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Root Iron Localization and Proteomic Impairment in Anaerobic Rice Cultivars Exposed to Excess Iron

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Authors' contributions

This work was carried out in collaboration between all authors. Author TS designed the study, developed the conceptual framework, manage experimental analyses of the study, wrote the protocol and wrote the first draft of the manuscript. Author AB performed the statistical analysis. Authors RS and JB managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Plant faces iron (Fe) toxicity when the concentration of this mineral is high in soils under anaerobic conditions. Excess soil Fe^{2+} may cause severe impairment in rice photosynthesis, morphological parameters, and may induce oxidative damage with significant alteration in protein profiles. Present study aimed to investigate the extension of oxidative stress on exposure to excess Fe^{2+} ion and its effects on the rate of photosynthesis and modifications in protein profiles of rice cultivars with differential sensitivity. A pot experiment was conducted with three *Sali* rice cultivars, one conventional tolerant cultivar Mahsuri and two iron sensitive varieties Siyal Sali and Ranjit. Constant waterlog environment with four different Fe^{2+} doses +100 ppm, +200 ppm and +300 ppm treatments and a control without external Fe, were executed. Mahsuri displayed well adaptation to iron overload recording superior morphological parameters with better photosynthetic activity compared to Fe intolerant varieties. The SDS-PAGE results of leaf protein showed that Mahsuri had greater numbers of intense bands indicating more leaf proteins accumulation in all treatments. In contrast Ranjit and Siyal Sali expressed only few weak bands when supplemented with higher Fe^{2+} doses. Thus proteomic comparison between tolerant and sensitive cultivars after iron overload

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provides insight into the transcriptional regulation of the variety to tolerance response. These findings establish the foundations of introducing iron tolerance into farmers' friendly rice cultivars triggering better nutritional values.

Keywords: Iron toxicity; Oryza sativa; oxidative stress; photosynthesis, protein profile.

ABBREVIATIONS

GF	*: Grain Filling,*
kDa	*: Kilo Dalton,*
MW	*: Molecular weight,*
PI	*: Panicle initiation,*
ROS	*: Reactive Oxygen Species,*
SOD	*: Superoxide Dismutase.*

1. INTRODUCTION

Fe is an essential element in plants and involves in many physiological processes, but it becomes toxic when supplemented in excess. In plant tissues, Fe^{2+} participates in several cellular processes, such as respiration, chlorophyll biosynthesis and photosynthetic electron transport [1].It is a major constituent for the cell redox systems like heme-proteins, Fe-S proteins like feredoxin, aconitase and superoxide dismutase (SOD) [1]. The Fe (II)/Fe (III) redox couple plays an important role in plant growth by enhancing the enzymatic redox reactions [2]. Higher concentration of Fe^{2+} ion within plant cell may accelerate many redox reactions where Fe^{2+} acts as an electron donor [3].

In anaerobic soils environment Fe is reduced to its soluble form Fe^{2+} and can be taken up by rice plants. In waterlog environment and under low pH condition, the reduction of Fe^{3+} to Fe^{2+} become more significant. Higher concentration of ferrous ions in soil solution enhances its rate of adsorption at the root periphery and finally into the bulk of the plant which may cause oxidative damage either through the generation reactive oxygen species called true Fe toxicity or by impeding the uptake of other nutrients [4, 5]. Hence excess soil iron is a serious constraint to low land rice cultivation in terms of growth to grain quality components of the plants. Elevated amount of Fe^{2+} may cause several nutritional disorders [5, 6, 7] that affect yield attributing parameters and grain quality components of rice plant [8]. The generation of reactive oxygen species and extent of their toxic effect depends on the presence of Fenton's catalyst, such as ferrous ions, which give rise to extremely reactive OH^{-}ion and hydroxyl free radical with H_2O_2 and O^{2-} [9]. The reactive oxygen species cause irreversible damage to membrane lipids, proteins and nucleic acids. They oxidized chlorophyll and subsequently reduced leaf photosynthesis [10,11], thereby lead to yield reduction [8].

