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Iron Toxicity, Tolerance, and Quantitative Trait Loci Mapping in Rice (*Oryza sativa* L.)

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Abstract: ***Background*: An experiment was conducted in the rainout shelter comprising of five varieties/ genotypes of Indica rice, exposed under different concentrations of two different forms of iron viz., ferrous ($FeSO_4$) and ferric ($FeCl_3$). Visual scoring scale was used to screen the genotypes and effects of excess iron were examined on different vegetative traits in which root weight and shoot weight were found to be more sensitive to excess iron concentration of both forms andeffect of iron on different genotype examined. *Main body*: In this experiment, five varieties/ genotypes of Indica rice, exposed under different concentrations of two different forms of iron viz., ferrous ($FeSO_4$) and ferric ($FeCl_3$).In two different forms of iron, ferrous form was found to be toxic than ferric form but high amount of ferric chloride without chelating agent can be more toxic in hydroponics condition. On the basis of visual scoring, we identified 4 genotypes tolerant (Dagad Deshi, IBD-1, RRF 127, and RRF 105) and Swarna as susceptible genotype for both form ferrous and ferric iron. Cross of Swarna and IBD-1 was used for development of F_4 generation and QTLs were identified on the basis of genotypic and phenotypic data obtained from F_4 generation. A total of thirteen QTLs have been identified using interval mapping (IM) approach. These QTLs are major and minor QTLs based on R^2 or phenotypic variance explained (PVE %). In composite interval mapping approach, a total of twenty-four major and minor QTLs were detected, of these QTLs, ten were the major QTLs. RM 152 and RM 264 markers on chromosome number 8 are highly significantly (PVE>10) associated with the variation of two traits shoot length and Fe^{+3}content in shoot. *Conclusion*: Significant differences among genotypes for various traits associated with iron tolerance under different doses of iron. In general, high dose of iron have toxic effect on genotypes. In sources of iron ferrous form of iron was noticed as more toxic form but high amount of ferric iron without chelating agent become more toxic than ferrous. Root weight and shoot weight were found to be more sensitive to excess iron***

Keywords: Rice, iron toxicity, tolerance, iron concentration, QTLs

1. Introduction

Rice is India's preeminent crop and is one of the chief grains and staple food of the people of all over the world. India is one of the world's largest producers of rice, accounting for 20% of all world rice production and contains high nutritional values and calorific value. (Bouman *et al.*, 2002). Most of the land approximately 129-million-hectare world land is come under rice cultivation but there is major problem of toxicity and deficiency of nutrient and it has been reported that its accounts for reduction of 100 million hectare from whole world. (Becker and Asch 2005). Iron is an important micronutrient performed many works like chlorophyll synthesis, maintenance of structure and function of chloroplast, helpful in biological processes such as photosynthesis, chlorophyll synthesis, respiration, nitrogen fixation, uptake mechanisms (Kim and Rees, 1992). Absorption of iron take place in two form, first one ferrous ($Fe^{+2)}$ and second one ferric ion ((Fe^{+3}) but the ferrous (Fe^{2+}) ion is majorly absorbed form of iron and it can cause nutrient imbalance or nutrient hampering condition in plants and accounts for indirect toxicity, which is more commonly found in lowland rice. (Fageria*et al.*, 2006 and Fageria*et al.*,1987).On other hand, in Fe^{3+} form of iron has transported across the plant root membrane by chelating agents (Phytosiderphores) and commonly this absorption occurs in upland condition, but this is low absorbing ion. So, aerobic rice often suffers from micronutrient deficiency and mainly Fe deficiency, this problem take place due to low release of Fe chelators (phytosiderophores) by rice. (Kreye *et al*. 2009 and Takagi 1976). Iron toxicity can be considered as a complex nutrient disorder, as its accounts for deficiencies of other nutrients by interfering with other important nutrients (P, K, Ca, Mg, and Zn).Iron toxicity cause significant grain yield losses, there is 18 percent soil are suffered from iron toxicity. (Das *et al*, 2014 *and* Vose,1982) which can change the healthy physiology of soil and also soil process of*eg*. Soil redox potential, soil pH, soil fertility status, and finally significant grain yield (GY) reductions (Audebert, 1885). In severe iron toxic soil conditions, more than a 50% GY reduction is reported, however, before grain filling iron toxicity can attack in early vegetative stage and can cause complete crop failure (Audebert 1885 and Chérif *et al*, 2009).So, it become important to understand the genetic of Fe absorption, uptake,its emphasis on crops and their interactions with other micronutrients. However, not much attention has been given to the development of rice varieties with tolerance to Fe toxicity(Kar and Panda, 2018, Bashir *et al*,2014 and Dos *et al* 2017), mainly because ofcomplex nature of trait that is governed by several component traits and is much affected by environmental factors. Here, we conducted this study with the objective of analyzing differential behavior of rice varieties for Fe^{+2} and Fe^{+3} uptake and to see the effect of different concentration of iron under nutrient solutions and understanding the behavior pattern of the iron toxicity by development of mapping population.

2. Material and Method

Different experiments were performed in the rain out shelter by taking twelve genotypes. Five genotypes (Dagaddeshi, IBD-1, R-RF-127, Swarna and R-RF-110) from twelve genotypes were selected on the basis of previous iron toxicity experiment performed in Department of Plant Molecular Biology and Biotechnology, IGKV, Raipur.

Screening System against iron toxicity:For screening of iron toxicity, experiments were conducted using three type of screening setup using disposable cups, glass plate and hydroponics setup with two iron forms (Fe^{+2} and Fe^{+3}) at the doses of 0, 40, 80, 120, 160, 200, 400, 600 and 800 ppm.

Disposable cup experiment/ Glass plate experiment:
Disposable cups and glass plates (rhizotron) were filled with sterilized soil. Seeds were directly sown in the soil. The solution of both forms of iron i.e., ferrous sulphate and ferric chloride were applied every week. and observation were recorded after 30 days of germination.

Hydroponics Experiment: For conducting hydroponics, healthy seeds of selected five genotypes were germinated in Petri-plates and were subsequently transferred in glass tubes filled with Yoshida nutrient solution, all nutrient were fixed in solution except iron concentration was given in form of ferrous sulphate ($FeSo_4$) and ferric chloride ($FeCl_3$) in different doses (0, 40, 80, 120, 160, 200, 400, 600 and 800 ppm). There was one controlled condition in which iron was present in optimum dose according to the standard dose of Yoshida nutrient solution (Yoshida 1976). The pH of nutrient solutions (4.5) was adjusted on every two days and nutrient solutions were changed every week. Plants were exposed to iron stress condition for 30 days of sowing. After 30 days sowing harvesting and observation recording was done. plant shoot and root were harvested separately and washed with ion free distilled water. Plant materials were dried at about 60^0c and dry matter was determined. Data were taken on different vegetative traits like shoot length, root length, fresh shoot weight, dry shoot weight, fresh root weight and dry root weight. Iron content in shoot and root was also estimated. Simultaneously visual scoring (0-9 scale) was done during the vegetative growth of plants. The recorded data were subjected to the factorial CRD analysis. Least square equation was used to find out the optimum dose.

