processes like respiration, photosynthesis and glycolysis. Correspondingly non-enzymatic antioxidants like glutathione and ascorbate were lessened under Fe-deficiency and Cd exposure but less pretentious when Fe was present.

Alterations in non-enzymatic cellular antioxidants like ascorbate and glutathione under oxidative stress are well documented (Foyer et al., 1976). Our results showed a decrease in ascorbate and glutathione levels in absence of iron. However, the decrease was more prominent in presence of Cd. Decrease in levels of ascorbate and glutathione in presence of iron and cadmium and increase under cadmium exposure suggest its active participation in mitigating oxidative stress and protective role of iron for Cd tolerance (Schutzendubel et al., 2001).

Our study revealed that in absence of iron and presence of Cd photosynthetic pigments decreased but in presence of iron it is less affected as compared to that of control. Chlorophyll is a vital pigment for photosynthesis in plants. Recent studies suggest that the formation of light harvesting complex is disturbed in Cd treated leaves (Horvath et al., 1996), due to the inhibition of LHC protein synthesis at transcriptional level. Same results were also reported in barley leaves, where considerable amount of chlorophyll was broken down after the first day of Cd treatment. It is likely that the catabolism of chlorophyll starts with the chlorophyll still bound to the membrane protein within the chloroplast, with the removal of phytol tail by an enzyme, chlorophyllase. The Mg atom is then removed by Mg dechelatase, the ring is opened by a dioxygenase and the binding protein is released for degradation. The remaining chlorophyll catabolite is then transported to the vacuole where further metabolism takes place. Similar results were analyzed in *Brassica juncea* under iron deficiency and cadmium stress (Qureshi et al., 2010).

The length of root and shoot is affected by oxidative stress due to effect on certain proteins responsible for growth and various metabolic activities under Cd exposure and Fe-deficiency. But iron help to curtail such effects under Cd stress. Fe and cadmium exhibited inhibitory effect on plant growth, plant height and biomass accumulation in a dose- and time-dependent manner (Qureshi et al., 2010, Qadir et al., 2004).

Protein profile in our study showed that under Fe-deficiency and Cd-stress the expression of protein bands were under-expressed owing to increase in oxidative

stress but less exaggerated in absence of Cd which overwhelms the proteins accountable for photosynthesis and stress tolerance (Vincent et al., 2007). This may be due creation of reactive species in chloroplast and mitochondria. Moreover this might be due to inactivation of certain genes encoding for that particular type of a protein which is over-expressed and under-expressed. We also analyzed these proteins from certain literatures which suggest that these proteins might be the stress responsive related proteins and photosynthetic proteins which protect the plant from oxidative stress. Qureshi et al., (2010) studied the protein profile in *B. juncea* followed by Blue Native Page (BNP) and mass spectrometry under iron deficiency and Cd stress and they found over and under expressed proteins as chloroplast precursors and other stress responsive proteins which supports our findings. Although we analyzed our observations based on the molecular weight and found that these proteins might be the proteins like chloroplast precursor proteins essential for photosynthesis, molecular chaperons such as HSPs which are produced in the plants when they are in extreme stressed environmental conditions. In supposition from our results showed that Cd stress grounds oxidative stress by eliciting with Fe-deficiency which causes Fenton reactions and ominously causes disorder of metabolic progressions which is conquered by metalothionine and Phytochelatins. Nevertheless, Fe plays a important role in contending these stresses.

Conclusion

Summarizing the results the study indicated that Fe-deficiency prompt Cd ions causing unavoidable reactive species. The adaptive responses are different under iron-deficiency and cadmium stress this strategy relies more on thiol induction and metal binding peptides leading to metal tolerance and iron make a big difference in providing stress tolerance. There is also an increased antioxidant enzyme activity under cadmium stress in presence of iron thus providing act as better scavenging property potential to leaf. Plant metal tolerance is not a simple execution of single mechanism, instead expression of metal tolerance may be an integration of all possible mechanism(s) that a plant possess and operate as indicated by up-regulation of proteins from various protein families. It may also be concluded that *Brassica juncea* can be used as hyperaccumulator and it can act as a biomarker for monitoring Cd and mineral deficiencies present in the crop field so as to increase certain crop yield.

References

[1] Abdel-Ghany S, Garifullina GF, Zhang L, Pilon-Smith EAH and Pilon M (2005). Iron-sulfur cluster biogenesis in chloroplast. Involvement of the scaffold protein CpIscA. *Plant Physiol.* 138:161-72.
[2] Aebi H (1984). Catalase in vitro methods *Enzymol.* 105:121-126.
[3] Aghaei K, Ehsanpour AA, Shah AH and Komatsu S (2009). Proteome analysis of soybean hypocotyl and root under salt stress. *Amino acids.* 36: 91-98.