Although numerous literatures reported the mechanisms that control the iron uptake behaviour or those involved in iron translocation throughout the plant, iron toxicity in rice and varietal selection still needs further study in regional level. In the present investigation, an attempt has been made to focus on root iron localization and oxidative damage in rice protein profile caused by iron overload in anaerobic environment. Titabor is a famous rice growing area of North – Eastern India. In waterlog soil, Fe toxicity is a major constraint for rice growth in this area. To check the oxidative damage in leaf protein profile under higher iron concentration, we chose a package of three rice cultivars, grown popularly in *Sali* rice season in Titabor. Ranjit and Siyal Sali are two iron sensitive varieties and Mahsuri, a tolerant rice variety to higher soil iron concentration [7,6,8]. Although the various physiological and biochemical analysis have been investigated to identify the effects of iron overload on rice and proposed different mechanism towards iron tolerant cultivars, the protein profile study has remained limited on effect of iron overload in rice plants. In this research we aimed to investigate the banding pattern of different protein molecules and their comparative study that may be a competent approach to assess the oxidative stress caused by iron overload on different cultivars. The study also aimed to explore the oxidative damage triggered by Fe toxicity on some morphometric parameters and photosynthetic process in rice cultivars with differential sensitivity as stress indicators.

2. MATERIALS AND METHODS

A pot experiment was conducted during monsoon rice season in the year 2015-2016 with three rice (Oryza sativa-L) cultivars viz. Mahsuri, Ranjit (high yielding varieties) and Siyal Sali (traditional tall variety). Artificial Fe toxic environments in the experimental pots were developed with soils collected from a rice field located at Titabor (soil type - sandy clay loam, total soil iron 345 ppm and soil pH 5.4), a well known rice growing area in North- Eastern India. The rice seedlings were grown in the pots in four different levels of Fe^{2+} like control (+0 ppm external Fe^{2+}), +100 ppm, +200 ppm and +300 ppm Fe^{2+} in the form of $FeSO_4.7H_2O$. Doses were added in the pots once in a week after transplanting up to flowering stage maintaining uniform waterlogged environment. Treatments were replicated four times in a randomized block design. All the reagents used in the analysis are of analytical grade (Merck GR).

2.1 Morphological Variables

Some morphological parameters like number of tillers and leaves per pot, total number of panicles and fraction of sterile panicles, panicle length and filled grains per panicles were recorded by using standard methods [12] as visual symptoms to document the differential impact of iron stress on the chosen varieties.

2.2 Rate of Photosynthesis

Leaf relative greenness was measured by LI-6400XT Portable Photosynthesis System. Four readings were averaged to represent the mean chlorophyll index value of each replicate. All non-invasive sampling was performed on a part of the leaf that was equivalent to one-third the length of the entire leaf.

2.3 Perls' Stain for Ferric Iron in Root

Four randomly collected root samples from each of the three varieties grown in four different Fe^{2+} concentrations were cut into approximately 50-mm sections with a Spencer type microtome (MEDIMEAS MRM 1120A) and treated with Perls' stain according to established methods for mammalian tissues [13]. Briefly, equal amounts of solutions of 4% (v/v) HCl and 4% (w/v) potassium ferrocyanide were mixed immediately prior to use. Roots were collected before harvesting and carefully cleaned and washed with very dilute HCl (2%) and double distilled water. Finally roots were dried from alcoholic environment and Perls' staining was observed immediately in root cross-sections, after staining samples were fixed in 4% (w/v) para formaldehyde and imbedded in 4% (w/v) low-melt agarose.

2.4 Protein Quantification and One Dimensional SDS-PAGE

Total soluble proteins in the leaf tissues were estimated in Grain Filling stage by standard method [14]. Total protein profile analysis was conducted to identify the proteomic response of the cultivars to iron overload. Protein samples were stored at -20°C and 20 µg of each sample was analyzed by SDS-PAGE, according to Laemmli's method [15].