Equation used of least square method was as: $Y= a+ b X$

Where normal equation for $a = \sum Y= na + b \sum x$

Where normal equation for $b = \sum XY= a\sum x + b \sum x^2$

Development of mapping population
Genetic material consisted of 48 individual lines derived from the F_4 of cross between the Swarna X IBD-1. The Swarna was observed as susceptible parent and on other hand IBD-1 was noticed as tolerant. The F_4 population was initially developed in the view of QTL mapping for tolerance of iron toxicity (Aluko et al. 2004). Therefore, both parents were chosen for their degree of resistance to Fe toxicity which was confirmed by the experiment. For screening of the F_4 population and the parents, they were grown hydroponically under high Fe conditions (120 ppm Fe^{+3} and 200 ppm of Fe^{+2}) as these doses were determined as optimum dose at which plant can sustain and also F_4 population was grown in field for taking observations on yield traits.

QTL Analysis
Plant Materials and PCR Marker Mapping 48 F_4 lines from a cross between Swarna and IBD-1 were selected for QTL mapping. A linkage map was constructed with 10 simple sequence repeat (SSR) markers. For QTL analysis, composite interval mapping (CIM)was applied using Windows QTL Cartographer ver. 2.5. A LOD score >3.0 was used as the empirical threshold for interval mapping (Yano and Sasaki 1997). Secondly, multiple interval mapping (MIM)was applied using the WinQTLCart to compensate a residual variance that was not explained by the CIM.

3. Result and Discussion

Disposable cup experiment/ Glass plate experiment:
In disposable cups and glass plates (rhizotron), all twelve genotypes were directly sown in sterilized soil and all genotypes were exposed to iron solution (Fe^{+2} and Fe^{+3}) details of these two experiment was not presented in this paper becausemixed performance was observed for all genotypes andleaf bronzing symptom was not clearly seen, it may be due high buffering capacity of soil which can adjust easily 40 and 80 ppm of iron solution and the another reason is that the soil we have used comes under red yellow soil. Thissoil is having low cation exchange capacity resultant high leaching losses. Due to high leaching losses, it become difficult to determine the iron effect on plant (CUCE 2007). In this way, we concentrated on hydroponics experiment, and on the basis of overall all performance of all genotypes for all vegetative traits we selected five genotypes (Dagaddeshi, IBD-1, R-RF-127, R-RF-110 and Swarna) for our final hydroponics experiment.

Hydroponics Experiment:
Observations recorded in hydroponics experiment, showed that genotype Dagaddeshi was found with superior performance at 40 ppm and controlled condition of ferric form (Fe^{+3}) respectively for shoot length and root length and variety Swarna was reported as susceptible variety for the sametraits at 200 ppm of ferric form of iron (Fe^{+3}). For the trait fresh shoot and root weight, genotype IBD-1 was found with superior performance respectively at controlled condition and 40 ppm of ferric chloride and Swarna was noticed with low performance at 200 ppm of ferric form (Fe^{+3}) for fresh shoot weight and R-RF-110 for fresh root weight. For dry shoot weight genotypes Dagaddeshi, IBD-1, R-RF-127 and R-RF-110 (0.35) found with superior performance at controlled condition and all genotypes found sensitive for 200 ppm of iron especially for Fe^{+3}. On other hand for dry root weight genotype IBD-1 was found with good performance at 40 ppm of ferrous form of iron (Fe^{+2}) and all genotypes namely dagaddeshi, IBD-1, R-RF-110 and Swarna (0.15) found sensitive for dry root weight at both form of iron (Fe^{+2} and Fe^{+3}). Above 200 ppm of iron, at 400 ppm, in Fe^{+2} form, only 50% plants survived after 4 weeks

of stress and in case of Fe^{+3} form, all the plant died after 2 weeks. At 600 ppm, in Fe^{2+}, the survival rate was about 70%, 30%, and 0% after 1, 2 and 3 weeks of stress, respectively. Thus, an increase trend of mortality was observed at the end of the month. In case of Fe^{+3}, all plants showed mortality/ died within 2 weeks. At 800 ppm, plants survived only for one week in Fe^{+2} and in Fe^{+3} plants died within 2 days. Thus, none of the data were available for this experiment.

After analysing the result of hydroponics experiments, we found that in all five rice varieties/genotypes four genotypes Dagaddeshi, IBD1, RRF110 and RRF127 were superior and Swarna was noticed as inferior genotype. Dry shoot weight and dry root weight was found more sensitive for higher dose of iron for both form and a decreasing trend for all vegetative traits were seen as there was increase in the doses of iron.

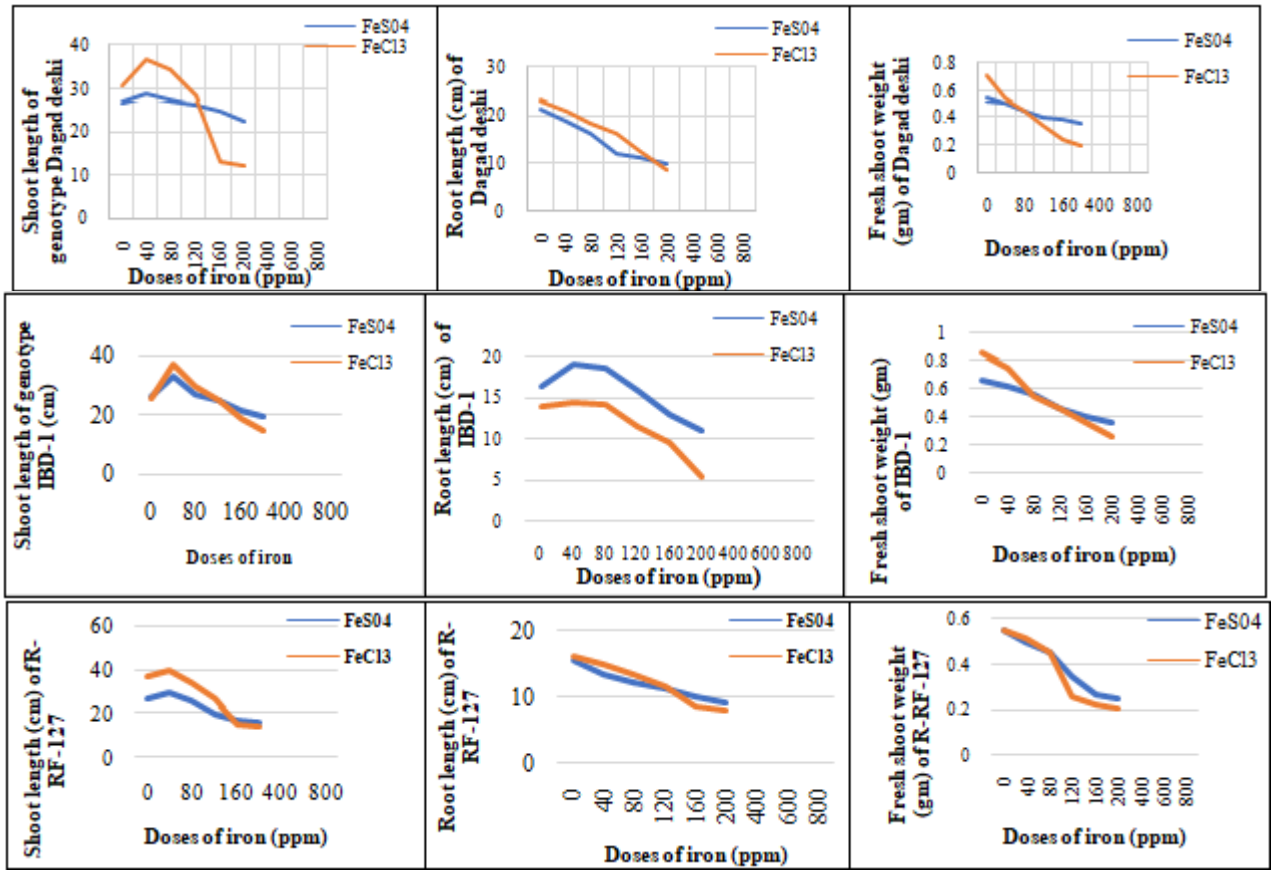
At the starting point of experiment, we found that Fe^{+2} form of iron is toxic than Fe^{+3} form, when we increase doses from controlled condition (0 ppm) to 80 ppm, but with higher doses (from 120 to 200 ppm) Fe^{+3} form became more toxic than Fe^{+2}. In two form of iron ferrous (Fe^{+2}) form of iron was reported as more toxic form as compared to the ferric form of iron (Fe^{+3}).In most of the cases, Fe^{+2} was reported as more toxic as compared to Fe^{+3}, the most probable reason behind more toxicity of Fe^{+2} is that Fe^{+2} ion is easily soluble in water compared to ferric ions (Fe^{+3}) at 25°C (Takagi, 1976), Fe^{+2} ions exist in highly soluble and reactive form in the plant tissues with Fe overload and even numerous Fe^{+2} ions might exist in free form in the cytosol, directly absorption of them by rice roots causes severe Fe toxicity reactions such as. Ferrous toxicity inhibits cell division and elongation of the primary roots and subsequently, the growth of lateral roots (Li *et al.*, 2015). The high deposition of ferrous form of iron in roots can be seen and browning of leaves is also common in the genotypes which were exposed to ferrous sulphate.

