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

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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 (2^{nd} dimensional), PMF (peptide mass finger printing), RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase), RUBP (ribulose-bis phosphate), 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 (Helliwel et al., 1999). Ultimately plant growth and development such as root/shoot elongation and biomass accumulation is affected. Fe is known to be essential for many physiological and biochemical processes including photosynthesis, respiration, DNA synthesis and N_2-fixation. The legume-rhizobia symbiosis is particularly sensitive to iron deficiency (Tang et al., 1991). Fe deficiency has been reported worldwide in a number of agriculturally important crops, with deficiency usually occurring in alkaline soils and being attributed to the lowered availability of Fe at high pH in calcareous soils (Qureshi et al., 2010; Muneer et al., 2011). Fe-deficiency elicits a large stimulation of Cd influx in plants (Qadir et al., 2004) and adversely affects them at different levels. Fe-deficiency plays a role in enhanced heavy metal absorption which includes induction of divalent cations such as Cd^{2+} and Zn^{2+}, probably due to random substituting of Cd with other essential ions (e.g., Zn, Fe or Ca) into the prosthetic groups of some proteins. Hence, Fe-deficiency during Cd-stress may lead to severe stress in plants by altering the activities of some important enzymes and cofactors. Although the toxic effects of Cd and interaction between Cd and Fe have been widely documented in several oil seed plants (Qureshi et al., 2010; Muneer et al., 2011) less information have been reported for leguminous plants such as *Vigna radiata*. In this study we hypothesize that Fe nutrition modulates ascorbate-glutathione cycle and proteome under Cd stress in leaves of

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 alleviate 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), RuBP/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 RuBP/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 phyochelatins and metalothionines (Sànita di Toppi 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 1. Identification of differentially expressed proteins from leaves of *Vigna radiata* by LC/MS-MS.

spot no	Acession no.	Homology	peptides	score	mr/*pI*	species
S5	gi/131384	Oxygen evolving enhancer protein 1	79	189	35100/6.25	*Pisum sativum*
	gi/147791852	Oxygen evolving enhancer protein 1	80	109	33441/5.87	*Vitis vinifera*
	gi/115436780	Oxygen evolving enhancer protein 1	56	107	35068/6.10	*Oryza sativa*
	gi/119952178	Oxygen evolving enhancer protein 1	82	80	35344/6.48	*Bruguiera gymnorhiza*
S6	gi/131974	RuBisCO	30	110	52139/6.23	*Harpagophytum qrandidieri*
S7	gi|266856	Oxygen evolving enhancer protein 2	50	117	27946/8.28	*Solnum lycopersium*
	gi/266856	Oxygen evolving enhancer protein 2	17	122	28614/8.58	*Spinacia oleracea*
	gi/11134035	Oxygen evolving enhancer protein 2	60	107	28158/8.27	*Solnum tuberosum*
S8	gi/1143427	Heat Shock protein 70	71	220	75480/5.15	*Cucumis sativa*
	gi/115487998	Heat Shock protein 70	43	178	74269/5.11	*Oryza sativa*
	gi/145388994	Heat Shock protein 70	30	159	73137/5.23	*Pennisetum string*
	gi/399942	Heat Shock protein 70	30	155	75583/5.22	*Pisum sativum*
	gi/116061511	Heat Shock protein 70	33	127	60098/6.59	*Ostreococcus tauri*
S9	gi|68565782	RuBP/oxygenase activase	73	192	52450/6.38	*Larrea tridentata*
	gi|29429152	RuBP/oxygenase activase	54	189	48079/6.29	*zea mays*
	gi|125535371	RuBP/oxygenase activase	37	176	40176/8.48	*Oryza sativa*
S10	gi|3687652	RuBP/oxygenase activase	48	162	40176/8.48	*Datisca glomerata*
	gi|15450379	RuBP/oxygenase activase	33	102	41045/7.59	*Arabidopsis thaliana*
	gi|10720248	RuBP/oxygenase activase	39	85	52405/5.69	*Phaseolus vulgaris*
S11	……..	Rubber elongation factor protein	11	48	48342/8.19	*Hevea brasiliensis*
S12	gi|147811098	PR protein	8	53	16518/4.83	*Vitis venifera*
	gi|60418924	PR protein	55	288	14582/5.04	*Vigna radiata*
	gi|130829	PR protein	48	239	16361/4.72	*Phaseolus vulgaris*