To study the leaf protein profile, unidirectional SDS-PAGE was carried out in a mini vertical system with 12% separating gel and 5% stacking gel. The protein contents obtained from 1g dry leaf sample of each treatment were loaded in sample wells along with 10 µl sample buffer containing bromophenol blue as tracking dye. A medium range (Molecular weight ranges 14 — 95 kDa as shown in Fig. 1) protein marker (from srl chemicals Research Laboratories Pvt. Ltd) was also incorporated into the gel to compare the molecular weight of the bands shown by the three cultivars with contrasting levels of tolerance to Fe toxicity. Total protein electrophoresis results were documented in a digital photograph.

2.5 Statistical Analysis

Statistical analyses of experimental data were carried out by using ANOVA (SPSS software). Analysis of variance was carried out to test the significance of treatment effect. F-test, coefficient of variance and critical difference were calculated by standard method [16].

Mid-Range _Protein Marker: 14-95 kDa
(Code: 38220090, BLM003)
Bromophenol Blue as the tracking dye.

Purified proteins	molecular weight
Lysozyme	14 kDa
Trypsin Inhibitor	20 kDa
Chymotrypsinogen A	25 kDa
L-Lactate dehydrogenase	35 kDa
Ovalbumin	43 kDa
Urease	47 kDa(faint band), 95 kDa
Bovine Serum Albumin	66 kDa

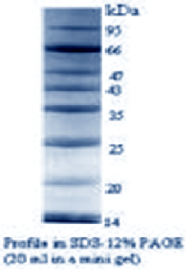


Fig. 1. Identity of protein marker

3. RESULTS AND DISCUSSION

3.1 Photosynthesis

The rate of photosynthesis in PI stage is higher than the GF stage for all the cultivars. A significant varietal factor was observed in leaf photosynthetic process when plants were subjected to different iron levels. Increases in soil iron concentrations due to Fe^{2+} treatments led to changes in rate of photosynthesis in Ranjit and Siyal Sali more significantly compared to Mahsuri. The deleterious effect of higher iron on photosynthetic rate of Ranjit and Siyal Sali became more pronounced at 300 ppm Fe^{2+} concentrations (Fig. 2). These two cultivars showed similar decreasing trend on the rate of photosynthesis in PI stage as well as in GF stage. However, variety Mahsuri showed higher photosynthetic rate in both growth stages indicating its better response to iron overload.

Pereira et al. [17] has already reported that iron overload causes nutritional disorders and severe impairment in rice photosynthesis. Evidently rice cultivars differ widely in their ability to tolerate excess iron. In present investigation, increase in soil iron concentration by supplementation of Fe^{2+} ions showed distinct reduction in rate of photosynthesis in Ranjit and Siyal Sali. A sharp drop in the rate of photosynthesis in Ranjit and Siyal Sali can be attributed to both stomatal and non-stomatal limitations through biochemical and photochemical impairment after severe stress. Supplementation of higher iron to the growth medium accelerates the formation of ROS through Haber- Fenton's reactions that render the oxidative damage to leaf greenness pigments and intensify the number of bronzed leaves in iron sensitive varieties [7]. Pinto et al [11] proposed that light energy partitioning impairment and oxidative damage which is more severe in sensitive cultivars, may take place before the non-stomatal limitation because of the Fe overload in the roots. Mahsuri recorded better photosynthesis activity even at 300 ppm Fe^{2+} might be due to lower accumulation of shoot iron. These results are consistent with the findings of Saikia and Bhuyan [6] who observed lower shoot iron concentration in Mahsuri compared to other two varieties and proposed the iron avoidance mechanism through lesser iron translocation from root to shoot that operates in Mahsuri. Pinto et al, [11] also observed similar result in their experiment that the reduced pigment index after seven days of Fe overload for the sensitive BR IRGA 409 cultivar compared to EPAGRI 107 is related to degradation that is mediated by oxidative stress. The resistance to excess iron toxicity exhibited by the EPAGRI 107 cultivar may involve a mechanism of avoidance, which prevents high levels of iron in shoots. They proposed that the higher sensitivity to excess iron in some rice cultivars is caused by the severe impairment of photosynthesis by non-stomatal limitation as triggered by oxidative damage and its consequent inability to reduce excess energy by regulated dissipation.