But the question is that why Fe^{+3} become more toxic with increasing iron doses (when doses increased up to 120 ppm). In fact, in case of ferric form of iron (Fe^{+3}), genotypes were hardlysurviving for one month at 200 ppm of Fe^{+3}, and all genotypes showed mortality above 200 ppm of iron either for Fe^{+3} or for Fe^{+2}. The reason behind more toxicity of Fe^{+3} at higher doseswas that, rice plants have a weak ability in Fe^{+3} reduction and phytosiderophore secretion. So, in response to Fe deficiency rice expresses the divalent metal transporters *OsIRT1* and *OsIRT2* in the root epidermis, these Fe^{+2} transporters (*OsIRT1* and *OsIRT2*) and *OsNRAMP1* gene were found to be dramatically up-regulated in the roots, 30-fold for *OsIRT1*, and 64-fold for *OsIRT2* in response to iron deficiency (Cheng *et al.*, 2007). It has been suggested that *OsNRAMP1* plays a role in Fe uptake and translocation from roots to the aerial parts, including rice grains. Hence, hydroponics with higher ferric form of iron without chelating agent may causes iron deficiency due to low solubility of ferric chloride, this led to induction of divalent iron transporters *OsIRT1* and *OsIRT2* and *OsNRAMP1* gene. In fact, in rice plants without the ability to synthesize PS, these Fe^{+2} transporters were found to be dramatically up-regulated in the roots. Surprisingly, these plants even accumulated more Fe in roots and shoots. In aerobic soils (where Fe is limiting) and in hydroponic solution, these plants died due to high reduction of ferric form to ferrous form by the reduction strategy (Nugraha*et al.*, 2016) hence, the reduction strategy with high Fe^{+3} without chelating agent or in Fe^{+2} limited conditions can causes death of plants. Another reason of early death of plants may be due to highly corrosive nature of ferric chloride; aqueous solution of ferric chloride without chelating agent undergoes hydrolysis and form hydrates, acid and precipitate as ferric hydroxide due to higher amount of ferric chloride. The chelating agent prevent precipitation, so in absence of chelating agent with high ferric iron causes precipitation, that causes root hair to die, this dead root can be seen with highest visible iron hydroxide content. The behavior of aqueous solution of ferric chloride undergoes hydrolysis and higher amount of ferric chloride is responsible for formation of hydrochloric acid, this strong acid release H^+ ion in the solution, hence, as a result, the solution becomes acidic and every one unit drop in the pH, Fe^{+3} becomes a 1000-fold more soluble (Richard *et al.*, 2005). Highly soluble ferric iron is corrosive in nature and also corrosive to tissues resulting death of plant cell and ultimately plant death.

After conduction of experiment, we reached at some points like decreasing trend of all vegetative traits was observed with increasing iron doses. Among five genotypes/ varieties Dagaddeshi, IBD-1, RRF127 and RRF110 were observed with superior performance (tolerant) and Swarna (susceptible) with low performance under both iron form. At first ferrous from was observed more as toxic form than ferric but ferric form of iron was found to be more toxic form as there is an increase in doses of ferric form of iron. In case of ferric form of iron (Fe^{+3}), genotypes survived with difficulty at 200 ppm of Fe^{+3} but in ferrous form all genotypes survived at 200 ppm, keeping in view these doses we chosen 120 ppm of ferric form and 200 ppm for ferrous form for further conduction of experiment.

Table 1: Performance of various genotype under different concentration of ferrous and ferric form of iron

