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Original Research Article

Iron Overload in the Root Environment of Rice (*Oryza sativa- L)* with a Miserable Nutrients Specification.

Abstract:

In waterlogged soils under low pH, Fe^{2+} availability increases and may reach toxic levels. The conditions of iron toxicity are quite well established over the World. The physiological effects of Fe^{2+} within plant with subsequent plants' nutrients status are well documented in many literatures. Despite our current knowledge of the processes and mechanisms involved, iron toxicity, a function of growth conditions and the cultivar types remains as an important constraint to rice production, together with nutrients deficiency in the regional levels. To screen Fe tolerant cultivars and thus to evaluate the mechanisms involved in response to excess Fe, experiment was carried out with rice cultivars – Ranjit, Siyal Sali and Mahsuri, grown by developing artificial Fe toxic conditions in the soils of experimental pots applying different Fe^{2+} concentrations (control- normal soil iron from rice field, +100, +200 and +300 ppm respectively). The study of plants' biochemical parameters confirmed the resistance of Mahsuri plants to Fe excess. With steady recovery of neutral pH and better chlorophyll contents, the root and shoot nutrients of Mahsuri were found to be higher compared to the plants of other two varieties when exposed to excess Fe. Except Fe and N in roots and shoots, the excess of Fe caused a negative impact on other nutrients in these vulnerable cultivars. Plants of Ranjit and Siyal Sali seem to be affected directly by Fe toxicity and also by the pseudo Fe toxicity, whereas Mahsuri seems to make use of the exclusion /and or avoidance mechanism to Fe overload.

Key Words: toxicity, nutrients, Oryza sativa- *L*, investigation, vulnerable

Introduction:

Iron is essential for plant growth and development[1]. In anaerobic acid soils, however, high concentrations of ferrous (Fe^{2+}) ions may lead to Fe toxicity due to excessive Fe uptake[2], which can result in yield reductions from 12 to 100 percent[3]. Excess Fe can be extremely toxic, as it reacts with oxygen and catalyses the production of free radical species. In waterlogged soil iron toxicity may disrupts or over expresses a number of metabolic routes

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can bring about nutrient disorder in rice cultivars. The expression of iron-toxicity symptom requires the excessive uptake of Fe^{2+} by roots and its acropetal translocation via xylem flow into the leaves.

In North East India, a major portion of the rice is grown under lowland conditions[4], and Assam is the highest rice producing state, where all rice is grown in waterlogged soils. Use of tolerant rice cultivars retaining better nutrients level is the best alternative and inexpensive technologies for rice production on Fe toxic soils of this area[4,5].

Although several research work have been conducted worldwide to identify adaptive responses of different rice genotypes still rate of nutrients absorption (ionic competition for absorption) and their availability (in favourable oxidation states) under higher iron concentrations is a matter of debates. Under anaerobic conditions O_2 release from rice roots, oxidise Fe^{2+} to polymeric oxy-hydroxide which coats on roots surface preventing the uptake of Fe^{2+}, Mn^{2+} and also acts as P reservoir[6]. Silveira et al (2007)[6] had also cited that except for Mn, no other nutrients seemed to have impaired uptake due to Fe toxicity in the vulnerable cultivar (I409 plants) and not in the resistant one (E108 plants).

The Fe^{2+} concentrations in the soil solution that reportedly affect lowland-rice yields can range from 10 to >2000 mg per liter[7]. Iron-induced yield reduction is frequently associated with a poor nutrient status of the soil[8]. Hence, many workers suggest that excess Fe^{2+} may result in lower uptake of other essential nutrients due to chemical interactions in soil ($ZnFe_2O_3$, K-Fe complex). Sahrawat (2004, 2010)[3, 9] has reported the possibility of “pseudo Fe toxicity” (Fe toxicity symptoms induced by nutrients deficiency) and “true Fe toxicity” (caused by excessive Fe^{2+} uptake) in rice grown at higher iron concentration.

Plant’s tolerance to excess Fe might be the effect of Fe avoidance and/or tolerance to high internal Fe concentration. Such avoidance and/or tolerance capacity to Fe overload is a genotypic function[6,10]. To sum-up, the conditions of iron toxicity are quite well established all over the World. The physiological effects of Fe^{2+} within the plant with subsequent plants nutrients status are well documented in many literatures. In spite of our current knowledge of the processes and mechanisms involved, iron toxicity remains an important constraint to rice production in regional level where selection of cultivars having the ability to maintain high levels of essential micro and macro nutrients under Fe toxic condition is a successful approach for lowland rice cultivation in acid soil. To screen Fe tolerant cultivars and thus to evaluate the mechanisms involved in response to excess Fe, experiment was carried out with

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rice cultivars – Ranjit, Siyal Sali and Mahsuri, grown by developing artificial Fe toxic conditions in the soils of experimental pots applying different Fe^{2+} concentrations. In this work we were studied the differential responses of three rice cultivars to iron excess by evaluating the influence of Fe nutrition on other nutrients uptake, their elemental concentrations in rice roots and shoots, to help the investigation of mechanisms involved in resistance to Fe toxicity.