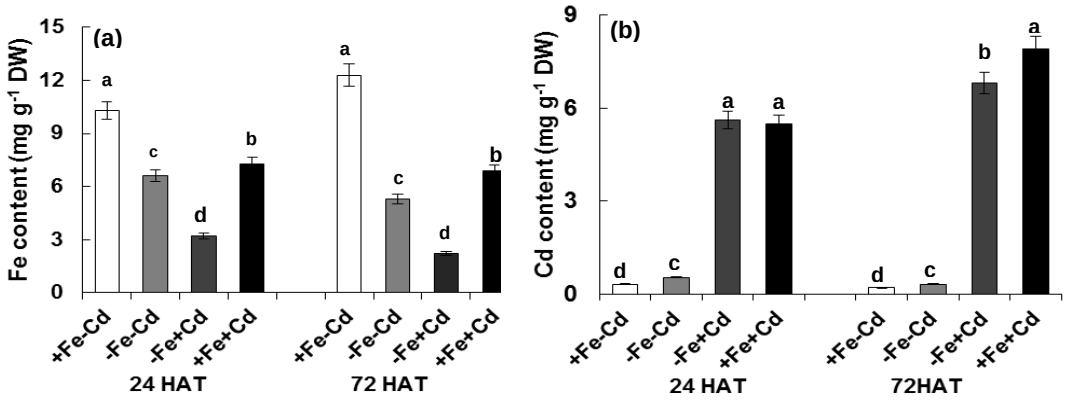

Fig 1. Changes in the content of (a) Fe (b) Cd as affected by four Fe/Cd combined treatments: sufficient in Fe but absence of Cd (Fe/+Cd, control; white bars), deprived Fe and absence of Cd (-Fe/-Cd); dark grey bars), deprived Fe and presence of Cd (-Fe/-Cd; light grey bars), and sufficient Fe but Cd 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.

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 lipoxygenase may also contribute to the decreased level of chlorophyll with Cd treatments (Somashekaraiah 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 photoxidative 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 H_2O_2 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 tubrusum, Oryza 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 vinifera* 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

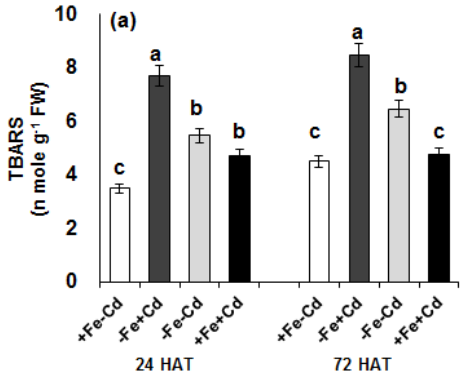

Fig 2. Changes in lipid peroxidation (TBARS) in leaves as affected by four Fe/Cd combined treatments: sufficient in Fe but absence of Cd (Fe/+Cd, control; white bars), deprived Fe and absence of Cd (-Fe/-Cd); dark grey bars), deprived Fe and presence of Cd (-Fe/-Cd; light grey bars), and sufficient Fe 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.

