Involvement of Fe nutrition in modulating oxidative stress and the expression of stress responsive proteins in leaves of Vigna radiata L.

Sowbiya Muneer¹, ², Javed Ahmad¹ and M. Irfan Qureshi¹* 

¹Proteomics and Bioinformatics Lab, Department of Biotechnology, Jamia Millia Islamia, New Delhi-110025, India 
²Department of Animal Science, Institute of Agricultural Science and Technology, College of Agriculture and Life Science, Chonnam National University, Gwangju 500-757, Korea

*Corresponding author: mirfarq@gmail.com

Abstract

Cadmium (Cd) is deleterious to all forms of life which in plants has competition with iron (Fe) for root uptake. This study investigated the protective role of iron (Fe) and its interaction with cadmium (Cd) in leaves of Vigna radiata L. (Green gram). To characterize the significance of Fe nutrition under Cd exposure, biochemical and gel based proteomic analysis was performed in the leaves of Vigna radiata exposed to Fe and Cd combined treatments: Fe-sufficient (+Fe/-Cd), and Fe-deficient (-Fe/+Cd) soils by comparing them with -Fe/-Cd treated plants and with non Cd exposed (+Fe/-Cd) plants. –Fe/+Cd led to an elevation in lipid peroxidation (TBARS) which was further increased in –Fe/+Cd plants. However, in +Fe/+Cd plants oxidative stress in terms of TBARS was minimized by 30%. Biochemical parameters such as total chlorophyll, total ascorbate and glutathione concentration reduced in –Fe/-Cd plants which was more reduced in -Fe/+Cd plants, whereas increased in +Fe/+Cd plants. Enzymatic activities such as APX, SOD, CAT, and GR activities were reduced in –Fe/-Cd plants which was more severe in –Fe/+Cd plants and in –Fe/+Cd enzyme assays were increased. Furthermore, proteomic analysis revealed 238 spots were reproducibly detected on 2D gels of all the four treatments among which 40 proteins were differentially expressed. Eight proteins of interest from differentially expressed proteins were identified and were classified as photosynthetic pigments, chloroplast precursor, stress responsive proteins, protein destination and secondary metabolism. These results indicate that Fe nutrition is involved in alleviating oxidative stress and expression of stress responsive proteins under Cd-stress.

Key words: Cd-stress, Fe-deficiency, mass spectrometry, oxidative stress, proteomics.

Abbreviations: APX (ascorbate peroxidase), SOD (superoxide dismutase), CAT (catalase), GR (glutathione reductase), TBARS (thiobarbituric acid), MS (mass spectrometry), 2D (2nd dimensional), PMF (peptide mass finger printing), RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase), RUBP (ribulose-bisphosphate), PR (pathogenesis related protein).

Introduction

Cadmium is a non-essential element that negatively affects plant growth and development (Clara et al., 1998; Muneer et al., 2011). Cd is released into the environment by power stations, heating systems, metal working industries or urban traffic and used in electroplating, pigments, plastic stabilizers and Ni-Cd batteries (Sanita di Toppi et al., 1999). It is recognized as significant major pollutant due to its considerable solubility and high toxicity (Pinto et al., 2004). Among several mechanisms of inducing toxicity, Cd can also alter the uptake of minerals by plants through its effect to reduce the availability of minerals from the soil, or through a reduction in the population of soil microbes (Maroco et al., 2002). Cd is well known to induce oxidative stress in plants (Balestrasse et al., 2001) because it is involved in several types of reactive oxygen species (ROS) generating mechanisms (Stohs et al., 1995). Although some of them may function as important signaling molecules that alter gene expression and modulate the activity of specific defense proteins (Alscher et al., 2002) most ROS can be extremely harmful to organism at high concentration. ROS can oxidize proteins, pigments, lipids, and nucleic acids, often leading to alterations in cell structure and mutagenesis (Helliwell et al., 1999). Ultimately plant growth and development such as root/shoot elongation and biomass accumulation is affected.
Vigna radiata. To test this hypothesis the response of related enzymes of ascorbate glutathione cycle and changes in expression of proteins in leaves were studied 24 and 72 hours after Fe/Cd treatment sufficient in Fe but absence of Cd (+Fe/Cd, control), Fe deprived and Presence of Cd (-Fe/+Cd), deprived Fe and absence of Cd (-Fe/Cd) and presence of Fe but Cd stressed (+Fe/+Cd).