Materials and methods:

An artificial Fe toxicity conditions in the experimental pots were developed with soils collected from a rice field located at Titabor of state Assam, India (soil type-sandy clay loam, total soil iron 345ppm, pH 5.4, available phosphorus $18.1 kg.ha^{-1}$, nitrogen $460 kg.ha^{-1}$, potash $127 kg\ ha^{-1}$ and organic carbon 1.2%). The experiments was conducted with three rice (Oryza sativa-L) varieties viz. Mahsuri, Ranjit (high yielding varieties) and Siyal Sali (traditional tall variety) and were grown in four different levels of Fe^{2+} -- control (normal soil + 0 ppm), +100 ppm, +200 ppm and +300 ppm in the form of $FeSO_4.7H_2O$. Treatments were replicated four times in a randomized block design (Fisher and Yates, 1957). Thirty days old seedlings of uniform vigour were transplanted at the rate of three seedlings per pot. The Fe^{2+} solutions were added in the pot one week after transplanting at an interval of seven days till panicle initiation stage. A uniform waterlogged environment was maintained with distilled water in the pots throughout the experimental period.

pH record: pH of soil solutions were recorded (from each pot) *in-situ* at an interval of seven days from days after transplanting (n=5 for each pot) with the help of a digital pH meter.

Total Leaf chlorophyll: Extraction and estimation was done by spectrophotometric method[12].

Nutrients analysis: The K content was determined flame photo-metrically from mineral solution obtained after tri-acid digestion[13]. P was estimated from mineral solution converting phosphate to phosphomolybdic acid and finally reducing with hydroquinone. The blue colour developed was measured in a spectrophotometer (Systronics UV-VIS Spectrophotometer 118) at 660nm[13]. The total nitrogen in roots and shoots were determined by Micro-Kjeldahl's method[14] with 0.5g powdered sample after digesting with concentrated H_2SO_4 and catalyst mixture. N content was determined by titrating the distillate with 0.1N HCl.

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Mineral solution was prepared by digesting 1gm dry samples in tri-acid mixture and extracted with concentrated nitric acid. Fe, Mn, Zn & Cu were determined by Atomic Absorption Spectrophotometer (Chemito, AA 203D) from mineral solution using separate primary standard for each micro-nutrient[14].

Statistical analyses of experimental data were carried out by using 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[15].

Results and Discussions:

The pH of the growth medium has significant impact on the properties of soils and consequently on the nutrient uptake by crop plants. The pH of soil solution is thought to be best for plant growth when kept between 5.5 and 6.5. Plant growth in acid soils may be limited by pH-induced Fe^{2+} toxicity as acidity increases the solubility of Mn and Fe in acid soils[7]. In such adverse pH condition plants suffer from ionic imbalance through a competition between the similarly charged ions for binding and carrier sites. Although acidic injury is negligible in a medium at a pH above 4, lower pH in acid soils is one of the factors responsible for growth retardation, empires mineral nutrients in plants[16]. In our investigation we detected an interesting relation between soil pH vs varieties and also these variables with Treatments (Figure 1). The initial pH rested in between 5 to 5.5 irrespective of treatments. Here Mahsuri considered being efficient variety which showed recovery of pH after sharp drop in the initial period. Similar improvement of pH was not observed in varieties Ranjit and Siyal Sali. The varieties Ranjit and Siyal Sali could not recovered the initial pH (pH=5.2) up to 70 days after transplanting (DAT), rather a decreasing trend was detected for Ranjit at 300 ppm Fe^{2+} in the medium (Figure 1D). Of course Mahsuri showed a differential behaviour in the change of pH and showed better recovery at different growth stages. At maximum tillering stage (MTS) and panicle initiation stage (PIS) we observed a sharp increase in soil pH (pH =6) and a quick revival after 70 DAT (as straight line) at 300 ppm Fe^{2+} (Figure 1D). A sound recovery in the pH in the efficient plants might lower the reduction of Fe^{3+} to Fe^{2+} on root surface through the release of other reductants and the plants sustain better physiological, biochemical activities. At nearly neutral pH solubility of Fe in the rooting medium is reduced by the fast oxidation of ferrous-Fe, favours the formation of iron plaque and hence iron immobilization occurs in the roots. Although we conducted the experiment

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with similar soil environment, different pH curves were documented by the three varieties which signify that pH variation in water saturated soil is also a varietal function.