3.2 Iron Localization in Roots

In the present investigation, we employed a qualitative assess of iron localization in the root tissue by iron staining process. Perls' stain is an acidic solution of potassium ferrocyanide, which reacts with ferric iron to form an insoluble blue precipitate of ferric ferrocyanide. The insoluble nature of the precipitate formed indicates that the blue colour will not diffuse after staining and is a precise reflection of iron localization in living tissue.

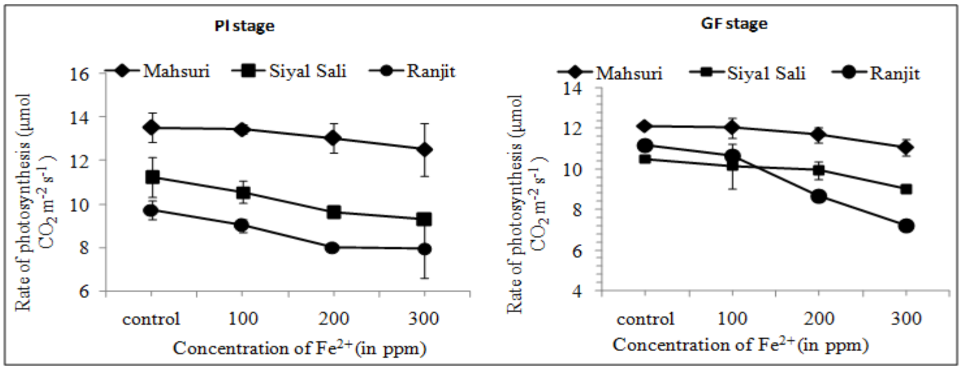


Fig. 2. Rate of photosynthesis (µmol CO_2 $m^{-2}s^{-1}$) in Mahsuri, Siyal Sali and Ranjit at different growth stages under different Fe^{2+} levels

The vertical bars represent the standard error

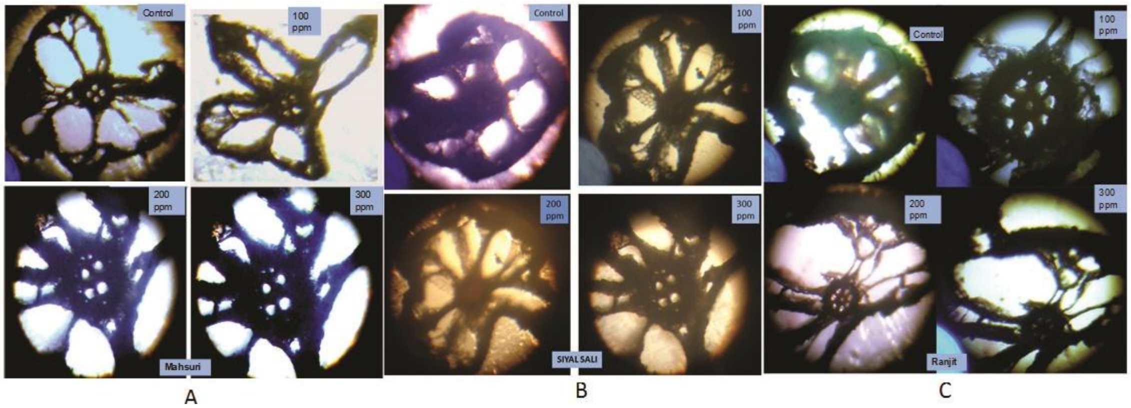


Fig. 3. Root section pictures displaying iron localization in the roots of three rice cultivars at different Fe^{2+} levels. Fig. 3 (A) represents iron localization in the roots of Mahsuri with distinct blue Perls' stain at 200 ppm and 300 ppm Fe^{2+} concentrations. Fig. 3 (B) and (C) represents iron localization in the roots of Siyal Sali and Ranjit