Results

Fe and Cd content
Fe content under Fe deprivation in absence of Cd (-Fe/-Cd) reduces by 35% and 56% for 24 and 72 hours after sampling respectively when compared to control (Fig 1a) and reduction was observed more in Fe deprived plants under Cd exposure (+Fe/+Cd) by 68% and 82%. Whereas in Fe-sufficient plants in presence of Cd (+Fe/Cd) the Fe content was increased respectively to 41% and 43% 24 and 72 hours after treatment. Cd content was observed negligible in Fe deprived plants in absence of Cd (-Fe/Cd) whereas in presence of Cd in absence (-Fe/+Cd) or presence (+Fe/+Cd) of Fe (Fig 1b) the Cd content was increased significantly to large level.

Production of reactive oxygen species
Lipid peroxidation was studied in terms of TBARS and it was significantly increased under Fe-deficient condition (-Fe/-Cd), and was significantly more increased in Cd exposed under Fe deficient (-Fe+Cd) plants (Fig. 2) when compared with control plants (+Fe/Cd).

Changes in total chlorophyll, ascorbate and glutathione
Fe deficiency significantly decreased total chlorophyll content by 70% and 78% which was further more affected in –Fe/Cd treated plants by 33% and 34% 24 h and 72 h, respectively, after sampling (Fig 3a). Whereas in +Fe+Cd treated plants, chlorophyll content was increased significantly by 16.6% 24 h after sampling while remain unchanged in +Fe+Cd treated plants 72 h after sampling. Total ascorbate concentration was reduced by 10% and 8% 24 h and 72 h respectively after sampling in –Fe/Cd treated plants which was further more reduced in –Fe/Cd plants by 33% and 34% (Fig 3b). While in +Fe+Cd treated plants total ascorbate concentration was increased by 47% and 52% 24 h and 72 h, respectively. Total glutathione concentration in –Fe/Cd treated plants was decreased by 31% and 28% 24 h and 72 h after sampling, and was more affected by 54% and 63% in –Fe/Cd treated plants (Fig 3c), however, +Fe+Cd alleviated this effect and was increased by 47% and 52% after 24 h and 72 h respectively.

Enzyme assays
The response of all antioxidative enzymes examined in this study was in exquisite contrast between Fe-sufficient and Fe-deficient condition. Cd-exposure increased APX, SOD, CAT and GR activity under Fe-sufficient (Fig. 4a, 4b, 4c, 4d), while inversely decreased under Fe-deficient condition at all-time courses of measurement when compared with control. At 72 h of Cd-exposure, APX, SOD, CAT and GR activity was increased by 46%, 29%, 3% and 11% respectively under Fe-sufficient soil compared to those of control, but decreased by 31%, 66%, 48% and 35% respectively under Fe-deficient soil.

Proteomic analysis
Proteome map and peptide mass finger printing (PMF)
A proteomic analysis of leaf was performed to understand the defense-associated responses to Cd toxicity and the protective role of Fe in Vigna radiata. Proteomic data showed that some of the important primary metabolism pathways were strongly affected by Cd treatment (+Fe+Cd) particularly in Fe deficient plants (-Fe+Cd). Approximately 238 spots were reproducibly detected on 2D gels (Fig 5, representative image of 2D gel) of leaf proteins. Among these 40 protein spots were differentially expressed, analyzed by LC/MSMS. Eight of the differentially expressed spots (Fig. 6) were identified with sufficient confidence and were identified as oxygen evolving enhancer protein 1 (Spot S5), RuBisCO (Spot S6), oxygen evolving enhancer protein 2 (Spot S7), HSP70 (Spot S8), RubBP/oxygenase activase (Spot S9, S10), rubber elongation protein (Spot S11) and pathogenesis related protein (Spot S12). The ion score and matched peptide of the identified proteins are shown in Table 1. Cd exposure in Fe deficient plants caused a negative effect on light phase of photosynthesis via down regulation of oxygen evolving enhancer protein 1, 2, and RuBP. However an over expression of HSP70 and RubBP/oxygenase was observed in +Fe+Cd treated plants. On the other hand Fe deficiency lead to up regulation of the expression of Pathogenesis related protein and a rubber elongation factor protein. To further classify the proteins Map man ontology were used (Beven et al., 1998) which classifies the proteins as chloroplast precursor, photosynthetic pigment proteins, stress responsive proteins, protein destination and secondary metabolism proteins (Fig 7).