A marked reduction in chlorophyll contents were observed at 300 ppm and 200 ppm Fe^{2+} in the growth medium (Figure 2). Maximum chlorophyll content was recorded in the plants grown in control soil iron. Total leaf chlorophyll content was found to be reduced in the varieties Ranjit and Siyal Sali grown at higher level of iron (Figure 2). Interactions between levels of Fe^{2+} and varieties were also found to be significant on the total chlorophyll content. Mahsuri recorded relatively higher chlorophyll content irrespective of the treatments compared to Ranjit and Siyal Sali (Table 1). Mahsuri might have been able to maintain higher chlorophyll content through chloroplast development[4]. Ranjit recorded lower chlorophyll at 200ppm and 300ppm Fe followed by Siyal Sali. Our findings also revealed the variation of total chlorophyll in different growth stages. Mahsuri sustained stable leaf chlorophyll content at different growth stages, quite reverse to the other two varieties (Figure 3). Perhaps, severe damage in cell structural components in early growing stages due to Fe^{2+} mediated ROS, might be the reason of rapid reduction of chlorophyll content in these varieties.

The detrimental effect of Fe^{2+} became more pronounced when its concentrations increase in waterlogged soil. In waterlogged soil, excess uptake of Fe^{2+} by the roots and its acropetal translocation into the leaves must have catalyzed the generation of active O_2 species or free radicals which could render the peroxidation of chloroplast membranes, damage cell structural components and impair the plants' physiological processes and subsequently lead to a decrease in chlorophyll content in the sensitive varieties.

As expected, plants grown in higher soil Fe^{2+} had higher Fe concentrations than those grown under control Fe levels, both in roots and shoots (Figure 4). Of course higher Fe concentrations were found in roots than in shoots. At 200 & 300 ppm Fe^{2+}, higher concentrations of total Fe were detected in the plants of Ranjit and Siyal Sali, more susceptible to Fe toxicity, both in roots and shoot; with shoot concentrations nearly 2 times higher than in Mahsuri plants.

Expression of some plant ferritin isoforms can be induced by Fe overload[17] and iron storage inside ferritin could be related to Fe overload tolerance in some rice cultivars[6]. Surprisingly, lower shoot Fe in the present investigation could not define the tolerance capacity of the cultivars to ferritin expression. Audebert and Sahrawat (2000)[18] reported that Fe tolerant cultivar absorbed less Fe or translocated less Fe from root to shoot, a mechanism

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involved in cultivar differences in Fe toxicity tolerance. Here we suggest that Mahsuri plants are more resistant to excess Fe due to the possible induction of avoidance and / or exclusion mechanisms, allowing the plant to keep lower Fe amounts in its tissues and reducing Fe translocation to shoots. Moreover a large concentration of root Fe compared to shoot Fe concentrations might also be attributed to the formation of root plaque in the form of *Compound B* (goethite and lepidocrocite) as stated by Silvaira et al. (2007)[6].

The root Zn concentrations for the three cultivars were found higher up to 1.5 times than shoot (Figure 5 A and D). Marked treatment effects were predicted in root and shoot Zn concentrations. Here Zn concentration decreases both in roots and shoots of the tree cultivars with the increment of Fe^{2+} treatments. An apparent difference observed in Zn concentration under Fe excess was a higher Zn concentration in shoots of Mahsuri plants than in Ranjit and Siyal Sali plants. Shoot Zn concentration in Mahsuri was about 6 times higher than Ranjit and also above 2 times compared to Siyal Sali when the plants exposed to 300 ppm Fe^{2+}.

Iron (III)-oxides are known to have a strong zinc-binding tendency. In waterlogged soil environment, Zn becomes available in the process of iron oxide reduction[19]. At the same time the plaque formation resulting from Fe re-oxidation around the rice root can reduce the concentration of soluble Zn in the rhizosphere by forming sparingly soluble $ZnFe_2O_4$ complex[20]. Moreover reduced Fe can also exert a direct antagonistic effect on Zn uptake[20]. Sometimes it might also happened that the Fe plaque can lead to higher or lower Zn concentrations in shoots, depending on the size of plaque layer[21]. In the present work, the lower shoot Zn content compared to root in excess Fe^{2+} may be referred to the root Fe plaque formation that seem to be acting either as a Zn reservoir or preventing Zn uptake. On the other hand a better shoot Zn content in Mahsuri indicates its tolerant capacity to higher Fe^{2+} levels than the other two cultivars which may attributes to up regulation of some *ZIP* genes in Mahsuri plants[22, 23].

Mn concentration in shoots were higher than roots in all the three cultivars, but a considerable reductions were observed in both roots and shoots subjected to higher Fe levels, with Ranjit reaching lowest levels of Mn concentration in roots and shoots (Figure 5 B and E).Of course, shoot Mn content in Mahsuri was significantly higher than other two cultivars at 300 ppm Fe^{2+}. Precipitation of Mn in the Fe plaque may have resulted in its lower absorption by the cultivars where highest Fe concentrations were found. Such negative interactions between Fe and Mn have also been reported in plants[24].