	Traits	Shoot length		Root length		Fresh shoot weight		Fresh root weight		Dry shoot weight		Dry root weight		Iron in shoot		Iron in root	
	Doses (ppm)	$FeS0_4$	$FeCl_3$	$FeS0_4$	$FeCl_3$	$FeS0_4$	$FeCl_3$	$FeS0_4$	$FeCl_3$	$FeS0_4$	$FeCl_3$	$FeS0_4$	$FeCl_3$	$FeS0_4$	$FeCl_3$	$FeS0_4$	$FeCl_3$
Dagaddeshi	0	26.80	30.80	21.20	23.10	0.55	0.70	0.65	0.45	0.3	0.2	0.30	0.20	36.30	36.30	39.30	38.50
	40	28.80	36.80	18.50	20.80	0.51	0.55	0.60	0.60	0.35	0.41	0.35	0.41	262.50	255.50	261.00	274.00
	80	27.50	34.70	16.30	18.50	0.45	0.45	0.55	0.45	0.32	0.34	0.32	0.34	576.10	547.10	583.00	602.20
	120	26.10	28.50	12.20	16.20	0.40	0.35	0.45	0.45	0.30	0.16	0.30	0.16	752.90	780.90	753.00	786.00
	160	24.60	13.00	11.30	12.60	0.38	0.25	0.35	0.25	0.15	0.15	0.15	0.15	1013.70	992.70	993.70	1113.30
	200	22.10	12.20	9.90	8.60	0.35	0.20	0.30	0.23	0.15	0.15	0.15	0.15	1223.90	1443.90	1323.90	1396.10
IBD-1	0	26.70	25.70	16.30	13.80	0.65	0.85	0.65	0.63	0.30	0.20	0.30	0.20	32.30	30.30	34.20	23.65
	40	33.10	37.00	19.00	14.30	0.60	0.75	0.45	0.75	0.35	0.38	0.35	0.38	275.50	265.50	354.10	286.50
	80	27.00	29.80	18.50	14.00	0.55	0.55	0.35	0.37	0.32	0.35	0.32	0.35	563.10	521.10	561.90	618.10
	120	24.90	25.30	15.80	11.50	0.45	0.45	0.25	0.35	0.3	0.16	0.30	0.16	756.90	756.90	778.80	776.90
	160	22.10	18.90	13.00	9.50	0.40	0.35	0.24	0.28	0.15	0.15	0.15	0.15	890.70	913.70	875.60	915.60
	200	19.80	15.10	11.00	5.30	0.35	0.25	0.23	0.19	0.15	0.15	0.15	0.15	1313.90	1458.90	1213.90	1393.90
R-RF-127	0	27.30	36.80	15.50	16.20	0.55	0.55	0.55	0.65	0.30	0.20	0.30	0.20	28.30	26.30	30.30	32.60
	40	30.00	39.60	13.40	15.00	0.50	0.51	0.65	0.65	0.35	0.39	0.35	0.39	329.50	316.50	453.60	342.50
	80	26.70	33.70	12.10	13.40	0.45	0.45	0.62	0.55	0.32	0.37	0.32	0.37	512.10	499.10	567.60	543.10
	120	19.70	26.40	11.20	11.70	0.35	0.25	0.35	0.45	0.30	0.34	0.30	0.34	676.80	666.90	779.60	682.80
	160	16.90	15.30	10.10	8.70	0.27	0.22	0.32	0.35	0.15	0.15	0.15	0.15	913.70	916.70	937.70	984.90
	200	16.40	14.10	9.20	8.00	0.25	0.20	0.25	0.25	0.15	0.15	0.15	0.15	1126.90	1226.90	1137.90	1386.00
Swarna	0	22.00	22.00	17.90	12.50	0.3	0.7	0.7	0.3	0.20	0.30	0.20	0.30	33.30	33.30	36.30	36.90
	40	20.60	18.30	14.80	11.50	0.25	0.3	0.3	0.25	0.30	0.35	0.30	0.35	553.50	453.50	572.50	556.50
	80	18.20	18.20	14.50	11.20	0.29	0.25	0.25	0.29	0.16	0.32	0.16	0.32	582.10	517.10	617.30	597.10
	120	16.30	15.80	12.50	10.40	0.25	0.23	0.23	0.25	0.16	0.3	0.16	0.3	792.80	779.90	879.10	813.10
	160	14.50	13.20	8.60	9.10	0.2	0.22	0.22	0.20	0.15	0.15	0.15	0.15	1022.70	1122.80	998.70	986.60
	200	11.30	10.60	8.40	4.30	0.22	0.21	0.21	0.22	0.15	0.15	0.15	0.15	1461.90	1502.20	1503.20	1516.30
R-RF-110	0	27.90	28.20	15.70	18.00	0.43	0.45	0.35	0.55	0.25	0.4	0.25	0.40	30.30	28.30	29.30	26.80
	40	25.70	25.80	12.70	16.20	0.35	0.55	0.34	0.5	0.25	0.35	0.25	0.35	376.50	360.50	455.10	383.50
	80	22.00	24.30	11.20	13.70	0.34	0.5	0.32	0.45	0.22	0.32	0.22	0.32	566.10	512.10	576.10	663.50
	120	19.70	22.30	9.40	12.90	0.32	0.45	0.32	0.25	0.18	0.25	0.18	0.25	676.90	679.90	781.30	686.90
	160	18.20	13.70	8.00	7.30	0.32	0.25	0.25	0.15	0.15	0.15	0.15	0.15	924.40	1024.80	985.40	1034.70
	200	13.30	11.10	7.60	5.40	0.25	0.15	0.35	0.55	0.15	0.15	0.15	0.15	1260.90	1332.90	1263.90	1292.20



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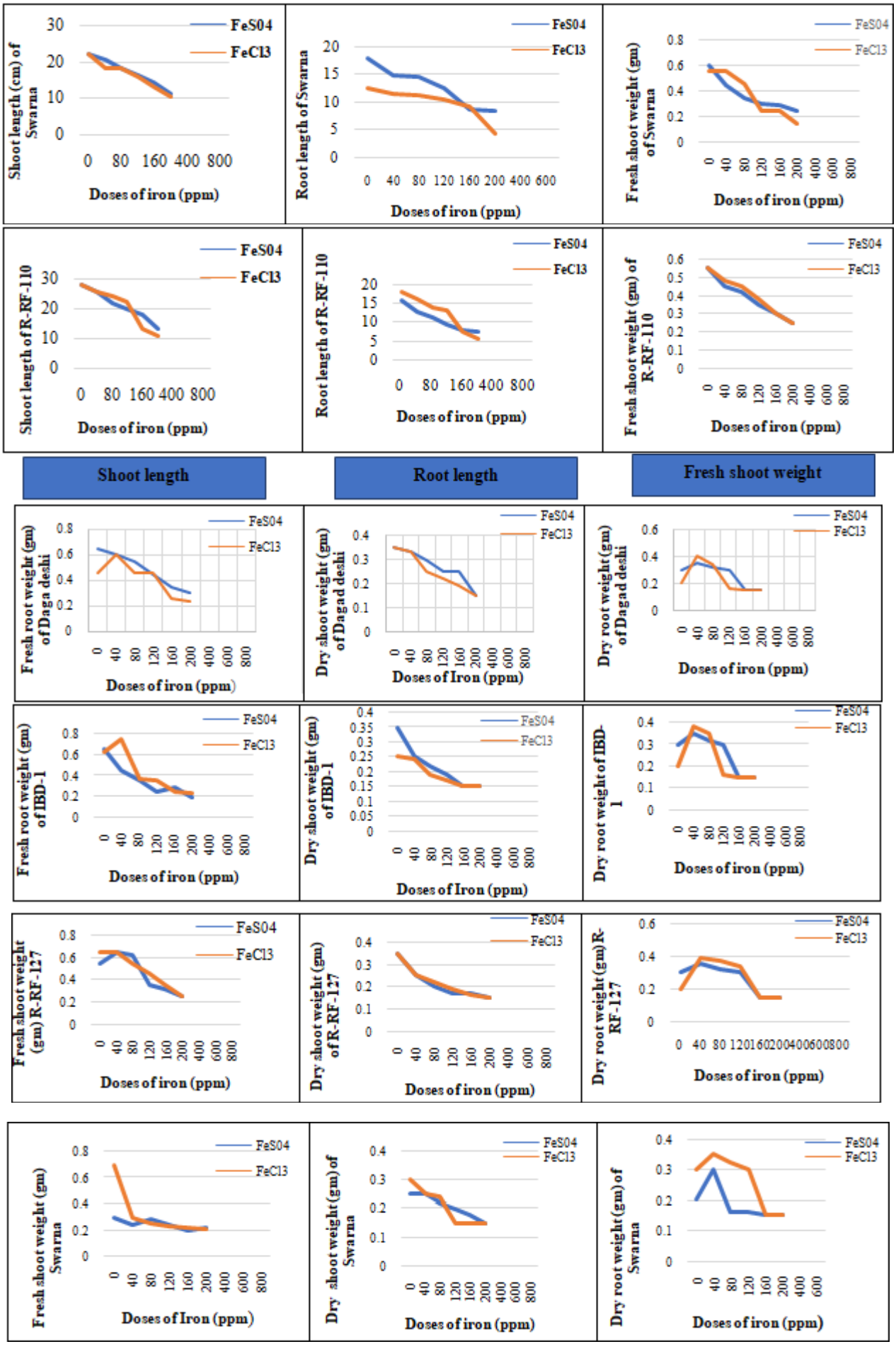