Discussion
The present study investigated that Cd negatively affect the growth and development of plants which in turn was alleviated by Fe nutrition. The present data showed that the degree of decrease in Fe content by Cd exposure was significantly increased by Fe supply (Fig 1), whereas total Cd content in was negligible in Fe deprived plants and increased significantly in Cd treated plants in presence or absence of Fe. The decrease in Fe accumulation in leaf tissues under Cd exposure might be due to inhibition of Fe uptake as a consequence of the reduction of phytosidrophores (PS) release in the roots (Astolfi et al., 2010; 2012). An enhanced accumulation of Cd content in Fe deprived or Fe sufficient plants under Cd exposure might be due to reduction phychelatins and metallothionines (Sànita di Toppì et al., 1999). The toxic effects of Cd in plants can be recognized by the induction of Oxidative stress leading to enhanced production of Reactive oxygen species (ROS) and toxic substances. Exposure to Cd elicited an oxidative burst in Vigna radiata indicated by an increase in the TBARS content (Fig 2). Particularly in Fe deficient plants the elevation in TBARS content was more pronounced in our experiment. Plants possess efficient systems for scavenging active oxygen species and eliminating toxic substances, alleviating their damage to the organism (Lee et al., 2009; Muneer et al., 2011; 2012). This may be due to formation of ROS (reactive oxygen species) in chloroplast and peroxisomes which leads to the formation of lipid radicals and reactive aldehydes due to removal of hydrogen from unsaturated fatty acids (Muneer et al., 2011; Muneer et al., 2012). Reactive oxygen species have been characterized as key factors in response of plants to both biotic and abiotic stresses (Hart et al., 1998). Our
<table>
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<th>peptides</th>
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study reflects that iron deficiency as well as cadmium stress impairs the plant ability to counter attack ROS. Further the presence of Cd under Fe deficiency enhanced the intensity of oxidative stress. Fe might help in controlling the formation of oxyradicals under both stresses because Fe is important for expression of proteins which leads to conversion of singlet oxygen into hydrogen peroxide and finally into water.