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Except Mahsuri, lower Cu concentrations were recorded in roots and shoots of Ranjit and Siyal Sali when submitted to Fe excess. It has been suggested that the Fe plaque could act as a Cu reservoir in plants, increasing Cu absorption[25]. But in our experiment, reduction of Cu content in roots and shoots of Ranjit and Siyal Sali (Figure 5 C and F) might be due to formation of Fe plaque, being able to act as a barrier to Cu absorption[6] or preferential uptake of Fe^{2+} on Fe overload, supported by highest shoot Fe concentrations. Mahsuri, on the other hand only the variety that could sustain better Cu concentrations by active absorption through roots and dynamic translocation to shoots even 300 ppm Fe supplementation. The varietal differences in shoots Cu and Zn concentrations may also be attributed to higher Cu/Zn SOD activities in tolerant plants in Fe overload[5].

The nitrogen concentrations for the plants grown in higher Fe were high than grown under control Fe levels, both in roots and shoots (Figure 6 C and F). Of course rate of increment in shoots were higher than roots. The percentage increase of shoots nitrogen concentration in Mahsuri plants was higher than the other two varieties. The variations pattern of nitrogen concentrations were similar to that of root and shoot Fe concentrations in the cultivars. Since in water saturated acidic soil Fe^{3+} and NO_3^- act as electron acceptors, a strong ionic competition of Fe^{2+} and NO_2^- might developed around roots' periphery and accelerated the uptake of nitrogen adduct along with Fe. Earlier studies have also demonstrated a direct relation in the uptake of Fe and N in wheat plant or seed and external supply of N at different phonological stages[26,27,28].

Moreover, the uptake and transport of metals in plant is regulated by some special N loaded transporter proteins situated in different tissues of root, stem, leaf and reproductive parts. Many of them, like the proteins of *NRAMPs* and *ZIP* family are specific in transporting iron[29,30]. Thus the rice plants grown in higher soil Fe^{2+}, the superior uptake rate of Fe from soil and their translocation to leaf and to grain is facilitated by transporter proteins, which might be considered as the possible mechanism of higher N supplement to plants.

Phosphorus concentration decreased considerably in roots and shoots of Ranjit and Siyal Sali plants submitted to excess Fe but not in roots of Mahsuri plants (Figure 6 A and D). A decreasing trend of phosphorus concentration also observed in the shoots of Mahsuri plants when exposed 200 & 300 ppm Fe^{2+}, was suggesting the limited P translocation to the shoots of all the cultivars.

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The concentration of phosphorus in the soil solution depends mainly on soil pH, and a decrease in pH can reduce P concentration by causing precipitation of amorphous Fe-phosphate polynuclear complexes with high surface area. In the present investigation we proposed that at low soil pH, higher amounts of Fe(III) oxides may be accumulated in the roots that can absorb anions such as phosphate and control the uptake of apoplast P into the simplast[31,6]. Our shoot data seems to agree with Howeler (1973)[32], who states that, the root's apoplastic precipitation results in lower P absorption by the plant.

Potassium is a common macronutrient in plants that activates many enzymes involved in photosynthesis, respiration and plays important roles such as starch and protein synthesis, cell expansion, and stress alleviation[33,34]. However, higher Fe concentration in the medium plays an antagonistic role in plants' K uptake. Mehraban et al, (2008)[34] reported lower root and shoot K concentration under high Fe nutrition. In the present experiment K concentration decreased in the roots and shoots of Ranijt and Siyal Sali under excess Fe^{2+} in comparison to the control treatment (Figure 6 B and E) which may be considered as the consequence higher Fe nutrition and formation of Fe—K complex in soil solution. In contrast the plants of Mahsuri sustained stable K concentrations in roots and shoots, where it would be expected due to its higher sustainability to excess Fe toxicity.

CONCLUSION:

The variability observed in the results of soil pH, leaf chlorophyll contents and all the major nutrients in roots and shoots under excess Fe^{2+} indicate the differential tolerance capacity among the cultivars. Although, root and shoot Fe and N concentrations showed positive correlation among the cultivars, the remarkable shoot Fe concentrations with simultaneous reduction in leaf chlorophyll contents explains the oxidative damage in the plants of Ranjit and Siyal Sali due to Fe^{2+} induced reactive oxygen species, OH^{-} radicals through Fentons' reactions. On the other hand, variety Mahsuri probably with its tolerable shoots Fe concentration and radical pH recovery, sustained ionic balance around root surface and thereby showed a positive respond to Fe overload. This variety recorded superior nutrients status even at 300ppm and may be conspired as Fe tolerant cultivars. With deferred pH recovery, low leaf chlorophyll and reduced root and shoot nutrients level, the plants of Ranjit and Siyal Sali exhibited Fe susceptible nature when grown in Fe^{2+} excess medium. Moreover, except Fe and N, all other nutrients seemed to have impaired uptake due to Fe toxicity in these susceptible cultivars compared to Mahsuri. Thus the plants of these cultivars appear to be affected by direct Fe toxicity as well as by pseudo Fe toxicity"-- Fe toxicity

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symptoms induced by nutrients deficiency. The Mahsuri cultivar seems to keep up mostly on avoidance and/or exclusion of Fe uptake into the plant and decreased translocation to shoots, being able to maintain higher nutrients levels in roots and shoots.