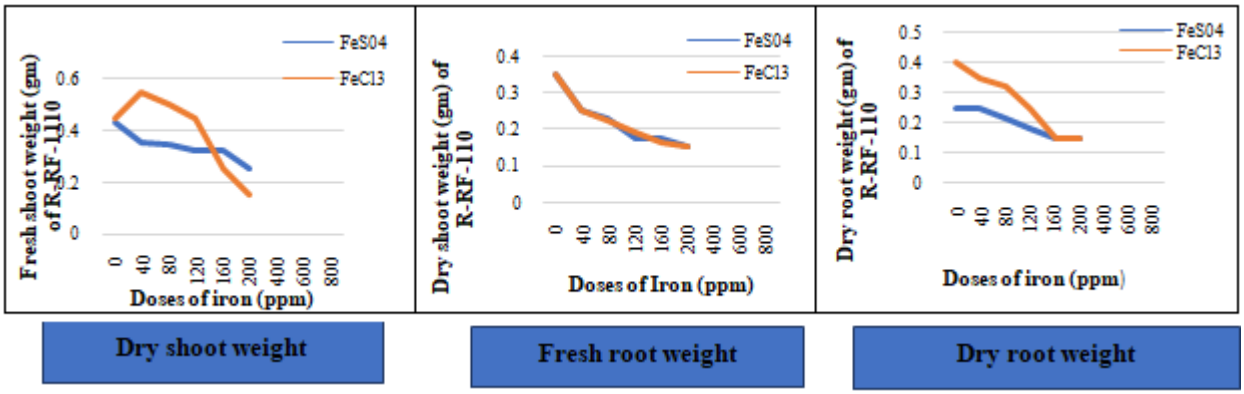


Figure: Performance of genotypes (Dagaddeshi, IBD-1, R-RF-127, Swarna and R-RF-110) at different doses (Controlled, 40, 80, 120, 160, 200, 400, 600 and 800 ppm) of iron form ($Feso_4$ and $FeCl_3$).

Table 2: least quare equation for determining the optimum dose of iron

	Shoot length			
Genotypes	**$Feso_4$**	**R^2**	**$Fecl_3$**	**R^2**
Dagaddeshi	$y = -0.4161x^2 + 1.8411x + 25.85$	$R^2 = 0.9514$	$y = -1.5643x^2 + 6.0757x + 28.46$	$R^2 = 0.8728$
IBD-1	$y = -1.9886x + 32.56$	$R^2 = 0.6541$	$y = -1.2911x^2 + 5.8432x + 24.43$	$R^2 = 0.7965$
R-RF-127	$y = -0.25x^2 - 1.13x + 30.58$	$R^2 = 0.8541$	$y = -0.7286x^2 - 0.4343x + 40.22$	$R^2 = 0.9251$
Swarna	$y = -0.1179x^2 - 1.2807x + 23.42$	$R^2 = 0.9951$	$y = -0.0804x^2 - 1.5718x + 23.07$	$R^2 = 0.9699$
R-RF-110	$y = -0.0839x^2 - 2.2068x + 30.13$	$R^2 = 0.982$	$y = -0.525x^2 + 0.1379x + 28.38$	$R^2 = 0.9604$
	Root length			
Dagaddeshi	$y = 0.2089x^2 - 3.8111x + 25.07$	$R^2 = 0.9829$	$y = -0.2446x^2 - 1.1275x + 24.29$	$R^2 = 0.9981$
IBD-1	$y = -0.5839x^2 + 2.7389x + 14.87$	$R^2 = 0.9202$	$y = -0.5411x^2 + 2.0904x + 12.29$	$R^2 = 0.9931$
R-RF-127	$y = 0.1214x^2 - 2.0586x + 17.28$	$R^2 = 0.994$	$y = -0.0554x^2 - 1.3725x + 17.81$	$R^2 = 0.9803$
Swarna	$y = 0.0018x^2 - 1.9582x + 19.61$	$R^2 = 0.9466$	$y = -0.4107x^2 + 1.475x + 10.9$	$R^2 = 0.939$
R-RF-110	$y = 0.2393x^2 - 3.2864x + 18.64$	$R^2 = 0.9951$	$y = -0.2304x^2 - 0.9732x + 19.15$	$R^2 = 0.9704$
	Fresh shoot weight			
Dagaddeshi	$y = 0.0038x^2 - 0.0674x + 0.619$	$R^2 = 0.9917$	$y = 0.0089x^2 - 0.1625x + 0.85$	$R^2 = 0.998$
IBD-1	$y = - 0.0629x + 0.72$	$R^2 = 0.9878$	$y = 0.0071x^2 - 0.1729x + 1.03$	$R^2 = 0.9915$
R-RF-127	$y = 0.0005x^2 - 0.0692x + 0.629$	$R^2 = 0.9762$	$y = -0.0806x + 0.6453$	$R^2 = 0.9167$
Swarna	$y = -0.0018x^2 - 0.0761x + 0.66$	$R^2 = 0.9263$	$y = -0.0886x + 0.6767$	$R^2 = 0.9255$
R-RF-110	$y = -0.0018x^2 - 0.0478x + 0.596$	$R^2 = 0.9912$	$y = -0.0018x^2 - 0.0478x + 0.596$	$R^2 = 0.9912$
	Fresh root weight			
Dagaddeshi	$y = -0.0036x^2 - 0.0493x + 0.71$	$R^2 = 0.9869$	$y = -0.0187x^2 + 0.0698x + 0.445$	$R^2 = 0.8016$
IBD-1	$y = 0.0191x^2 - 0.2169x + 0.831$	$R^2 = 0.9636$	$y = 0.0077x^2 - 0.1552x + 0.855$	$R^2 = 0.7976$
R-RF-127	$y = -0.0152x^2 + 0.0274x + 0.591$	$R^2 = 0.8069$	$y = -0.0089x^2 - 0.0232x + 0.7$	$R^2 = 0.9866$
Swarna	$y = -0.0002x^2 - 0.0156x + 0.309$	$R^2 = 0.6647$	$y = 0.0377x^2 - 0.3412x + 0.941$	$R^2 = 0.8779$
R-RF-110	$y = 0.0016x^2 - 0.0401x + 0.451$	$R^2 = 0.8654$	$y = -0.0286x^2 + 0.13x + 0.37$	$R^2 = 0.952$
	Dry shoot weight			
Dagaddeshi	$y = -0.005x^2 - 0.0019x + 0.354$	$R^2 = 0.9472$	$y = 0.0018x^2 - 0.0539x + 0.41$	$R^2 = 0.9764$
IBD-1	$y = 0.0082x^2 - 0.0955x + 0.428$	$R^2 = 0.9756$	$y = 0.003x^2 - 0.0438x + 0.299$	$R^2 = 0.9563$
R-RF-127	$y = 0.0107x^2 - 0.1113x + 0.442$	$R^2 = 0.9777$	$y = 0.008x^2 - 0.0934x + 0.425$	$R^2 = 0.9762$
Swarna	$y = -0.002x^2 - 0.0071x + 0.263$	$R^2 = 0.984$	$y = 0.0052x^2 - 0.0688x + 0.369$	$R^2 = 0.9172$
R-RF-110	$y = 0.008x^2 - 0.0934x + 0.425$	$R^2 = 0.9762$	$y = 0.008x^2 - 0.0934x + 0.425$	$R^2 = 0.9762$
	Dry root weight			
Dagaddeshi	$y = -0.013x^2 + 0.0521x + 0.277$	$R^2 = 0.8484$	$y = -0.0145x^2 + 0.0667x + 0.221$	$R^2 = 0.4563$
IBD-1	$y = -0.0146x^2 + 0.0702x + 0.208$	$R^2 = 0.473$	$y = -0.0146x^2 + 0.0702x + 0.208$	$R^2 = 0.473$
R-RF-127	$y = -0.013x^2 + 0.0521x + 0.277$	$R^2 = 0.8484$	$y = -0.0291x^2 + 0.1752x + 0.095$	$R^2 = 0.7296$
Swarna	$y = 0.0004x^2 - 0.0225x + 0.26$	$R^2 = 0.4088$	$y = -0.013x^2 + 0.0521x + 0.277$	$R^2 = 0.8484$
R-RF-110	$y = -6E-17x^2 - 0.024x + 0.284$	$R^2 = 0.9333$	$y = -0.0005x^2 - 0.0511x + 0.457$	$R^2 = 0.9577$

Different graphs showing behaviour of all genotypes for various vegetative traits at different doses of both forms Fe^{+2} and Fe^{+3}. Here by fitting least square method we found that the physical optimum value was 40 ppm for almost all of the trait as on this value all plants were survived well, further to see the effect of doses on different traits we calculate the how much percent change occurs with each increasing doses and the result of percent change was presented below.