As shown in Fig. 3, iron is accumulated to high levels in the central vascular tube of Mahsuri roots. This is in contrast to Ranjit and Siyal Sali roots, where iron levels in root are low enough that little blue colour forms with Perls' stain. Excess iron accumulated in these varieties might have translocated to shoots and thus provide the iron mediated oxidative damage in the plants [6]. Excess root iron accumulation in resistant varieties is in agreement with the works published earlier [5,6] and was documented that the roots of all varieties recorded excess iron when grown in waterlog acid soil with higher soil iron concentration but the extent of translocation to shoots was a varietal function. They proposed that Mahsuri plants were more resistant to excess Fe due to the possible induction of avoidance and / or exclusion mechanisms and formation of root plaque, allowing the plant to keep lower shoot Fe contents. Green and Rogers [13], in their experiment reported that root tissue from *frd3* mutants grown under iron-sufficient conditions showed significantly higher levels of the ferritin protein than wild-type root tissue and validated the consistency of their result with the higher iron levels demonstrated by elemental analysis and by Perls' stain.

3.3 Morphological Observations

Iron toxicity is one of the vital growth limiting factors in anaerobic rice ecosystem. When it is present in excess, facilitates the overproduction of reactive oxygen species (ROS) [18] and thus accelerates oxidative damage in the plants. In this study, we observed substantial variations in growth parameters among the three cultivars when subjected to iron overload.

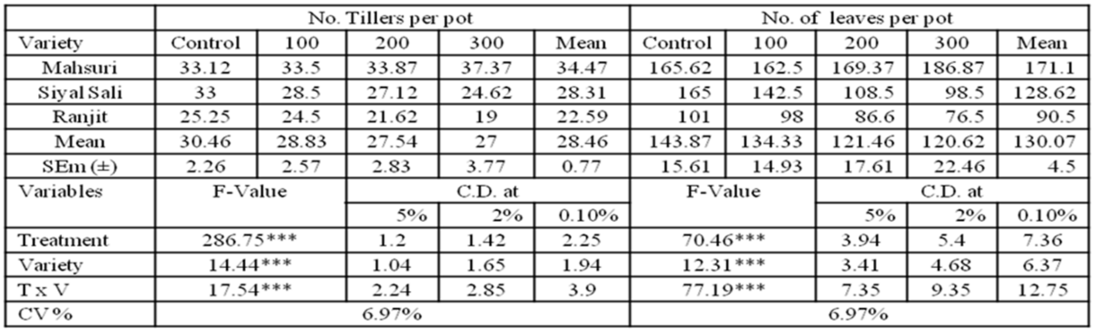
The results of all the morphological parameters demonstrated the distinct effect of different iron levels on the cultivars. The tiller numbers and leaf numbers per pot was maximum in Mahsuri even at 300 ppm Fe^{2+} iron (Table 1A). This variety also recorded superior performance in panicle numbers; panicle length and filled grain per panicle on iron overload (Table 1B and C). Ranjit and Siyal Sali displayed a significant reduction on morphometric parameters at 300 ppm iron in the growth medium. These two varieties recorded lower numbers of filled grains per panicle with simultaneous increase in the number sterile panicles with the increment Fe^{2+} concentrations (Table 1B and 1C). The reduction in growth and yield parameters in Ranjit and Siyal Sali might be the result of ferrous ion mediated oxidative damage and / or nutrient imbalance in the plants due to higher iron inflow in iron excess condition. Such decreasing trend of growth parameters in sensitive varieties on iron overload have also been reported by many investigators [19,20,21,22,23]. On the other hand, Mahsuri which showed luxuriant vegetative growth even at 300 ppm concentration of Fe^{2+} ion recorded better results of those parameters. Saikia and Bhuyan [6] had already suggested that Mahsuri plants exhibited stable nutrient status, expressed Fe avoidance mechanism and thus decreased iron translocation to shoots when coping with Fe overload.

3.4 SDS PAGE Profile

In this study we have acknowledged substantial variations in protein profile among the cultivars exposed to excess iron. The protein pattern was studied in rice leaves collected during the time of grain filling. Total Proteins obtained from 1g fresh leaves of all the three cultivars grown under different iron concentration were used for SDS PAGE analysis. Distinct treatments effects were observed in protein-bands profile of the cultivars. In Mahsuri, a similar band pattern was observed regardless of treatments. Even at 300 ppm iron, the variety Mahsuri displayed eight protein bands and two of them are highly intense. Protein bands of 14kDa and 20 kDa were detected in Mahsuri at 300 ppm iron which were absent in control as well as other treatments (Fig. 4).