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Figure and Table Captions:

Figure 1: Varietal impacts of iron treatments on pH change in the soil solutions at different growth stages.

Figure 2: Varietal impacts of iron treatments on leaf chlorophyll contents at different growth stages (in mg g^{-1} FW). The vertical bars represent the standard errors.

Figure 3: Varietal change on leaf chlorophyll contents at different growth stages (in mg g^{-1} FW). The vertical bars represent the standard errors.

Figure 4: Varietal impacts of iron treatments on roots and shoots Fe. The vertical bars represent the standard errors.

Figure 5: Varietal impacts of iron treatments on roots and shoots Zn (A, D), Mn (B, E) and Cu (C, F) (in µg g^{-1} DW). The vertical bars represent the standard errors.

Figure 6: Varietal impacts of Fe treatments on root and shoot P (A, D), K (B, E), and N (C, F) (in µg g^{-1} DW). The vertical bars represent the standard errors.

Table 1: Varietal impacts of iron treatments on leaf chlorophyll contents (in mg g^{-1} FW) at different growth stages.* Significant at 5%, ** Significant at 1% level and *** significant at 0.1% level of probability.

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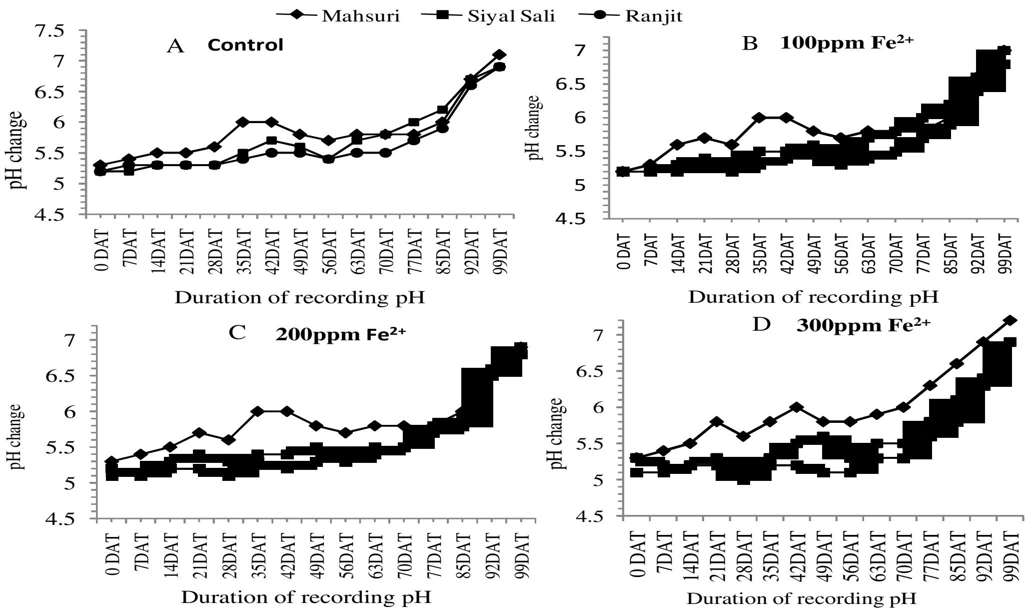

Figure 1: Varietal impacts of iron treatments on pH change in the soil solutions at different growth stages.

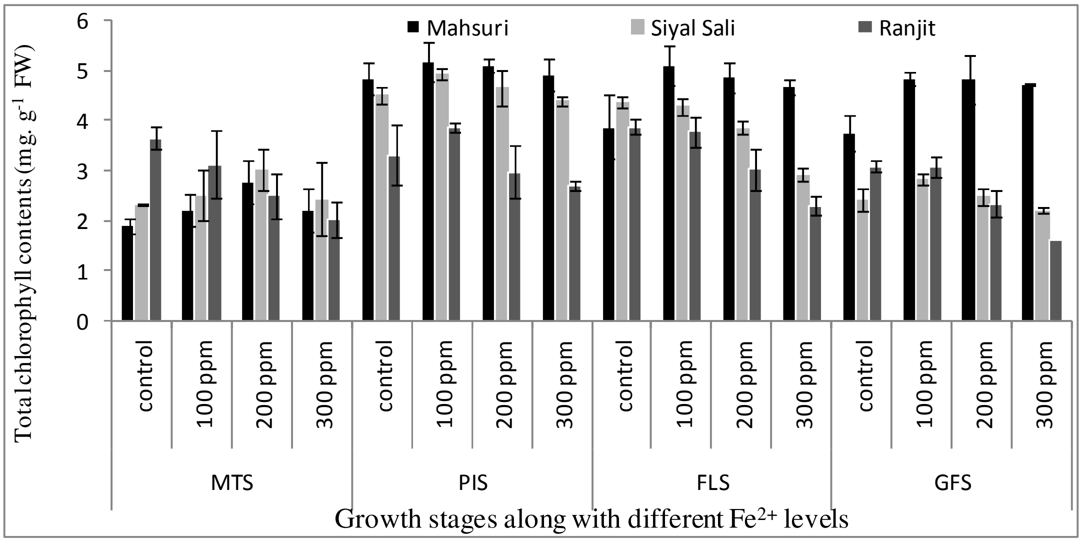

Figure 2: Varietal impacts of iron treatments on leaf chlorophyll contents at different growth stages (in mg g^{-1} FW). The vertical bars represent the standard errors.

