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Differential responses by upland and lowland cultivars of rice (*Oryza sativa* L.) under iron toxicity

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ABSTRACT

Iron is an important micronutrient for plants along with the food crops. However, in fluctuating environmental condition it can bring abiotic stress to plants. Among food crops, rice is mostly cultivated in the country. On the basis of growing conditions, especially the lowland rice cultivars, they undergo stress from iron toxicity. The experiment was conducted in the department of Life Science and Bioinformatics, Silchar during khari to season of 2017. In the present work, two contrasting varieties: Khandagiri (Upland cultivar) and Chilarai (lowland cultivar) were grown under excess iron treatment with concentration of 5, 10 and 15 mM. The dry weight of Chilarai had decreased upto 65% at 15 mM at 72 hours whereas, it was 47.8% in case of Khandagiri. At 15 mM, H_2O_2, MDA and superoxide radical content of Khandagiri increased by 60.65%, 46% and 48.7% whereas, in Chilarai, it increased by 75%, 86% and 59.4% in roots, respectively. Similar increase was observed in case of shoots and the content was recorded higher in Chilarai. Expression of OsIRT1 and OsIRT2 was reported in both the varieties however, expression of OsTOM1 was negligible under Fe^{+2}stress. Between these two cultivars, Chilarai was recorded to be more susceptible to iron toxicity.

Keywords: *Oryzasativa*, ROS, iron (II), varietal differences, gene expression

INTRODUCTION

Rice (*Oryza sativa* L.) is the main food crop of India and it has become a self-sufficient nation in food grains since green revolution. Depending upon the altitude and climatic changes, rice has different growing systems and therefore, rice is of two types: lowland and upland varieties. The lowland rice varieties are mostly grown under waterlogged condition where irrigation is continuous and proper along with the maintenance of deep water condition. Upland varieties, on the other hand, grow in sloped condition having un-bunded land where the water runoff easily. These lands therefore, need supplemental irrigation system (Saito *et al,* 2018). Among all the micronutrients, iron is one such element which plays a pivotal role in its respiration, cell division, and electron transport chain and chlorophyll synthesis. Any fluctuation from its normal range may result into metal stress in plants. Occurrences of iron toxicity for the crops depend on the number of factors: type of soil, amount of exchangeable Fe^{+2} and soil pH. The water logged condition provides a low amount of oxygen in the rhizosphere. This is due to the utilization of oxygen by the microbes of soil and also plant roots for its respiration. This environment leads to the reduction of Fe(III)to its most soluble form i.e. Fe(II)which is readily taken up by the plants (Asch *et al,* 2005).On the other hand, upland rice varieties are not affected by the presence of Fe^{2+} in the medium due to continuous drainage. Therefore, mainly the iron remains in Fe (III) form. Reportedly, in case of iron homeostasis, plants follow two strategies for its uptake from the soil: Strategy I includes the reduction of Fe^{2+} in the rhizosphere and is transported inside the root cells through appropriate carriers. This strategy is mainly found in dicotyledonous plants. Strategy II includes the formation of complex of Fe (II) and chelators exudated by plants which also have specific carriers. Such strategies are mainly maintained in monocotyledons. Rice, being a monocotyledon, maintains Strategy II mechanism as they acquire iron from soil by secreting mugienic acids (MA) from their roots to solubilise Fe^{3+} in the soil. These chelators are better known as phytosiderophores. But under flooded environment the crop scan switch to Strategy I

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mechanism depending upon the availability of Fe^{+2} in the medium (Kobayashi and Nishizawa, 2012). Several iron transporters have been identified in rice plants those are actively involved in iron uptake as well as translocation. The iron regulated transporter, OsIRT1 belonging to the ZIP family is a major route for iron transportation as it can transport Fe^{+2} directly from the rhizosphere. In case of Strategy II, the phytosiderophores need to be transported from the plants outside the root cells. Carriers like Transporters of mugineic acids (TOM1), are the set of transporters which performs the job and was reported first in rice and barley (Nozoye *et al,* 2011). The MA-Fe(III) complex is in turn later taken up by the root cells with help of Yellow Stripe like protein (YSLs) (Kobayashi and Nishizawa, 2012).