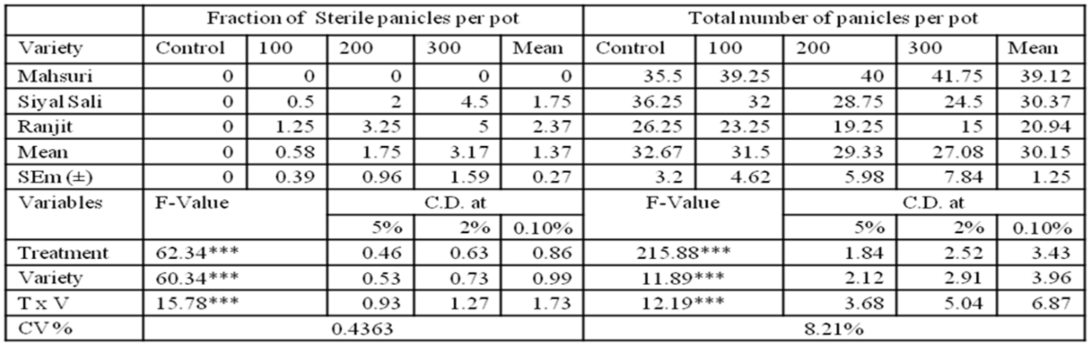
Mahsuri plants also expressed a protein band of molecular weight 24 kDa along with some doublet polypeptide bands of similar density and thickness that migrate close together. In nature such polypeptides typically form multiple- subunit proteins or are part of a "family" of isoenzymes that perform similar functions. In comparison to Mahsuri, other two varieties showed a depressed protein-bands profile on iron overload. These two varieties recorded only a fewer number of very weak bands under different iron levels (Table 2). In Siyal Sali and Ranjit most of the protein bands

Table 1. Effect of different levels of Fe^{2+} (in ppm) on (A) number of tillers and leaves per pot, (B) total number of panicles and fraction of sterile panicles, (C) panicle length and filled grains per panicles



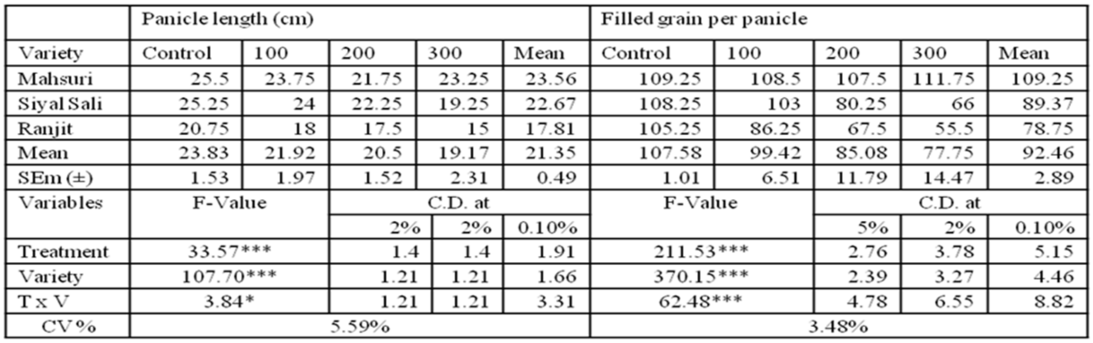
	No. Tillers per pot					No. of leaves per pot				
Variety	Control	100	200	300	Mean	Control	100	200	300	Mean
Mahsuri	33.12	33.5	33.87	37.37	34.47	165.62	162.5	169.37	186.87	171.1
Siyal Sali	33	28.5	27.12	24.62	28.31	165	142.5	108.5	98.5	128.62
Ranjit	25.25	24.5	21.62	19	22.59	101	98	86.6	76.5	90.5
Mean	30.46	28.83	27.54	27	28.46	143.87	134.33	121.46	120.62	130.07
SEm (±)	2.26	2.57	2.83	3.77	0.77	15.61	14.93	17.61	22.46	4.5
Variables	F-Value		C.D. at			F-Value		C.D. at		
			5%	2%	0.10%			5%	2%	0.10%
Treatment	286.75***		1.2	1.42	2.25	70.46***		3.94	5.4	7.36
Variety	14.44***		1.04	1.65	1.94	12.31***		3.41	4.68	6.37
T x V	17.54***		2.24	2.85	3.9	77.19***		7.35	9.35	12.75
CV%	6.97%					6.97%				