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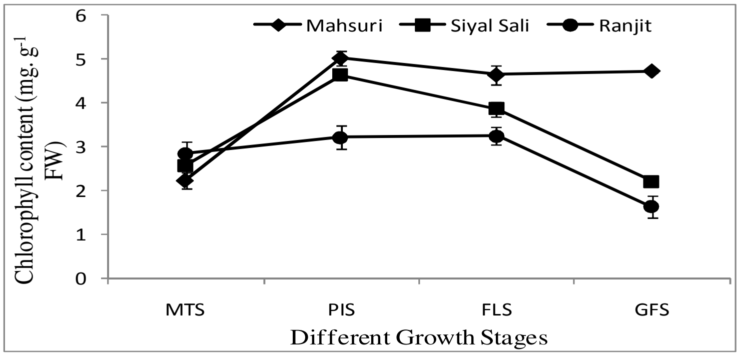

Figure 3: Varietal change on leaf chlorophyll contents at different growth stages (in mg g^{-1} FW). The vertical bars represent the standard errors.

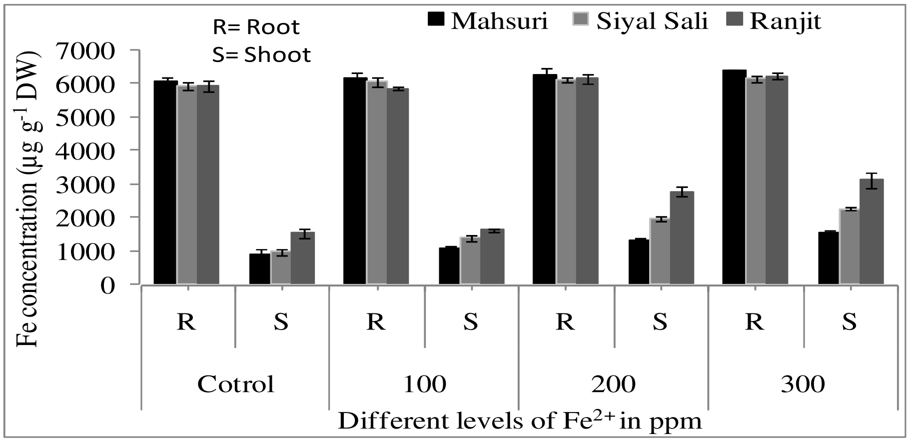

Figure 4: Varietal impacts of iron treatments on roots and shoots Fe. The vertical bars represent the standard errors.