Iron toxicity proves a major constraint in productivity as it acts as the growth limiting factor. The toxic effect can bring physiological and biochemical changes altering different pathways involved. These changes are due to the differential expression of functional genes and transcription factors which has been reported under various fluctuating iron content in the medium. The bronzing of the leaves are the common symptoms that are observed due to the high iron content in the soil. The leaves develop brown spots which initially starts developing from the tip. With extended stress, the browning starts spreading and curling of leaves are observed. The chlorophyll content is also reduced. The physiological parameters like root and shoot length along with their biomass is reportedly decreased under iron toxicity (Baruah *et al,* 2007). Excess of ferrous concentration in the plant cells can bring extensive biochemical changes. The foremost change that take place is the generation of reactive oxygen species (ROS) like hydroxyl radicals by the catalyzing effect of the ferrous ion. These free radicals bring oxidative damage to membrane lipids, proteins and nucleic acids (Hell and Stephan, 2003). A laboratory experiment was conducted to compare the response of three different concentrations of Fe(II) i.e. 5mM, 10mM and 15 mM in the medium to rice cultivars.

MATERIALS AND METHOD

The variety Khandagiri was collected from Orissa University of Agriculture and Technology (OUAT),Bhubaneshwar, Odisha. The variety Chilarai was collected from the Regional Rice Research Station (RARS) located in Karimganj district of Assam, India. An adequate amount of seeds from each varieties was taken and surface sterilised in 0.1% of mercuric chloride ($HgCl_2$) and thoroughly rinsed 3- 4 times for 5 minutes. The selected seeds were soaked in water for 12 hours, then placed in petriplates with moistened filter paper and incubated at 28 ± 2 °C for germination. Once, seedlings are visible, these were transferred to Hoagland solution with pH adjusted to 6.2 by using 0.1M NaOH or 0.1M HCl. The plants were grown for 7 days under 2200 Lux with photon flux density 220 µmol m^{-2} s^{-1} (PAR) with 14 hour of photoperiod. The solution was changed in 2 days intervals. On the 8^{th} day of the treatment, iron was supplied in the form of Ferrous sulfate at the concentrations of 5, 10 and 15mM. One control is maintained for each varieties. The pH of the stressed solution was maintained at 5.2.The length of root and shoot was measured at 24, 48 and 72 hours. Accordingly, the rate of root and shoot elongation were observed by measuring the root tolerance index (RTI) and shoot tolerance index (STI) at the highest concentration i.e 15 mM. Tolerance index (TI) for the plants were calculated by the formula:TI = (mean root or shoot length of stressed plant*100)/ mean root or shoot length of control plants.

The fresh weight of shoot and root was measured for 24, 48 and 72 hours. For the determination of dry weight, the shoots were separated from the roots and were washed with deionised water. The samples were then kept in the oven at 60 °C for 3 to 4 days. Relative decrease in shoot dry weight (RDSDW) and Relative decrease in root dry weight (RDRDW) were obtained by the formula:

RDSDW= (SDW of control – SDW under Fe^{2+})/SDW in control

Biochemical Analysis: The total peroxide content of the tissue was estimated by the procedure of Sagisaka (1976).The superoxide radical ($O^{\cdot-}$) was estimated by the protocol suggested by Eltsner and Heupel (1976) by monitoring the nitrate formation from hydroxylamine. The lipid content in the form of Malonaldehyde (MDA) was estimated by the

protocol suggested by Heath and Packer (1968). Chlorophyll content measured by the protocol suggested by Arnon, (1949). For the evaluation of the significant differences of growth among the various cultivars of rice, analysis of variance (ANOVA) was performed, and the means were compared through Student t-test at 0.05 probability using MS Excel, 2007.

Gene expression analysis: 100 mg of frozen tissue was thoroughly homogenized in liquid nitrogen using a pre-chilled Rnase free mortar and pestle. Total RNA was isolated using RNA sure plant mini Kit (Nulceo-pore). The first strand cDNA synthesis were performed using Revert Aid TM First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, MA, USA). The concentration of RNA was determined spectrophotometrically at 260 nm. The RNA purity was determined spectrophotometrically by means of the 260/280 ratio and checked electrophoretically using a garose (1.2%). Four pairs of PCR primers were designed to amplify the expression of OsIRT1, OsIRT2 and OsTOM1 along with actin. The PCR reactions were performed in a final volume containing1µl10xPCRbuffer,.8µl$MgCl_2$,.8µl dNTP (2.5mM), .6µl primers(5 µM), 1U taq polymerase, 1U template DNA (cDNA) for 30 cycles (94 ^{0}C for 30s,65 ^{0}C for 30s, 72 ^{0}C for 30s) followed by final extension of 5 min at 72 ^{0}C. Finally, 10 ml of reaction volume was separated using a garose (1.5% w/v) gel. Table 1 show the primers used.