Percent change with increasing doses of iron over controlled condition

For the trait shoot length value of percent change showed that firstly, shoot length increased from controlled condition for 40 ppm after that as well as doses increased shoot length get decreased with high percent change for both iron form Fe^{+2} and Fe^{+3} but for Dagaddeshi, IBD-1 and R-RF-127, shoot length value showing increased value from controlled condition up to 80 ppm after that they showed decreasing trend. Similarly, for dry shoot weight and dry root weight

decreasing trend was observed with increasing doses, all genotypes showed decreasing value of root length compared to controlled condition for both iron forms, only one genotype IBD-1 showed an increased value up to a dose of 80 ppm after that value get decreased with increasing doses, similarly fresh shoot & root weight also showed low performance with each increasing doses, beside all we observed that dry shoot weight and dry root weight was the most affected trait as it showed the lowest value at high doses of either form of iron.

Leaf bronzing Score:

Here selected five rice genotypes were taken for screening for their tolerance to iron toxicity. The extent of tolerance was assessed based on the degree of leaf bronzing on exposure to iron stress. The result attained was that in controlled condition (0 ppm), none of the variety showed bronzing, as it didn't have high level of iron either F^{+2} or F^{+3} form. As far as 40 and 80 ppm doses were concerned, the results indicate that leaf bronzing scores differed in 40 ppm dose as compared to 80 ppm. Four varieties, Dagaddeshi, Indira Barani Dhan 1, RRF 110 and RRF 127 showed moderate scores of tolerances. Among all the five genotypes, at a higher concentration of Fe (80 ppm) Dagaddeshi, R-RF-127, R-RF-105 and IBD-1 exhibited the LBI <6.0. Genotypes Swarna with a score of 7.6 considered highly susceptible to Fe stress. Further leaf bronzing score at 200 ppm showed that RRF 110 showed moderate tolerance followed by Dagaddeshi, IBD1 and RRF 127 and again Swarna reported with susceptible reaction.

Iron toxicity occurs when the rice plant accumulates a toxic concentration of Fe in the leaves (Sahrawat, 2010). The degree of leaf bronzing has been suggested to be a good measure of the severity of Fe toxicity (Fageria*et al*, 2003). At 40 ppm of Fe, genotypes Dagaddeshi, IBD1, RRF 110 and RRF 127 showed lower leaf bronzing score (<5) as per standard evaluation score (IRRI, 2006). Existence of such variability at genotypic level across genotypes in response to Fe toxicity has been reported earlier. Several rice cultivars have been reported to be tolerant/ resistant to this constraint (Gunawardena *et al*, 1982; Fageria and Rabelo, 1987; Fageria*et al*, 1990; De Datta *et al*., 1994; Sahrawat and Sika, 2002; Salirawat, 2004; Shimizu *et al*., 2005; Balasubramanian *et al*., 2007; Nozoe*et al*., 2008; Majeni*set al*., 2009; Sahrawat, 2010; Samaranayake *et al*, 2012 and Onaga *et al*., 2013a).

Table 3: Leaf bronzing Score for Selected five genotype

Genotypes	Leaf bronzing score		
	40 ppm of Fe^{+2}	80 ppm of Fe^{+2}	200 ppm of Fe^{+2}
Dagaddeshi	4.1	5.0	5.2
IBD-1	4.5	5.9	5.3
R-RF-110	4.4	4.7	4.9
R-RF-127	4.8	5.7	5.8
Swarna	7.6	9.0	9.0
Mean	5.08	6.06	6.04

On the basis of leaf bronzing score again we reached at same conclusion that genotypes Dagaddeshi, IBD-1, RRF127 and RRF110 were observed with superior performance at each dose of 40, 80 and 200 ppm of iron (Fe^{+2}). After identification of tolerant and susceptible genotypes, crosses were attempted in all possible combination b/w all tolerant and susceptible genotypes in which sufficient F_1 seeds were obtained from cross between Swarna/IBD-1. F_1 seeds were further sown to get F_2 seeds and finally F_4 seeds were obtained and 46 lines of F_4 generation were sown in the field (rain out shelter) along with the parents. Observations on all yield traits were recorded from 46 lines including parents. Simultaneously, hydroponics experiment was also conducted taking 120 ppm of Fe^{+3} (ferric chloride) and 200 ppm of Fe^{+2} (ferrous sulphate) as an optimum dose. The data on vegetative traits such as shoot length, root length, fresh shoot weight, fresh root weight, dry shoot weight and dry root weight of all 46 lines were recorded; moreover, iron content in shoot and root was also recorded.

QTL Analysis

The mapping population for generating phenotyping data in this study was F_4 segregating population developed by crossing two *indica* genotypes Swarna and Indira Barani Dhan 1 which showed a low level of difference at DNA levels. As high level of polymorphism attributable to wide variation facilitates in the construction of linkage maps and QTLs mapping. Many authors however emphasized the necessity of QTLs identification based on variation from the crosses between two related varieties belonging to same subspecies so as to make rice breeding fruitful (Yano and Saski, 1997; Redone and Mackill, 1996). Keeping this view in mind the present cross combination was used for this study.

Development of genotypic data based on SSR markers:

Total genomic DNA was extracted from 48 lines of rice along with both the parent using modified CTAB method. The DNA samples were then subjected to quantification using Nano Drop Spectroscopy. The quantity of the samples was found in the ranged from 412-4375 ηg/µl. DNA samples were then diluted with sterile water such that the final concentration of DNA becomes 50ηg/µl.

After standardization of the PCR protocol for SSR assay, the DNA of selected lines along with the parents was subjected to PCR based simple sequence repeat (SSR) technique to generate genotypic data using rice SSR primers and phenotypic data were recorded on vegetative traits like shoot length, root length, fresh shoot weight, fresh root weight, dry shoot weight, dry root weight (Hydroponics) and yield traits (field condition) from all 48 lines exposed to 120 ppm of Fe^{+3} and 200 ppm of Fe^{+2} and sown in field.

Parental polymorphism analysis using SSR primers:

A set of 112 primers were used in this study for amplification of genomic DNA of mapping population through PCR. Out of 112 SSR primers (Table 4.18), 10 primers showed parental polymorphism were selected (Table 4.19). Primer showing polymorphisms were further used for PCR amplification with all of the 48 lines along with parents using standardized PCR protocol. The markers were taken from previously published rice genetic and sequence maps (Singh *et al*., 2010, IRGSP, 2005; McCouch *et al*., 2002; Temnykh*et al*., 2001). PCR products were separated on 5 % PAGE gel containing 3µl/ 100 ml EtBr. The electrophoresis was carried for 1.5 hours at 125 volts to allow separation of amplified product.