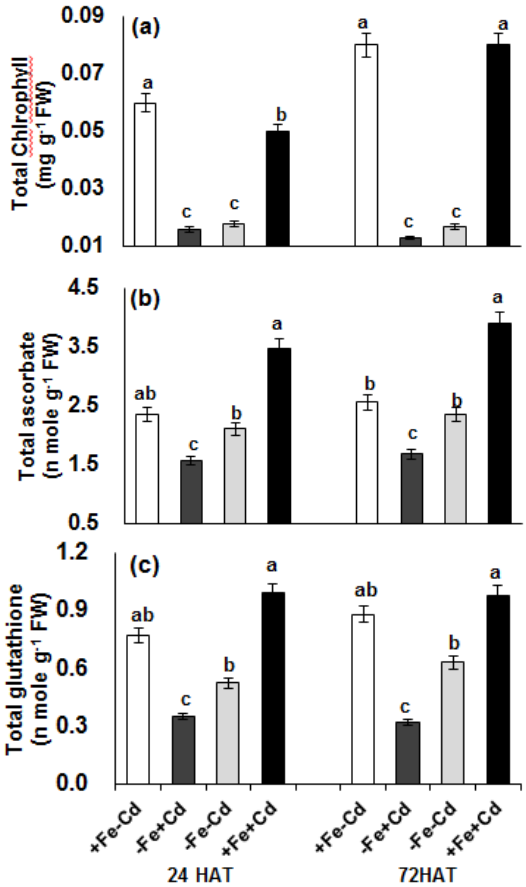

Fig 3. Changes in (a) total chlorophyll (b) total ascorbate and (c) glutathione (c) as affected by four Fe/Cd combined treatments: sufficient in Fe but absence of Cd (Fe/+Cd, control; white bars), deprived Fe and absence of Cd (-Fe/-Cd); dark grey bars), deprived Fe and presence of Cd (-Fe/-Cd; light 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.

abiotic stress plants are induced to express a set of genes including heat shock proteins (Hsps) (Georgiadis et al., 1992; Hendrick et al., 1993), a number of them are known as molecular chaperons (Boston et al., 1996; Lin et al., 2003; Wang et al., 2010). In our study HSP70 (Spot 8) were induced in response to Cd-stress particularly in Fe deficient conditions. Several studies have been reported on HSP70 which observed their involvement in heat stress initially, and various biotic and abiotic stresses later on (Sung et al., 2001). 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 the 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 (Maroco 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 CO_2 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 cysteine, methionine, histidine and tryptophan (Okushima et al., 2000). This protein has not been identified earlier in leaves of leguminous plants. But our results showed the expression of this protein under Cd-stress. A pathogenesis related protein (Spot S12), was also found to be over-expressed under Cd-stress in leaves of *V. radiata*. These proteins are defined as plant proteins that are induced in pathological or related situations (Pratt et al., 2003) and act as antifungal protein in leguminous plants. In our study PR protein was up regulated showing the highest score of 289 and maximum sequence coverage of 45%. In barley it has been shown that PR proteins are involved in the organization of the Hsp70/Hsp90 multiprotein complex (Swaney et al., 2008). Recently, a protein with homology to F23N19.10 has been detected as highly enriched in trichomes of tobacco leaves, as compared to the remaining leaf tissue, and authors assumed protective function of this protein (Wan et al., 2002). Possible roles of the identified protein from barley roots include protein stabilization and turnover as well as HSP-involved signal transduction processes (Wang et al., 2002; Winekoop et al., 2003). This suggests that PR protein play a significant role to combat cadmium stress in leguminous plants. Taking together all the data it suggests that Fe deficiency under Cd exposure (-Fe+Cd) caused severe oxidative stress in leaves of *Vigna radiata* which is alleviated by Fe. Further combined effect of Fe and Cd stressed conditions in leguminous plants may provide the identification of symbiosome proteins and their encoding