Table 1: Primers used for semi-quantitaive RT PCR analysis

Gene name	Primer
*Os*Actin	Forward: ATGGCTGACGGCGAGGACATC
	Reverse: CAATACCATGCTCGATCGGGTA
*Os*IRT1	Forward: AGCCGTGGTCGGAGTTCCC
	Reverse: ACCTGAACGACGACGCGG
*Os*IRT2	Forward: CGTTCGCGTCGGGCGTCAT
	Reverse: GCAGCCACGTCTGCTTGCC
*Os*TOM1	Forward: CTTGGTGCTCTAAATGGCA
	Reverse: CCGGTAGCCATATGCAGCT

RESULTS AND DISCUSSION

Morphological response

When toxicity hits seedling and vegetative phase of any growing plants,it could have adverse effect on morphological and physiological changes. During vegetative stage it brings reduced plant height and dry weight (Asch *et al,* 2005). With increasing concentrations from 5mM to 15 mM, Khandagiri and Chilarai underwent reduction in their both root and shoot length. Extending the stress up to 72 hours, the varieties showed inhibitory effect in its growth. Decline in length was observed in both the varieties from 48 to 72 hours (Table 2 and 3).

Table 2: Effect of iron stress on shoot length at 24, 48 and 72 hours. The values were represented as the mean ± SE (n=3). Difference between control plant and stressed plants were significant at P<0.05

Treatment	Chilarai			Khandagiri		
	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours
Control	9.474± 0.746	10.163± 0.634	10.399± 0.661	10.828± 1.089	11.212± 0.665	12.332± 0.995
5mM	8.487± 0.473	8.752± 0.376	7.899± 0.190	9.811± 0.089	8.201± 0.551	10.111± 0.212
10mM	7.734± 0.473	7.526± 0.160	7.574± 0.164	9.728± 0.332	7.677± 0.396	9.822± 0.312
15mM	7.798± 0.390	7.523± 0.290	6.154± 0.072	9.677± 0.476	7.141± 0.445	9.113± 0.244

This could be due to the shrinkage of the tissues. In the present study, the RTI and STI underwent significant changes with extending toxicity. RTI value of Chilarai decreased from 24 hours to 72 hours. However, Khandagiri became almost stable at 72 hours giving a higher value of RTI than Chilarai which is a lowland variety.Khandagiri showed 73.8% of STI whereas, Chilarai showed 59.1%. Hence, the physiological reports infer that the upland variety showed a higher degree of tolerance under iron toxicity (Table 4).

Table 3: Effect of iron stress on rootlength at 24, 48 and 72 hours. The values were represented as the mean ± SE (n=3). Difference between control plant and stressed plants were significant at P<0.05)

Treatment	Chilarai			Khandagiri		
	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours
Control	10.629± 0.385	10.000± 0.289	11.928± 0.486	11.822± 2.112	12.266± 0.332	13.00± 1.314
5mM	10.246± 0.688	9.159± 0.432	8.8211± 0.639	10.522± 0.1.221	9.264± 1.227	8.831± 0.228
10mM	10.725± 0.804	9.573± 1.199	7.991± 0.258	9.288± 0.433	8.724± 0.112	8.678± 1.344
15mM	10.117± 1.229	7.9901± 0.648	6.839± 0.179	8.634± 0.228	8.356± 1.332	8.586± 0.332

Another physiological symptoms that occur under iron toxicity is the reduction of dry mass accumulation. Hence, relative dry mass for the shoots were observed. The dry weight of Chilarai decreased upto 65% at 15 mM on the third day of stress. However, Khandagiri showed upto47.8% of decrease under the same period of stress (Table 5).Although, both the cultivars had reduced dry weight but the lowland variety showed a higher % relative decrease as compared to the upland cultivars. The dry weight of root was too much fluctuating due to its small amount. Therefore, in this study, only SDSDW has been considered.