In contrast Fe deficiency (-Fe-Cd) decreased total chlorophyll, ascorbate and glutathione concentration which was more severe in –Fe+Cd plants (Fig 3). This could be attributed to the interference of Cadmium with chlorophyll formation (Qureshi et al., 2010). Apart from inhibition of biosynthetic enzymes of chlorophyll formation, the increased level of free radicals of fatty acids produced from polyunsaturated fatty acids due to the higher activity of lipooxygenase may also contribute to the decreased level of chlorophyll with Cd treatments (Somasekhararajah et al., 1992). Recent studies, however, suggest that the formation of light harvesting complex is disturbed in Cd treated leaves (Horvath et al., 1996), due to the inhibition of LHC (light harvesting complex) protein synthesis at transcriptional level. Also, the newly formed free chlorophyll undergoes a photooxidative breakdown as it has been reported Cd treated barley or oat leaves (Chugh et al., 1999). However, the results of this study could be interpreted as the effect of interference of Cd with Fe uptake by roots and also the replacement of Mg in chlorophylls (Chugh et al., 1999). The Fe deprivation and Cd exposure leads to reduction of glutathione and ascorbate levels which might be due to glutathione depletion which in turn leads to a subsequent reduction in the ascorbate–glutathione cycle (Gomes-Júnior et al., 2006). Cd severely affected the activities of enzymatic antioxidants viz., APX, SOD, CAT and GR decreased in Fe deficient plants (-Fe-Cd). This could be due to over production of ROS which leads to oxidative injury such as membrane lipid peroxidation, protein oxidation, enzyme inhibition and DNA and RNA damage (Qureshi et al., 2010; Lee et al., 2009). Superoxide dismutase, the first enzyme in the detoxifying process, converts superoxide radicals to H₂O₂ at a very fast rate (Gratão et al., 2005). Our experiment revealed -Fe-Cd treated plants showed a decline in SOD activity (Fig 4b). This could be due to de-limitation of iron as metal ligand for SOD protein to make it active and iron acquisition in terms of microsiderophore utilization by plants which in turn was more severe in –Fe+Cd treated plants. While in +Fe+Cd treated plants SOD was increased to some extent which in turn may be associated with an induction of genes of SOD by superoxide-mediated signal transduction (Fatima and Ahamad, 2005). CAT and APX are important enzymes in leguminous plants because they are all heme containing enzymes so their activities are likely to be affected by Fe-deficiency (Ratanart et al., 1987; Deakin et al., 2009). In absence of Fe (-Fe-Cd), APX activity was decreased and was more severe in –Fe+Cd treated plants (Fig 3a). This could be due to reduced apoplastic fluid as well as at intracellular level. The diminished APX activity under Fe deficiency has been also studied in leaves of sunflower (Ranieri et al., 2001) which showed that due to iron deprivation specific APX activity may result as a consequence of the high quest for iron from APX molecule because it contains in addition to the heme group, also a non-heme iron atom (Grover et al., 2000). However, in presence of Fe (+Fe+Cd), APX was increased by 30% during first 24 h, which clearly reflects recovery of Heme groups. Similarly catalase and GR were decreased in –Fe+Cd plants. However, Fe-sufficient plants (+Fe+Cd) were not much affected. The reduction in CAT and GR activity may be due to GSH depletion and a subsequent reduction in the ascorbate–glutathione cycle (Gomes-Júnior et al., 2006). Several proteomic approaches have been discussed in leguminous plants under different abiotic stresses (Larrainzar et al., 2007; Qureshi et al., 2010). Cd exposure in Fe deficient plants (-Fe+Cd) caused a negative effect on the light phase of photosynthesis via down regulation of oxygen evolving enhancer protein 1(Spot 5) and 2 (Spot 7) which have also been identified in Spinacia oleracea, Solnum lycopersicum, Solnum tubrum, Oriza sativa under salinity and other abiotic stresses. Vincent et al., (2007) identified this protein in Vitis vinefera under water deficiency and saline conditions and found that this protein was down-regulated. Our results also revealed that these proteins were down-regulated under Fe-deficiency caused oxidative stress in chloroplasts and peroxisomes and break-down of the genes which are responsible for the formation of these proteins This suggests that Fe is important for normal synthesis of the proteins and essential to combat cadmium stress. The carbon fixation and Calvin cycle also showed numerous key proteins including RuBisCO less abundant in Cd stress. This protein was also found to be under-expressed in Vitis vinefera under water deficiency and salinity conditions (Wienkoop et al., 2003). A down-regulation of this protein has also been found in Phaseolus vulgaris under abiotic stresses. In response to