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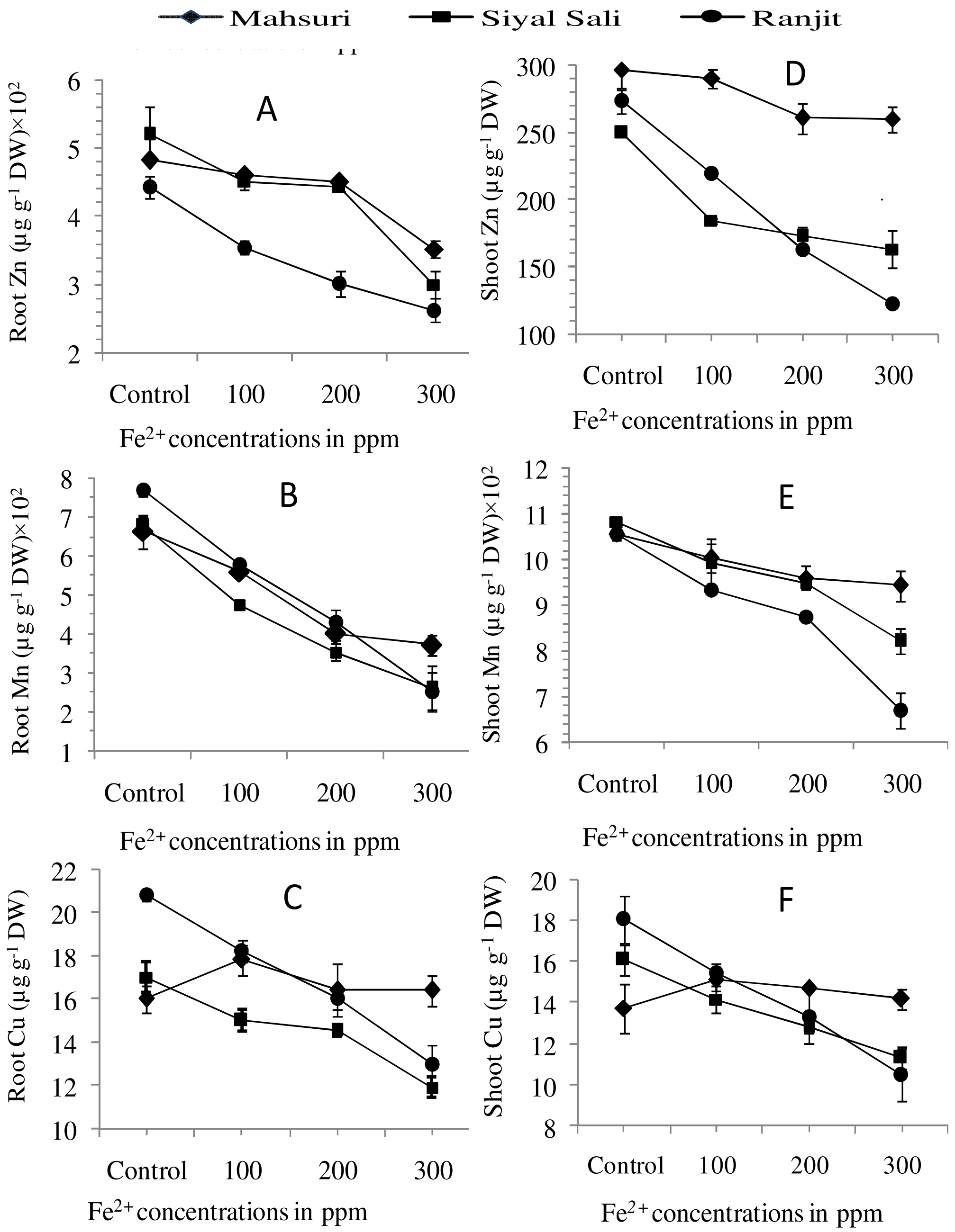

Figure 5: Varietal impacts of iron treatments on roots and shoots Zn (A, D), Mn (B, E) and Cu (C, F) (in µg g^{-1} DW). The vertical bars represent the standard errors.

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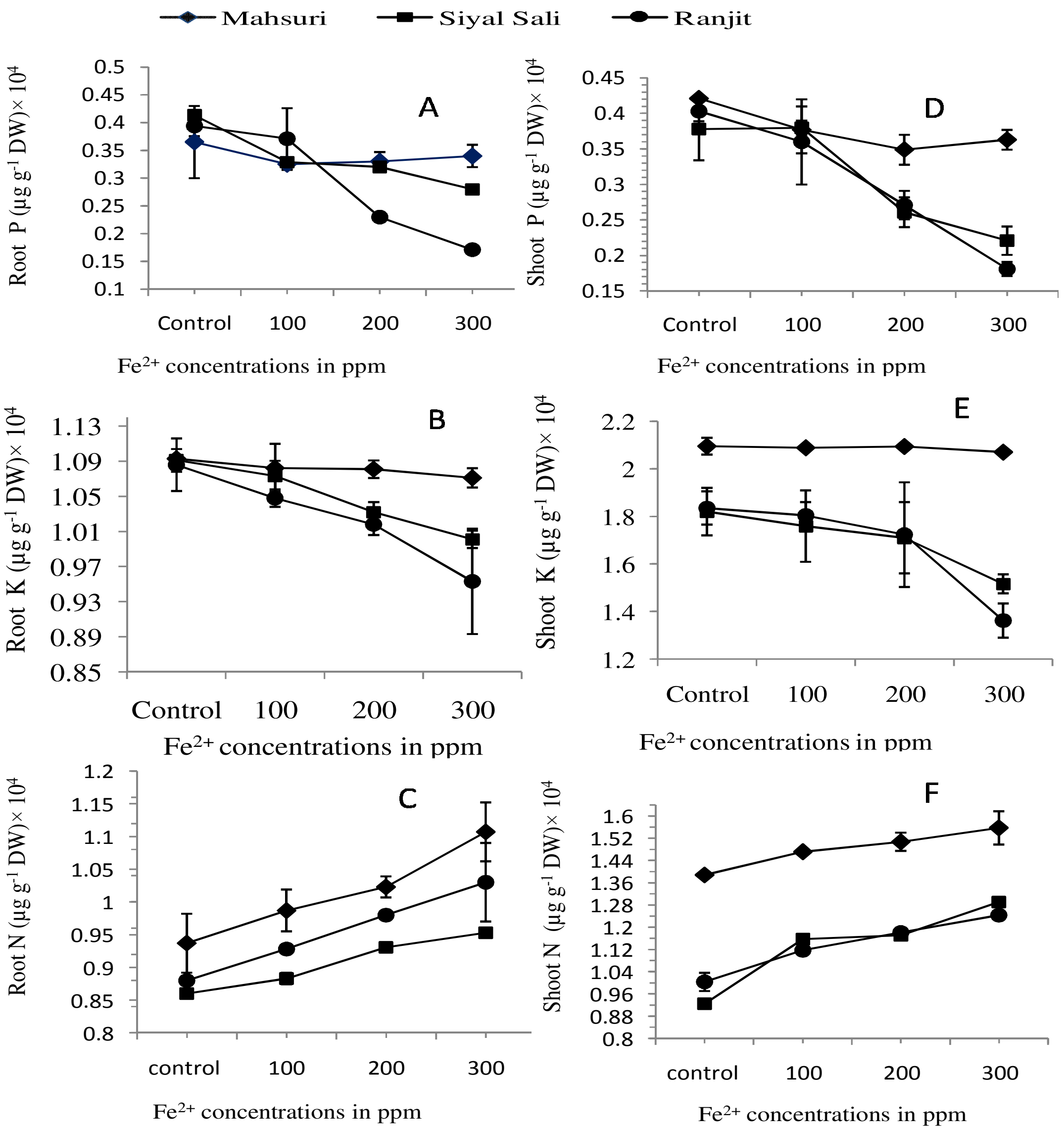