Table 4: The root tolerance index and shoot tolerance index of Chilarai and Khandagiri at 24 48 and 72 hours. The values were represented as the mean ± SE (n=3). Difference between control plant and stressed plantwere significant at P<0.05)

Duration	Chilarai		Khandagiri	
	Shoot tolerance index (%) at 15 mM	Root tolerance index (%)at 15 mM	Shoot tolerance index (%) at 15 mM	Root tolerance index (%)at 15 mM
24 hours	82.038± 0.371	95.182± 1.088	89.370± 0.316	73.03± 0.128
48 hours	74.023± 0.422	79.9± 0.662	63.69± 0.443	68.123± 1.125
72 hours	59.178 ± 1.029	57.335 ± 6.00	73.897± 1.524	66.046 ± 1.552

Biochemical Analyses

Hydrogen peroxide (H_2O_2) production gradually increased in Chilarai root with increasing Fe stress condition (5mM, 10mM, 15mM) for each time interval (24, 48, 72 hour). Similar type of result was observed in shoot tissue of Chilarai cultivar but amount of H_2O_2 production was higher in root than the shoot, whereas Khandagiri variety generates fewer amounts of ROS in both root and shoot than Chilarai. Maximum H_2O_2 production was observed at 15mM Fe stress condition at each time interval. Generation of Superoxide radical (O_2^-)under Fe stress condition also showed similar result like H_2O_2 which gradually increased with time and concentration, and the significant generation in both root and shoot of Chilarai cultivar at 15mM of stress condition. Lipid peroxidation is a hydroxyl radical mediated process where MDA is quantified as an indicator of peroxidation level in membrane. The application of iron (5mM, 10mM, 15mM) to both Chilarai and Khandagiri variety for 24, 48 and 72 h resulted in a significant increase in lipid peroxidation.MDA content was lower in Khandagiri variety compared to Chilarai variety at all levels of Fe treatment (Table 6 and 7).

Table 5: The relative decrease in shoot dry weight of Khandagiri and Chilarai at different time intervals. The values were represented as the mean ± SE (n=3). Difference between control plant and stressed plantwere significant at P<0.05)

Concentration of Fe (II) in mM	24 hours		48 hours		72 hours	
	Khandagiri	Chilarai	Khandagiri	Chilarai	Khandagiri	Chilarai
5	0.043±0.056	0.052±0.012	0.173±0.022	0.2±0.112	0.26±0.033	0.34±0.032
10	0.043±0.022	0.105±0.043	0.130±0.011	0.3±0.011	0.39±0.023	0.47±0.023
15	0.086±0.023	0.210±0.122	0.434±0.022	0.5±0.033	0.47±0.033	0.65±0.016

Enviornmental stresses including metal toxicity induces large amount of production of toxic ROS products like hydrogen peroxide and superoxide radicals and participates in the Fenton reaction producing hydroxyl radical that is damaging to the plant tissues (Dorlodot *et al,* 2005). It causes leaf senescence through lipid peroxidation. The variety Chilarai showed higher amount of H_2O_2, SOD and MDA content in comparison to Khandagiri inferring it is suceptible.

Table 6: The biochemical parameters recorded for shoots of Khandagiri and Chilarai at 72 hours. The values were represented as the mean ± SE (n=3). (*) represented the significance level at P<0.05

Concentration of Fe (II) in mM	Khandagiri			Chilarai		
	H_2O_2 content (µmol g^{-1} FW^{-1})	MDA content (µmol g^{-1} FW^{-1})	Superoxide radical content (µmol g^{-1} FW^{-1})	H_2O_2 content (µmol g^{-1} $FW^{-1)}$	MDA content (µmol g^{-1} FW^{-1})	Superoxide radical content (µmol g^{-1} FW^{-1})
0	1520.71±455.90	5.53±0.32	205.58±0.90	1561.11± 151.78	8.60± 0.18	213.98± 0.92
5	1413.94±68.91	8.64±0.19*	211.83±0.52	2507.02± 115.32*	10.81± 0.28*	330.29± 2.36*
10	2006.90±158.02	9.95±0.38*	249.16±0.57*	2822.12± 549.47	13.40±0.23*	418.28± 2.57*
15	2747.02±113.83	10.66±0.22*	331.59±8.85*	4011.97± 438.57*	15.19±0.12*	458.53± 4.66*

High ROS production also brings oxidative reactions in chloroplast. As a result, chlorophyll content were also recorded to be disturbed by the increasing concentration in the medium (Upadhyaya *et al,* 2007). A significant reduction in chlorophyll a and chlorophyll b content was observed in Chilarai variety as compared with Khandagiri variety. After exposure of Fe stress for 72h, the reduction of chlorophyll a content was 33.7% - 53.7 % in Khandagiri variety, whereas 27.6%-64.0% reduction was recorded in Chilarai variety. The reduction level of chlorophyll b was faster than chlorophyll a, and 69.4%-73.6% reduction level was observed in Khandagiri variety in comparison to 91.3%- 95.2% observed in Chilarai variety.