Fig 1. Changes in the content of (a) Fe (b) Cd as affected by four Fe/Cd combined treatments: sufficient in Fe but absence of Cd (Fe+/Cd, control; white bars), deprived Fe and absence of Cd (−Fe−/Cd; dark grey bars), deprived Fe and presence of Cd (−Fe+/Cd; light grey bars), and sufficient Fe but Cd exposure (+Fe+/Cd; black bars) for 72 h. Vertical bars indicate ±S.E of the means for n = 3. Means denoted by the different letter are significantly different at p < 0.05 according to the Tukey’s studentized range test.
This suggests that PR protein, which is assumed protective function of this protein under salt stress which showed two proteins in higher abundance and were hypothesized that HSP70 play an active role in maintaining protein metabolism (Wienkoop et al., 2003). Besides the known induction by heat and cold stresses, it also plays an important role in grain development and germination (Agarwal et al., 2002). During stress conditions, proteins are unfolded or misfolded and HSP 70 might be necessary to ensure proper protein aggregation in the mitigating oxidative stress and proper development of legumes. The accumulation of HSP70 in the Cd exposed Fe-deficient plants is in contrast to the previous findings, but it could confer tolerance to other stress factors, like drought, which has not been tested yet in V. radiata. Spot S9 and S10 were over-expressed and were found as RuBP/oxygenase activase. This catalyzes both the carboxylation of RuBisCO, which is the initial step in photosynthetic carbon reduction. Photosynthetic protein reduction is associated with oxidative stress (Moreno et al., 1999). In rice leaves Rubisco under ozone stress condition were down regulated (Marocco et al., 2002). Cui et al., (2005) reported a reduced activity under drought stress in grapevine. In our study Rubisco was up-regulated by cadmium stress but in presence of iron which explains that there was accumulation of this protein thus indicating the CO2 fixation which may be severely affected in plants under iron deficiency particularly after exposure to cadmium. Spot S11 was over-expressed and was found as rubber elongation protein. This is the protein first identified from rubber tree; it is a long chain amino acid having molecular mass of 14,600 Daltons, and lacks four amino acid residues in lipid peroxidation (a) glutathione (b) as affected by four Fe/Cd combined treatments: sufficient in Fe but absence of Cd (Fe/+Cd, control; white bars), deprived Fe and absence of Cd (-Fe/-Cd); dark grey bars), deprived Fe and presence of Cd (-Fe/+Cd; light grey bars), and sufficient Fe but Cd exposure (+Fe/+Cd; black bars) for 72 h. Vertical bars indicate ±S.E of the means for n = 3. Means denoted by the different letter are significantly different at p < 0.05 according to the Tukey’s studentized range test.

Fig 3. Changes in (a) total chlorophyll (b) total ascorbate and (c) glutathione as affected by four Fe/Cd combined treatments: sufficient in Fe but absence of Cd (Fe/+Cd, control; white bars), deprived Fe and absence of Cd (-Fe/-Cd); dark grey bars), deprived Fe and presence of Cd (-Fe/+Cd; light grey bars), and sufficient Fe but Cd exposure (+Fe/+Cd; black bars) for 72 h. Vertical bars indicate ±S.E of the means for n = 3. Means denoted by the different letter are significantly different at p < 0.05 according to the Tukey’s studentized range test.

Other studies on HSP70 have been published on barley grains under salt stress which showed two proteins in higher abundance and were hypothesized that HSP70 play an active role in maintaining protein metabolism (Wienkoop et al., 2003). Besides the known induction by heat and cold stresses, it also plays an important role in grain development and germination (Agarwal et al., 2002). During stress conditions, proteins are unfolded or misfolded and HSP 70 might be necessary to ensure proper protein aggregation in the mitigating oxidative stress and proper development of legumes. The accumulation of HSP70 in the Cd exposed Fe-deficient plants is in contrast to the previous findings, but it could confer tolerance to other stress factors, like drought, which has not been tested yet in V. radiata. Spot S9 and S10 were over-expressed and were found as RuBP/oxygenase activase. This catalyzes both the carboxylation of RuBisCO, which is the initial step in photosynthetic carbon reduction. Photosynthetic protein reduction is associated with oxidative stress (Moreno et al., 1999). In rice leaves Rubisco under ozone stress condition were down regulated (Marocco et al., 2002). Cui et al., (2005) reported a reduced activity under drought stress in grapevine. In our study Rubisco was up-regulated by cadmium stress but in presence of iron which explains that there was accumulation of this protein thus indicating the CO2 fixation which may be severely affected in plants under iron deficiency particularly after exposure to cadmium. Spot S11 was over-expressed and was found as rubber elongation protein. This is the protein first identified from rubber tree; it is a long chain amino acid having molecular mass of 14,600 Daltons, and lacks four amino acid residues in lipid peroxidation (a) glutathione (b) as affected by four Fe/Cd combined treatments: sufficient in Fe but absence of Cd (Fe/+Cd, control; white bars), deprived Fe and absence of Cd (-Fe/-Cd); dark grey bars), deprived Fe and presence of Cd (-Fe/+Cd; light grey bars), and sufficient Fe but Cd exposure (+Fe/+Cd; black bars) for 72 h. Vertical bars indicate ±S.E of the means for n = 3. Means denoted by the different letter are significantly different at p < 0.05 according to the Tukey’s studentized range test.