A



	Fraction of Sterile panicles per pot					Total number of panicles per pot				
Variety	Control	100	200	300	Mean	Control	100	200	300	Mean
Mahsuri	0	0	0	0	0	35.5	39.25	40	41.75	39.12
Siyal Sali	0	0.5	2	4.5	1.75	36.25	32	28.75	24.5	30.37
Ranjit	0	1.25	3.25	5	2.37	26.25	23.25	19.25	15	20.94
Mean	0	0.58	1.75	3.17	1.37	32.67	31.5	29.33	27.08	30.15
SEm (±)	0	0.39	0.96	1.59	0.27	3.2	4.62	5.98	7.84	1.25
Variables	F-Value		C.D. at			F-Value		C.D. at		
			5%	2%	0.10%			5%	2%	0.10%
Treatment	62.34***		0.46	0.63	0.86	215.88***		1.84	2.52	3.43
Variety	60.34***		0.53	0.73	0.99	11.89***		2.12	2.91	3.96
T x V	15.78***		0.93	1.27	1.73	12.19***		3.68	5.04	6.87
CV%	0.4363					8.21%				

B



	Panicle length (cm)					Filled grain per panicle				
Variety	Control	100	200	300	Mean	Control	100	200	300	Mean
Mahsuri	25.5	23.75	21.75	23.25	23.56	109.25	108.5	107.5	111.75	109.25
Siyal Sali	25.25	24	22.25	19.25	22.67	108.25	103	80.25	66	89.37
Ranjit	20.75	18	17.5	15	17.81	105.25	86.25	67.5	55.5	78.75
Mean	23.83	21.92	20.5	19.17	21.35	107.58	99.42	85.08	77.75	92.46
SEm (±)	1.53	1.97	1.52	2.31	0.49	1.01	6.51	11.79	14.47	2.89
Variables	F-Value		C.D. at			F-Value		C.D. at		
			2%	2%	0.10%			5%	2%	0.10%
Treatment	33.57***		1.4	1.4	1.91	211.53***		2.76	3.78	5.15
Variety	107.70***		1.21	1.21	1.66	370.15***		2.39	3.27	4.46
T x V	3.84*		1.21	1.21	3.31	62.48***		4.78	6.55	8.82
CV%	5.59%					3.48%				