Figure 6: Varietal impacts of iron treatments on roots and shoots P (A, D), K (B, E), and N (C, F) (in µg g^{-1} DW). The vertical bars represent the standard errors.

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Growth stages	Maximum Tillering Stage (MTS)					Panicle Initiation Stage (PIS)				
Treatment→ Variety ↓	Different levels of Fe^{2+}									
	control	100 ppm	200 ppm	300 ppm	Mean	control	100 ppm	200 ppm	300 ppm	Mean
Mahsuri	1.91 ±.15	2.01 ±.32	2.78 ±.42	2.22 ±.44	2.23±.18	4.84 ±.33	5.18 ±.40	5.09 ±.13	4.91 ±.31	5.01±.16
Siyal Sali	2.31 ±.02	2.51 ±.51	3.01 ±.42	2.43 ±.74	2.57 ±.23	4.5 ±.16	4.93 ±.11	4.65 ±.35	4.39 ±.10	4.62±.11
Ranjit	3.64 ±.23	3.13 ±.69	2.51 ±.45	2.02 ±.36	2.83 ±.27	3.31 ±.60	3.87 ±.10	2.98 ±.59	2.71 ±.51	3.22±.26
Mean	2.62	2.55	2.77	2.22	2.54	4.22	4.66	4.24	4.01	4.28
SEm(+)	0.26	0.16	0.07	0.06	0.07	0.23	0.23	0.32	0.33	0.23
Variables	F- Value	CD--	5%	1%	0.10%	F- Value	CD--	5%	1%	0.10%
Treatment	4.55*		0.32	0.44	0.6	3.82*		0.42	0.57	0.78
Variety	10.120***		0.28	0.38	0.52	60.08***		0.36	0.49	0.67
T **X** V	8.45***		0.55	0.76	1.04	3.50*		0.72	0.99	1.38
C V %	14.73					11.3				

Growth stages	Flowering Stage (FS)					Grain Filling Stage (GIS)				
Treatment→ Variety ↓	Different levels of Fe^{2+}									
	control	100 ppm	200 ppm	300 ppm	Mean	control	100 ppm	200 ppm	300 ppm	Mean
Mahsuri	3.87 ±.63	5.1 ±.38	4.86 ±.31	4.67 ±.16	4.63±.22	3.75 ±.37	4.84 ±.12	4.83 ±.49	4.72 ±.03	4.54±.18
Siyal Sali	4.36 ±.1	4.27 ±.16	3.85 ±.14	2.92 ±.14	3.85±.16	2.41 ±.23	2.84 ±.11	2.48 ±.17	2.21 ±.05	2.49±.10
Ranjit	3.87 ±.10	3.78 ±.14	3.02 ±.30	2.3 ±.42	3.24±.20	3.09 ±.20	3.07 ±.12	2.34 ±.20	1.62 ±.25	2.53±.18
Mean	4.03	4.38	3.91	3.3	3.91	3.08	3.59	3.22	2.85	3.18
SEm(+)	0.08	0.19	0.27	0.35	0.17	0.19	0.32	0.4	0.47	0.29
Variables	F- Value	CD--	5%	1%	0.10%	F- Value	CD--	5%	1%	0.10%
Treatment	8.35**		0.46	0.64	0.87	5.11**		0.41	0.55	0.76
Variety	26.340***		0.4	0.55	0.75	98.68***		0.35	0.48	0.65
T **X** V	4.41**		0.8	1.1	1.5	4.99**		0.7	0.96	1.31
C V %	13.85					14.83				

Table 1: Varietal impacts of iron treatments on leaf chlorophyll contents (in mg g^{-1} FW) at different growth stages.* Significant at 5%, ** Significant at 1% level and *** significant at 0.1% level of probability.