Fig 2. Changes in lipid peroxidation (TBARS) in leaves as affected by four Fe/Cd combined treatments: sufficient in Fe but absence of Cd (Fe/+Cd, control; white bars), deprived Fe and absence of Cd (-Fe/-Cd); dark grey bars), deprived Fe and presence of Cd (-Fe/+Cd; light grey bars), and sufficient Fe but Cd exposure (+Fe/+Cd; black bars) for 72 h. Vertical bars indicate ±S.E of the means for n = 3. Means denoted by the different letter are significantly different at p < 0.05 according to the Tukey’s studentized range test.
genes in legumes. This can definitely provide more but
important information for plants with better
with-standing potential under stress and being more N₂-fixing
efficient.

Material and methods

Plant material and Fe/Cd treatment

Seeds of Vigna radiata L. var. Pusa vishal were obtained from
IARI (Indian Agricultural Research Institute, Pusa, New
Delhi, India). Surface sterilized seeds were sown in a plastic
pot containing 4 kg of experimental soils: i) iron-deficient soil
collected from the Agricultural Soil Science Unit, IARI
(Indian Agricultural Research Institute) and ii) normal loamy
soil. The seedlings were grown in a phytotron with day/night
mean temperature of 25°C / 22°C under light intensity of 300
µm m-2 s-1 with 14 h of photoperiod. For Cd treatment, 50 μM
CdCl₂ per plants in liquid form was fed to 30-d-old plants
grown on the Fe-sufficient as well Fe-deficient soils. Sampling was performed at 24 and 72 h after Cd-exposure.

Chemical analysis of Fe and Cd content

For the determination of Fe content, about 1 g of dried
sample was digested with 50% perchloric acid and concentrated
H₂SO₄ at 350°C for 5 h. The digested samples were then filtered and diluted to a proper volume. The elemental content was determined by an inductively coupled plasma optical emission spectrometry, (ICP-OES, Thermo Elemental - IRIS Advantage, USA). For determination of Cd (500 mg) from all four treatments was digested in acid mixture (HNO₃/ HCl, 2/1, v/v), filtered (Whatman no. 42), and then diluted to appropriate volume. The Cd contents in the solution were estimated in ppm using a flame atomic absorption spectrometer (Video11; Thermo Jarrell Ash Corporation, Franklin, MA, USA).

Lipid Peroxidation

The lipid peroxidation level was determined by measuring
the concentration of malondialdehyde (Heath and Parker
1968). One gram of fresh tissue was ground in 0.1 %
trichloroacetic acid (TCA) and centrifuged at 10,000 g for 5
min. The mixture containing 1ml of supernatant with 4 ml of
0.5 % thiobarbituric acid (TBA) was heated at 99 °C for 30
min, cooled and centrifuged at 5,000 rpm 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.