[4] Allen RP, Webb RP, Schake SA (1997). Use of transgenic plants to study antioxidant defences, *Free Radic. Biol. Med.* 23:472–479.
[5] Andaluz S, Lopez-Millan AF, De las Rivas J, Aro EM, Abadia J and Abadia A (2006). Proteomic profile of thylakoid membranes and changes in response to iron deficiency. *Photosynth. Res.* 89:141-55.
[6] Anderson ME (1985). Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol.* 113:555-570.
[7] Aronon JD and Israelstam GF (1979). Copper enzymes in isolated chloroplast oxidase in *Beta vulgaris*. *Plant Physiol.* 42:287-292.
[8] Asada K (1999). The water-water cycle in chloroplasts: scavenging of active oxygen and dissipation of excess photons. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 50:601-639.
[9] Bowler C, Montagu MV, Inze D (1992). Superoxide dismutase and stress tolerance. *Ann Rev Plant Physiol. Plant Mol. Biol.* 43:83-116.
[10] Brad ford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding *Anal. Biochem.* 72:248-254.
[11] Chen Y and Bark (1982). Iron nutrition of plants in calcareous soils adv. *Agronomy.* 35:217-240.
[12] Cochran WG, Cox GM (1957). Experimental Designs, 2nd Edition. New York: John Wiley & Sons, Inc.
[13] Delledonne, M., 2005. NO news is good news for plants. *Curr. Opin. Plant Biol.* 8:390–396.
[14] Dhindsa RH, Plumb-Dhindsa P and Thorpe TA (1981). Leaf senescence correlated with increased level of membrane permeability, lipid peroxidation and decreased level of SOD and CAT. *J. Exp. Bot*. 32:93-101.
[15] Dong J, Wu FB and Zhand GP (2006). Influence of cadmium on antioxidant capacity and four microelement concentrations in tomato seedlings (*Lycopersicon esculentum*). *Chemosphere* 64:1659-1666.
[16] Fagioni M, D'Amici GM, Timperio AM and Zolla L (2009). Proteomic analysis of multiprotein complexes in the thylakoid membrane upon cadmium treatment. *J. proteome Res.* 49:49-55.
[17] Foyer CH, Lopez Delgado H, Dat JF, Scott IM (1997). Hydrogen peroxide and glutathione associated mechanism of acclamatory stress tolerance and signalling, *Physiol. Plant.* 100:241–254.
[18] Heath RL and Packer L (1968). Photoperoxidation in isolated chloroplast I. Kinetics and stoichiometry of fatty acid. Peroxidation. *Arch. Biochem. Biophys.* 125:189-198.
[19] Hiscox JD and Israclstam GF (1979). A method for interaction of chlorophyll from leaf tissue without maceration. *Can. J. Bot.* 57:1332-1334.
[20] Kato Y, Sakaguchi M, Mori Y, Saito K, Nakamura T, Bakker EP, Sato Y, Goshima S and Uozumi N (2001). Evidence in support of a four transmembrane-poretransmembrane topology model for the *Arabidopsis* thaliana Na^+/K^+ translocating AtHKT1 protein, a member of the superfamily of K+ transporters. *Proc. Natl. Acad. Sci. USA.* 98:6488-6493.

[21] Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.
[22] Lindsay, WL and Schwab AP (1982). The chemistry of Iron in soils and its availability to plants *J. of plant nutria.* 5:821-840.
[23] Lungon-Moulin N, Zhang M, Gadani F, Rossi L, Koller D and Krauss M et al., (2004). Critical review of the science and options for reducing cadmium in tobacco (*Nicotina tabacum* L.) and other plants. *Adv. Agron.* 83:111-80.
[24] Mittler R (2002). Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* 7:405-410.
[25] Qadir S, Qureshi MI, Javed S and Abdin MZ (2004). Genotypic variation in phytoremediation potential of *Brassica juncea* cultivars exposed to Cd stress. *Plant Sci.* 167:1171–81.
[26] Qureshi MI, Amici GMD, Fagioni M, Rinalducci S and Zolla L (2010). Iron stabilizes thylakoid protein-pigment complexes in Indian mustard during Cd-phytoremediation as revealed by BN-SDS-PAGE and ESI-MS/MS. *J. of Plant Physiol.* 167:761-770.
[27] Qureshi MI, M. Abdin MZ, Qadir S and Iqbal M (2007). Lead-induced oxidative stress and metabolic alterations in *Cassia angustifolia* Vahl. *Biologia Plantarum*. 51:121-128
[28] Qureshi MI, Muneer S, Bashir H, Ahmad J and Iqbal M (2010). Nodule physiology and Proteomics of stressed legumes. *Adv. Bot. Res.* 56:1-38.
[29] Römheld, V (1987). Different strategies for iron acquisition in higher plants. *Physiol. Plant* 70, 231–234.
[30] Sanita di Toppi, L and Gabbrielli R (1999). Response to cadmium in higher plants. *Environ. Exp. Bot.* 41:105-150.
[31] Schutzendubel A, Schwanz P, Teiichmamm T, Gross K, Langerfeld-Heyser R, Goldbold DL, Polle A (2001). Cadmium-induced changes in antioxidative systems H2O2 content and differentiation in scots pine roots, *Plant Physiol*. 127:887–898.
[32] Spiller SC and Terry N (1980). Limiting factor of photosynthesis II: iron stress diminishes photochemical capacity by reducing the number of photosynthetic units. *Plant Physiol.* 65:121-5.
[33] Van Hoewyk D, Abdle-Ghany SE, Cohu CM, Herbert SK, Kugrens P and Pilon M (2007). Chlroplast iron-sulfur cluster protein maturation requires the essential cysteine desulfurase CPNifs. *Proc. Natl. Acd. Sci. USA*. 104:5686-91.