C

**** Significant at 0.1% level of probability*

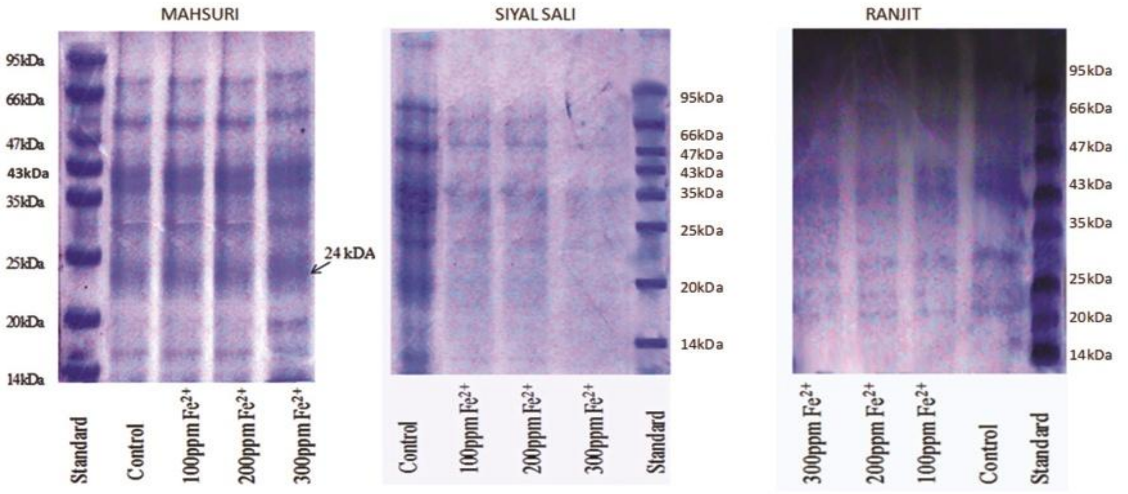


Fig. 4. The results showing the effect of different Fe^{2+} levels on total protein band profiles in the leaves of three rice cultivars during the grain filling stage by SDS-PAGE electrophoresis. The lane represented by Standard is the banding profile for Marker Proteins. The cultivars Mahsuri, Siyal Sali and Ranjit are shown at the top of each picture

Table 2. Band intensities in the leaves of three cultivars under different Fe^{2+} levels

Band Intensity range	Number of protein bands in the leaves of three varieties at different Fe^{2+} concentrations											
	Mahsuri				Siyal Sali				Ranit			
	Control	100 ppm	200 ppm	300 ppm	Control	100 ppm	200 ppm	300 ppm	Control	100 ppm	200 ppm	300 ppm
Highly intense	2	2	2	2	2	0	0	0	1	0	0	0
Midium intensity	2	2	2	5	2	0	0	0	3	1	1	0
Thin bands	2	2	3	1	2	3	3	2	1	3	3	2
Total bands	6	6	7	8	6	3	3	2	5	4	4	2

appeared in control plants was nearly vanished when plants were supplemented with external Fe^{2+} iron. Numbers of literatures are available that have mentioned the dominance of a resistant variety towards metal toxicity and demonstrated better protein profile under oxidative stress. Rout and Sahoo [24] reported that the disappearance and reappearance of some proteins and synthesis of other stress responsive proteins to Fe exposure indicate a direct relationship of metal stress induced proteomics. Similar observations were reported by Silveirs et al. [25] under iron stress in two rice (Oryza sativa L.) cultivars. They concluded that higher ferritin mRNA (MW: 24 kDa) protein accumulation in EPAGRI 108 plants compared to BR-IRGA 409 was one of the tolerance mechanisms of EPAGRI 108 plants to Fe overload.

Dwivedi et al. [26] also reported parallel observations in 13 rice genotypes under submergence and concluded that the tolerant *Swarna Sub 1* had one noble protein band of 26 kDa in submergence. The findings established the foundation of introducing submergence tolerance into agriculturally desirable cultivars of rice. The results of present investigation also support our earlier findings [6, 7, 8] that Mahsuri was a resistant cultivar in excess iron compared to other two varieties and included an exclusion and/or avoidance mechanism that displayed by this variety to iron overload. In those experiments, Mahsuri exhibited improved ant-oxidative enzyme activities, better nutrients status and grain biochemical components than Ranjit and Siyal Sali under higher iron levels. In this study, the Mahsuri plants expressed improved protein profile showing unusual protein band even at 300 ppm (quantification and sequencing is not included)iron in waterlog soil medium might be another reason for tolerance ability of the plant to excess iron.

4. CONCLUSION

From the present study we can conclude that iron sensitive rice cultivars exhibit a depressed photosynthetic rate with substantial growth reduction and may lead to the lower productivity in iron excess waterlog soil. Moreover, varietal changes in the occurrence of protein bands in iron stress conditions indicate that the tolerance ability of different rice cultivars is a genotypic function. There is a relationship between Fe-excess and alternation of protein patterns. A suppressive protein profile in the cultivars, Siyal Sali and Ranjit in iron excess condition may be reported as a pointer of intolerance to iron overload. On the other hand, Mahsuri recording superior morphological status and improved protein profile even at 300 ppm iron may be considered as Fe tolerant cultivar. Thus the analysis of protein profile seems to be a good indicator to estimate the tolerance ability in rice genotypes and hence their nutritional positions to iron stress environment.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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