Biochemical Parameters

Chlorophyll content was estimated by the method of (Hiscox
and Israelstam, 1979). One g of fresh leaves were collected in
glass vials to which 10 ml DMSO were added and were kept
in an oven at 65 °C for complete leeching of pigments for 1 h.
Optical density was recorded at 480, 645, 520 and 663 nm.
The chlorophyll concentrations in mg fresh samples were
calculated using formulae given by Arnon (1949). Ascorbate
determination was performed by method of Law et al., (1983). Five
hundred milligram of ground tissues was homogenized in 2
ml of phosphate buffer (pH 7.4), centrifuged at 10,000 g.
Resulting supernatant was divided into two parts each of 400
µl in separate tube. The resulting supernatants were added with
2 ml of 10% TCA and 2 ml of 5M NaOH (sodium
hydroxide) and were again centrifuged for 2 min. The
resulting supernatant of one tube was then added with 0.1 ml
ethylmelamide (0.5%) and other part of supernatant from
another tube was added with 0.2 ml reaction buffer (pH 7.4)
and 0.1 ml of 10 mM DTT. Both tubes were incubated for 30
min at room temperature. After incubation the reaction was
terminated by adding 0.4 ml of 10% TCA, 0.4 ml of ortho-
phosphoric acid, 0.44 ml of 4% bipyridyl and 0.2 ml of 3%
FeCl₃. The tubes were then vortexed and incubated for 60
min at 37 °C and absorbance was recorded at 525 nm. The
expression of total ascorbate was determined in n mole g⁻¹
FW. Glutathione was estimated by the method of Anderson
(1985). Five hundred milligram of fresh tissues was
homogenized in 5% sulphosalicyclic acid and centrifuged at
10,000 g. The resulting supernatant was added to 0.6 ml of
reaction buffer (pH 7.2) containing 150 mM K₂HPO₄, 150
mM KH₂PO₄, 1% PVP, 1ml TritonX100, 0.2 mM EDTA and
40μl of 0.15% dithionitrobenzoic acid (DTNB). The
absorbance was recorded at 412 nm. To the same tube 50 µl
of 0.2 mm NADPH and 2 µl of 0.5 mM oxidized glutathione
were added. The reaction was allowed to run for 30 min at
room temperature and absorbance was recorded at 412 nm.
The expression of total glutathione was determined in n mole
g⁻¹ FW.

Enzyme assays

Ascorbate peroxidase (APX) activity was estimated by measuring the decrease in absorbance at 290 nm (extinction coefficient of absorbance 2.8 mM⁻¹ cm⁻¹ for ascorbate) according to Nakano et al. (2006). One hundred milligram of fresh tissue were extracted in 100 mM K-phosphate (pH 7.0),
and then centrifuged at 15,000 g for 10 min. One unit of
enzyme was expressed as the amount necessary to decom-
Fig 4. Changes in antioxidant enzyme activities: (a) ascorbate
peroxidase (APX), (b) superoxide dismutate (SOD), (c)
catalase (CAT) and (d) glutathione reductase (GR) as
affected by four Fe/Cd combined treatments: sufficient in Fe
but absence of Cd (Fe+/Cd; control; white bars), deprived Fe
and presence of Cd (-Fe/-Cd); dark grey bars), and sufficient
Fe but Cd exposure (+Fe/+Cd; black bars) for 72 h. Vertical
bars indicate ±S.E of the means for n = 3. Means denoted by
the different letter are significantly different at p < 0.05
according to the Tukey’s studentized range test.
Proteins were separated in the first dimension on a nonlinear IPG strips, pH 4-7 and in second dimension on 12% polyacrylamide SDS-gel. Quantitative image represents differentially expressed proteins. 

Proteomic analysis

Protein extraction

One gram of leaf tissues was homogenized in phosphate buffer (pH 7.6) containing 40 mM tris, 0.07% βME (beta mercapto ethanol), 2% PVP (Polyvinylpyrrolidone) and 1% TritonX100 at 4°C using chilled pestle and mortar on ice. The homogenates were centrifuged at 15,000 rpm for 15 min. and proteins were precipitated with 10% TCA/acetone overnight at -20°C. The resultant precipitate was centrifuged at 15,000 rpm for 15 min and washed with 80% chilled acetone containing 2mM EDTA and 0.07% βME. The proteins pellet was solubilized in solubilisation buffer containing 9M urea, 2M thiourea, 4% CHAPS, 2% TritonX100, 50mM DTT and 0.2% ampholine (pH4-7).

Two-dimensional polyacrylamide gel electrophoresis

About 300-400 μg proteins were separated by 2-DE in the first dimension by isoelectric focussing on 11 cm IPG strip (pH 4-7) and the second dimension by SDS-PAGE on Protean II unit (Bio-Rad Hercules, USA) according to method given

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About 300-400 μg proteins were separated by 2-DE in the first dimension by isoelectric focussing on 11 cm IPG strip (pH 4-7) and the second dimension by SDS-PAGE on Protean II unit (Bio-Rad Hercules, USA) according to method given
by Qureshi et al., (2010). For first dimension the isoelectric focusing gel consisted of 8 M urea, 3.5% polyacrylamide, 2% Nonidet P-40, 2% Bio-Lyte, ammonium persulfate and tetramethylethylenediamine. Electrophoresis was carried out at 200 volt for 30 min, followed by 400 V for 16 h and 600 V for 1 h. After separation, SDS-PAGE as the 2nd dimension was performed using 12% polyacrylamide gel. The gels were stained with Coomassie brilliant blue R.