Identification of QTLs:

Two approaches were used for identification of QTLs. They were interval mapping (IM) and composite interval mapping (CIM). The feature of IM is that it uses two markers at a time known as flanking markers.

By taking interval mapping approach into consideration, total two major QTLs were identified on chromosome 1, 5 and 8 for the traits viz., dry root weight in $FeCl_3$; and dry root weight in $FeSO_4$ (FESODRW).In composite interval mapping approach, a total of twenty- four major and minor QTLs were detected, of these QTLs, ten were the major QTLs

For plant height, a significant and major QTL was observed on C#8 between markers RM 152 – RM 264 having the LOD value of 2.7 with PVE of 37.43%. The trait, shoot length in Fe^{+3} forms, the QTL was recorded on C#8 with 2.6 and 57.49 % of LOD and PVE. For dry shoot weight in Fe^{+3} forms, a significant and major QTL was observed on C#1 between markers RM 5 – RM 431 having the LOD value of 3.0 with PVE of 12.88%. Likewise, for dry root weight in Fe^{+3} forms, two QTLs were reported on C#1 and 5 with the LOD value of 6.0 and 7.0, and 13.21 and 10.20 % phenotypic variance explained, respectively.

For the trait, iron content in shoot in Fe^{+3} forms, a significant QTL was found on C#8 (LOD = 3.2; PVE = 60.67%). On the same chromosome, for the trait shoot length in Fe^{+2} forms were also observed (LOD = 2.9; PVE = 20.10%).

On chromosome #5, QTL for the traits namely, (LOD = 2.8; PVE = 21.12%) dry root weight in Fe^{+2} forms (LOD = 3.5; PVE = 21.73%) was detected. For shoot length in Fe^{+2} forms, a QTL was identified on chromosome #1 (LOD = 2.5; PVE = 10.16%).

Table 4: Putative QTLs with interval mapping (IM) in the F_4 population of Swarna x Indira Barani Dhan1

Trait[#]	C# [a]	Position	LR	Marker Interval	LOD	PVE (%)[b]	Add. effects[c]	Dom. effects[d]
4	1	0.3601	48.28	RM 5 – RM 431	10.0	3.77	-0.30	15.22
4	5	0.0501	49.38	RM 413 – RM 440	10.8	0.00	0.21	15.19
4	8	0.6301	46.84	RM 152 – RM 264	10.8	0.05	0.17	15.28
7	8	1.2301	25.68	RM 152 – RM 264	5.8	5.96	0.72	10.89
16	1	0.0601	15.37	RM 5 – RM 431	3.4	2.88	0.03	0.74
16	5	0.0901	15.86	RM 413 – RM 440	3.4	3.69	0.02	0.73
17	**1**	**0.3701**	**38.95**	**RM 5 – RM 431**	**8.0**	**17.96**	**-0.16**	**0.82**
17	5	0.4101	39.1	RM 413 – RM 440	8.0	2.92	0.15	0.83
17	8	1.0801	31.04	RM 152 – RM 264	6.0	1.38	0.07	0.89
30	8	0.7501	14.3	RM 152 – RM 264	3.2	0.25	-0.16	0.63
31	**5**	**0.0901**	**14.81**	**RM 413 – RM 440**	**3.2**	**27.76**	**0.10**	**0.51**

[#] 4 = Panicle length (PL); 7 = Number of unfilled spikelets (NUFS); 16 = Dry shoot weight in $FeCl_3$ (FECLDSW); 17 = Dry root weight in $FeCl_3$; 30 = Dry shoot weight in $FeSO_4$ (FESODSW); 31 = Dry root weight in $FeSO_4$ (FESODRW)

[a] C# = chromosome number

[b] The percentage of phenotypic variation explained by each QTLs

[c] Additive Effects

[d] Dominance Effects

Table 5: Putative QTLs with composite interval mapping (CIM) in the F_4population of Swarna x Indira Barani Dhan1

Trait	Chromosome	Position	LR	Marker Interval	LOD	PVE (%)	Additive	Dominant
1	1	0.3001	16.89	RM 5 – RM 431	3.5	3.11	-8.00	-26.45
2	5	0.6201	14.97	RM 413 – RM 440	3.2	2.65	4.40	-19.78
2	**8**	**1.1701**	**12.34**	**RM 152 – RM 264**	**2.7**	**37.43**	**4.14**	**-17.11**
4	1	0.2401	34.75	RM 5 – RM 431	7.0	1.31	-0.19	9.80
4	5	0.0401	42.69	RM 413 – RM 440	9.0	2.12	0.37	14.22
4	8	0.8301	35.01	RM 152 – RM 264	7.0	1.09	-0.19	10.03
6	5	0.0501	14.79	RM 413 – RM 440	3.2	1.09	1.49	66.44
7	8	0.1801	22.38	RM 152 – RM 264	4.8	0.18	0.29	11.46
7	8	0.4401	23.43	RM 152 – RM 264	4.8	1.20	0.86	11.49
7	8	1.2201	26.42	RM 152 – RM 264	6.0	6.94	0.77	11.32
12	**8**	**0.6401**	**12.14**	**RM 152 – RM 264**	**2.6**	**57.49**	**3.37**	**10.58**
16	**1**	**0.0701**	**14.28**	**RM 5 – RM 431**	**3.0**	**12.88**	**0.08**	**0.76**
16	8	0.2901	18.14	RM 152 – RM 264	4.0	1.17	-0.15	0.67
17	**1**	**0.3701**	**28.67**	**RM 5 – RM 431**	**6.0**	**13.21**	**-0.07**	**0.73**
17	**5**	**0.4701**	**36.55**	**RM 413 – RM 440**	**7.0**	**10.20**	**0.15**	**0.83**
17	8	0.8701	25.62	RM 152 – RM 264	5.5	8.97	0.03	0.75
17	8	0.9801	25.55	RM 152 – RM 264	5.5	7.81	0.03	0.75
18	**8**	**0.8501**	**14.82**	RM 152 – RM 264	**3.2**	**60.67**	**-172.72**	**153.00**
26	**1**	**0.0401**	**11.59**	**RM 5 – RM 431**	**2.5**	**10.16**	**1.26**	**-13.54**
26	**8**	**1.2201**	**12.78**	**RM 152 – RM 264**	**2.9**	**20.10**	**1.93**	**-13.11**
31	**5**	**0.1201**	**16**	**RM 413 – RM 440**	**3.5**	**21.73**	**0.10**	**0.56**