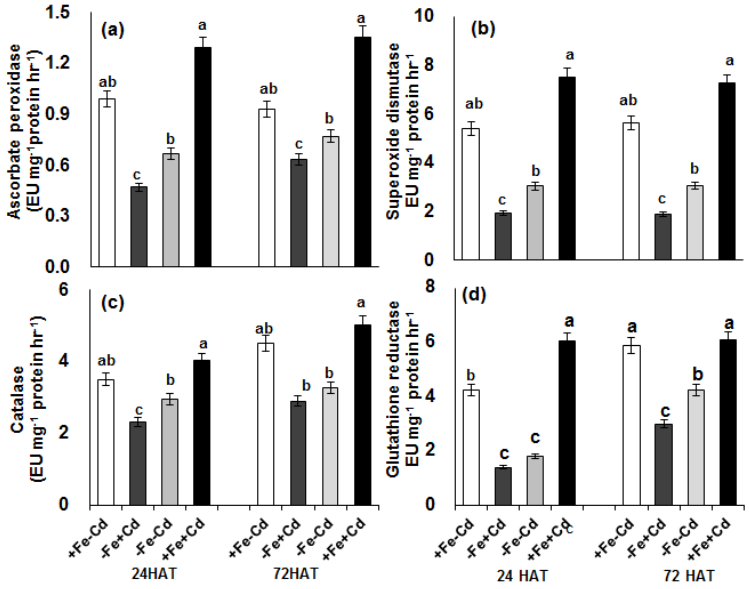

Fig 4. Changes in antioxidant enzyme activities; (a) ascorbate peroxidase (APX), (b) superoxide dismutase (SOD), (c) catalase (CAT) and (d) glutathione reductase (GR) as affected by four Fe/Cd combined treatments: sufficient in Fe but absence of Cd (Fe/+Cd, control; white bars), deprived Fe and absence 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 development of plants with better with-standing potential under stress and being more N_2-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 $CdCl_2$ 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 H_2SO_4 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 (HNO_3/ 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 Israclstam, 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 ^{0}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 was determined by the method of Law et al., (1983). Five hundred milligram of grounded 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% $FeCl_3$. The tubes were then vortexed and incubated for 60 min at 37 ^{o}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% sulphosalicylic 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 K_2HPO_4, 150 mM KH_2PO_4, 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 peroxidise (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-

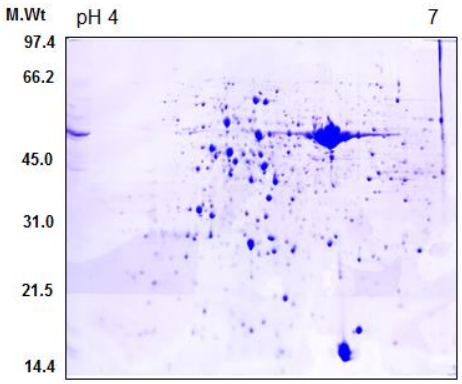

Fig 5. 2D references images of four Fe/Cd combined treatments as affected by four Fe/Cd combined treatments in leaves of *Vigna radiata*: sufficient in Fe but absence of Cd (Fe/+Cd, control), deprived Fe and absence of Cd (-Fe/-Cd), deprived Fe and presence of Cd (-Fe/-Cd), and sufficient Fe but Cd exposure (+Fe/+Cd) for 72 h. Proteins were separated in the first dimension on a nonlinear IPG strips, pH 4-7 and in 2nd dimension on 12% polyacrylamide SDS-gel. Quantitative image represents differentially expressed proteins.