**Image acquisition and data analysis**

Three replicates gels from each treatment were used for image acquisition and data analysis. Spot detection, spot measurement, back ground subtraction and spot matching were performed using PD-Quest software (Bio-Rad Hercules, V8.0.1, CA, USA) in automatic spot detection mode to review the annotations of spots statistically using one way ANOVA analysis automatically by the software. One of the gels was selected as a reference gel for spot matching. The amount of protein spot was expressed as the volume of spot, which was defined as the sum of intensity of all the pixels that make up the spot. In order to correct variability derived from CBB staining and to reflect the quantitative variation in intensity of protein spots, the spot volumes were normalized as a percentage of the total volume in all spots in the gel. The resulting data from image analysis were referred to PD-Quest software for querying protein spots that showed quantitative and qualitative variation. The $pI$ and $M_r$ of each protein were determined using 2-DE markers (Sigma).

**Protein digestion by trypsin**

The differentially expressed protein spots were excised manually from the gels (Shevchenko et al., 2007), and washed with distilled water three times by a centrifugation for 1,100 rpm at 22-24°C for 10 min. The protein spots were treated with 50% acetonitrile after washing and centrifuged for 10 min at 22-24°C at 1,100 rpm. The protein spots were then treated with 1M DTT and 50 mM ammonium carbonate, and incubated for 45 min at 38°C. After incubation protein spots were treated with 0.1 M IA and 55 mM ammonium carbonate, centrifuged again for 30 min at 22-24°C at 1,100 rpm and were vacuum dried for 15 min. The protein spot was then treated with 30 μl of 0.5 μg of trypsin and were kept at 37°C overnight before running on MALDI-TOF for peptide identification.

**LC/MSMS analysis**

Nanoflow electrospray ionization tandem mass spectrometric analysis of peptide samples was carried out using LTQ-OrbitrapVelos (Thermo Scientific, Bremen, Germany) interfaced with Agilent’s 1200 Series nanoflow LC system. The chromatographic capillary columns used were packed with Magic C18 AQ (particle size 5 μm, pore size 100A; Michrom Bioresources, Auburn, CA, USA) reversed phase material in 100% ACN at a pressure of 1000 psi. The peptide sample from each tube containing protein spot was enriched using a trap column (75 μm x 2 cm) at a flow rate of 3 μl/min and separated on an analytical column (75 μm x 10 cm) at a flow rate of 350 nl/min. The peptides were eluted using a linear gradient of 7-30% ACN over 65 min. Mass spectrometric analysis was carried out in a data dependent manner with full scans acquired using the Orbitrap mass analyzer at a mass resolution of 60,000 at 400 m/z. For each MS cycle, twenty most intense precursor ions from a survey scan were selected for MS/MS and fragmentation detected at a mass resolution of 15,000 at m/z 400. The fragmentation was carried out using higher-energy collision dissociation (HCD) as the activation method with 40% normalized collision energy. The ions selected for fragmentation were excluded for 30 sec. The automatic gain control for full FT MS was set to 1 million ions and for FT MS/MS was set to 0.1 million ions with a maximum time of accumulation of 500 ms, respectively. For accurate mass measurements, the lock mass option was enabled. The MS data obtained were analyzed by using mowers M/Z and MS/MS search was carried out using search algorithm against the NCBI. Search parameter included trypsin as the enzyme with 1 missed cleavage.

**Statistical analysis**

The experiment was carried out in randomized block design with three replicates. The mean and standard deviation was done for bar diagrams with percent variation. One way analysis of variance (ANOVA) was done with all the data to confirm the variability of the data and validity of results.

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**References**