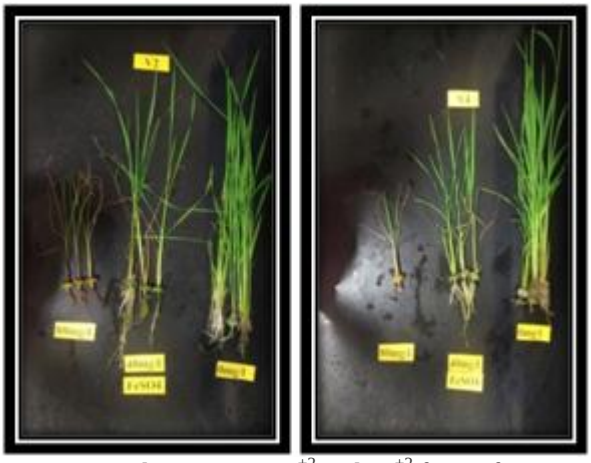
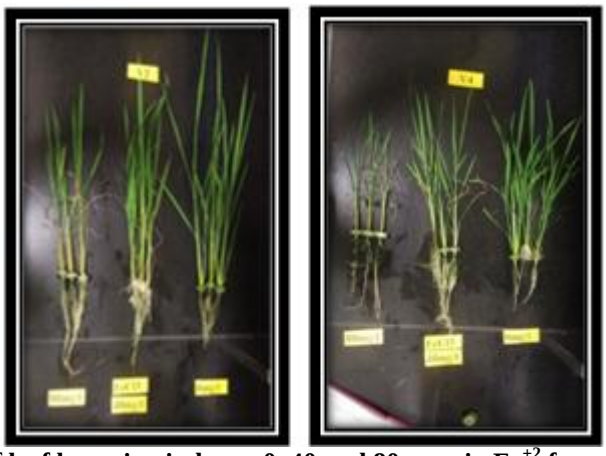
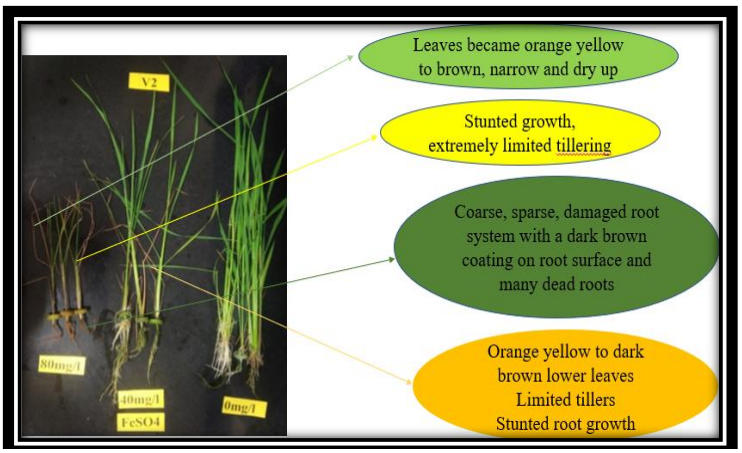
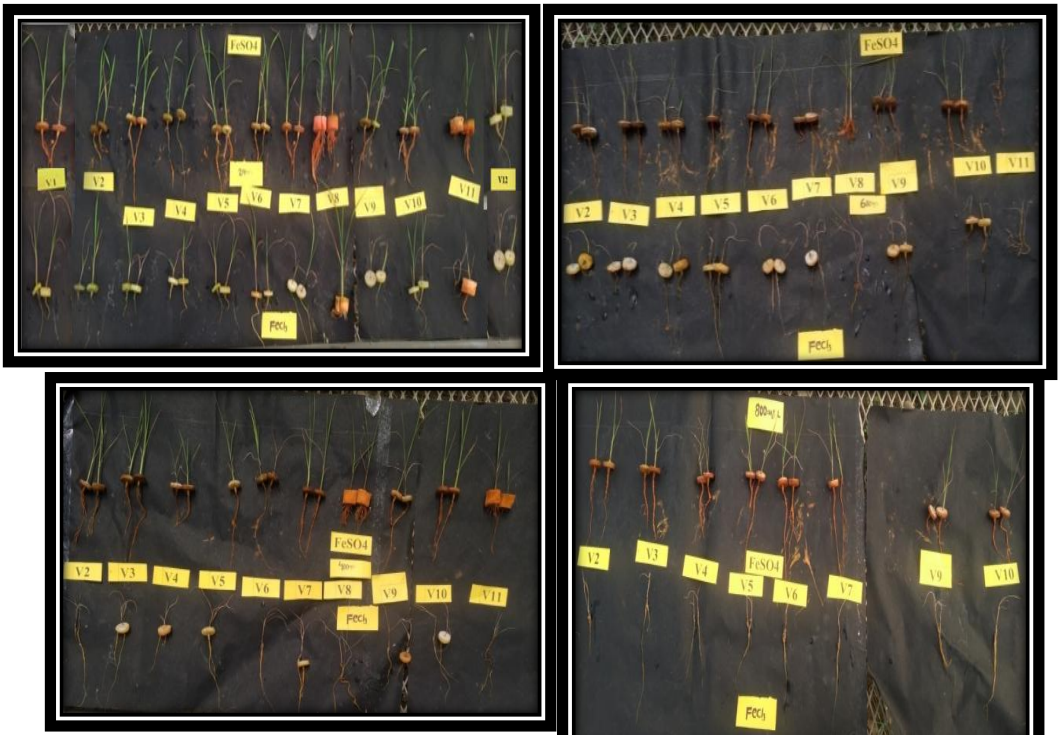


Figure: Leaf bronzing at 0, 40 and 80 ppm in Fe^{+2} and Fe^{+2} form of iron in Indira Barani Dhan 1



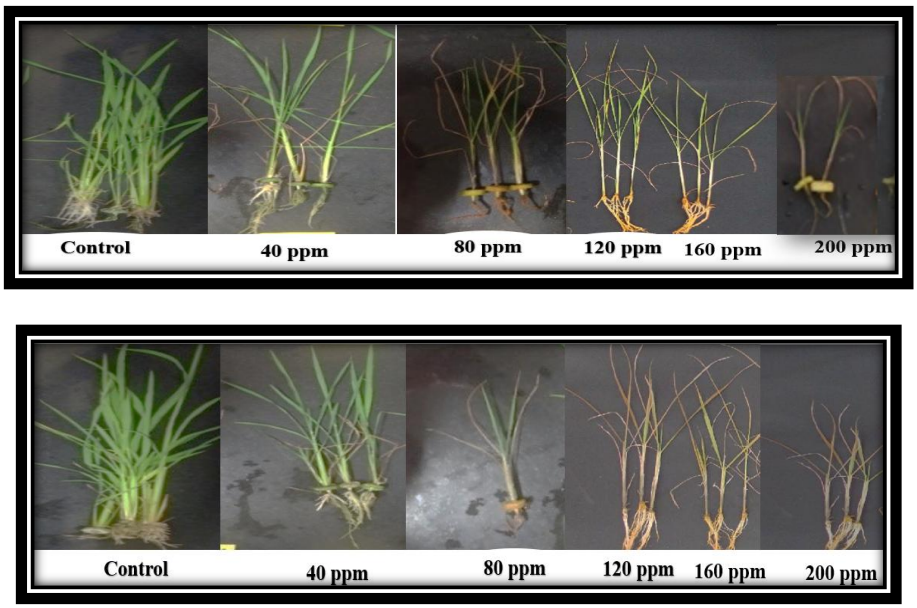
Visual scoring of leaf bronzing index at 0, 40 and 80 ppm in Fe^{+2} form of iron in IBD 1





V1 Dagaddeshi V2 = IBD 1; V3 = Samleshwari; V4 = Swarna; V5 = Sahbhagi; V6 = Danteshwari; V7 = RRF 127; V8 = MTU 1010; V9 = IR 64; V10 = RRF 110; V11 = Indira Aerobic V12 CR-Dhan-201

Figure: Screening of rice varieties at 200, 400, 600 and 800 ppm of Fe^{+2} and Fe^{+3} form of iron



Performance of Indira Barani Dhan 1 in Hydroponics (Yoshida) at 0, 40, 80, 120, 160, and 200 ppm of Fe^{+2} form of iron
Performance of Swarna in Hydroponics (Yoshida) at 0, 40, 80, 120, 160, and 200 ppm of Fe^{+2} form of iron

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