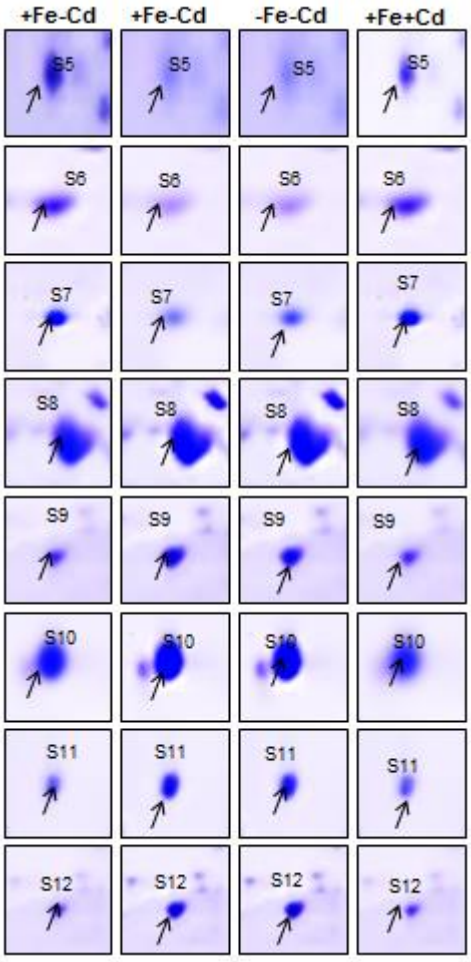

Fig 6. Enlarged expressions of differentially expressed proteins identified by LC/MSMS of four Fe/Cd combined treatments as affected by four Fe/Cd combined treatments in leaves of *Vigna radiata*: sufficient in Fe but absence of Cd (Fe/+Cd, control), deprived Fe and absence of Cd (-Fe/-Cd), deprived Fe and presence of Cd (-Fe/-Cd), and sufficient Fe but Cd exposure (+Fe/+Cd) for 72 h.

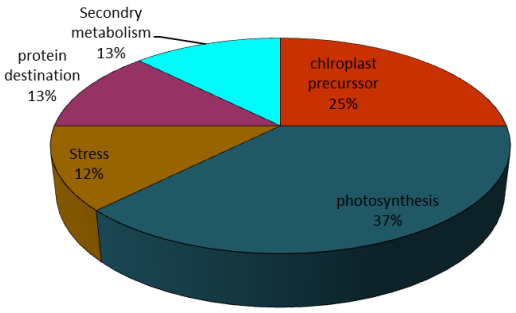

Fig 7. Functional classification of identified proteins from leaves analyzed by LC/MS-MS using Map Man ontology as described by Beven et al (1998).

pose 1 µmole of ascorbate per min. Superoxide dismutase (SOD) activity was determined by the method of Dhindsa et al. (1981) with minor modifications. Fresh tissues (200 mg) were extracted in phosphate buffer (pH 7.3) and centrifuged at 15,000 g. SOD activity in the supernatant was assayed by its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT) by reading the absorbance at 560 nm. One unit of enzyme activity was defined as the amount of enzyme required to inhibit 50 % of the NBT photo reduction in comparison with tubes lacking the plant extract. Catalase (CAT) activity was determined by the method of (Aebi et al. 1984). About 500 mg of grounded tissues were extracted in phosphate buffer (pH 7.3), centrifuged at 15,000 g for 20 min. The decrease in absorbance at 240 nm was recorded as a result of H_2O_2 degradation (extinction coefficient of 36 mM^{-1} cm^{-1}). One unit of enzyme determines the amount necessary to decompose 1 µm of H_2O_2 per min. Glutathione reductase (GR) activity was determined by the method of Rao et al. (1992). About 500 mg of fresh tissues were extracted in phosphate buffer (pH 7.0), centrifuged at 15,000 g for 10 min. The supernatant was immediately assayed for GR activity through glutathione-dependent oxidation of NADPH at 340 nm. One unit of enzyme determines its amount necessary to decompose 1 µmole of NADPH per min.

Proteomic analysis

Protein extraction

One gram of leaf tissues was homogenized in phosphate buffer (pH 7.6) containing 40 mM tris, 0.07% ßME (beta mercapto ethanol), 2% PVP (Polyvinylpyrrolidone) and 1% TritonX100 at 4ºC 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 -20ºC. The resultant precipitate was centrifuged at 15,000 rpm for 15 min and washed with 80% chilled acetone containing 2mM EDTA and 0.07% ßME. 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 (*pI* 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 tetramethylenediamine. 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 22-24ºC for 10 min. The protein spots were treated with 50% acetonitrile after washing and centrifuged for 10 min at 22-24ºC at 1,100 rpm. The protein spots were then treated with 1M DTT and 50 mM ammonium carbonate, and incubated for 45 min at 38ºC. After incubation protein spots were treated with 0.1 M IAA and 55 mM ammonium carbonate, centrifuged again for 30 min at 22-24ºC 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 37ºC 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 100Å; 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 × 2 cm) at a flow rate of 3 µl/min and separated on an analytical column (75 µm × 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 movers 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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