Table 7: The biochemical parameters recorded for roots of Khandagiri and Chilarai at 72 hours. The values were represented as the mean ± SE (n=3). (*) represented the significance level at P<0.05

Concentration of Fe (II) in mM	Khandagiri			Chilarai		
	H_2O_2 content (µmol g^{-1} FW^{-1})	MDA content (µmol g^{-1} FW^{-1})	Superoxide radical content (µmol g^{-1} FW^{-1})	H_2O_2 content (µmol g^{-1} FW^{-1})	MDA content (µmol g^{-1} FW^{-1})	Superoxide radical content content (µmol g^{-1} FW^{-1})
0	2029.80± 30.26	7.37± 0.14	172.27± 2.69	2365.60± 221.95	9.93± 0.35	173.33± 1.51
5	3941.39± 247.38*	10.11± 0.08*	225.99± 5.08*	7621.03± 87.15*	14.83±0.18*	293.6± 4.60*
10	4688.74± 62.75*	12.36± 0.27*	243.91±1.27*	8438.06± 240.59*	17.30±0.27*	379.91± 4.29*
15	5158.66±7.65*	14.58± 0.29*	335.83±3.08*	9795.40± 388.23*	18.52±0.25*	427.14± 7.09*

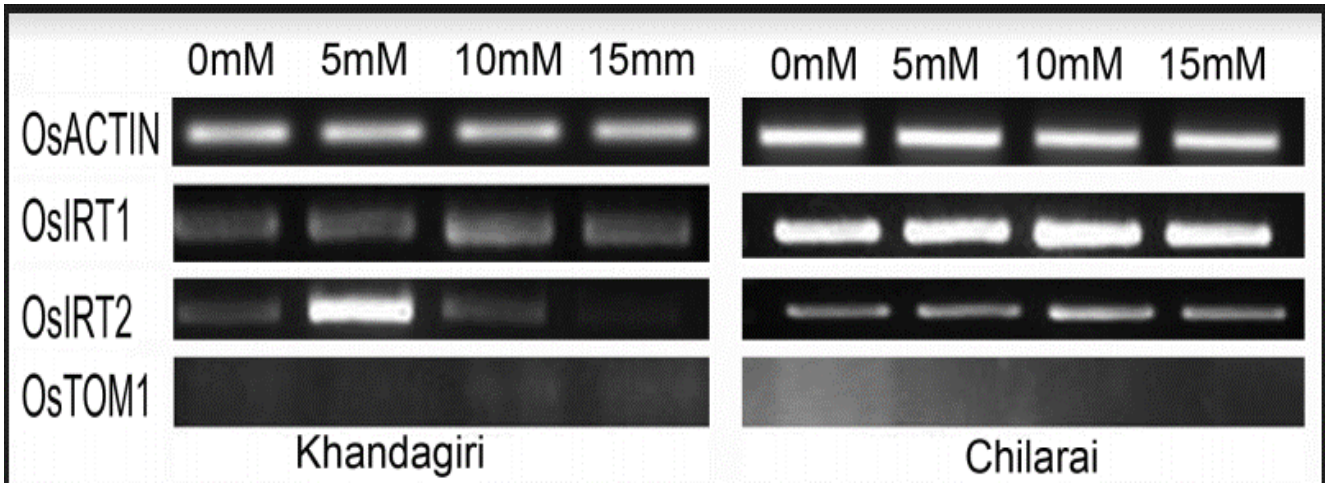
Gene Expression Analyses

We evaluated the expression of three major genes OsIRT1, OsIRT2 and OsTOM1 in rice shoots after 24hrs of Fe stress. OsIRT1 expression was found low in Khandagiri as compared to Chilarai. At 5 mM, expression of OsIRT2 was there in transcripts but decreased at higher concentrations. However, in Chilarai an increase in OsIRT2 expression was seen at all concentrations. Extremely low expression of TOM1 was observed for Khandagiri whereas negligible was recorded for Chilarai.

The transporters IRTs are mainly reported to be functional in Strategy I plants where it take up the ferrous ion directly inside the root cells. The genes OsIRT1 and OsIRT2 are the homologs of IRT1 derived from Arabidopsis (Connolly *et al,* 2002, Vert *et al,* 2002).In addition to root plasma membrane they are also expressed in leaves and stems, suggesting their active role in Fe translocation i.e. long distance.

Fe transport in rice plants. In this experiment OsIRT1 and OsIRT2 are expressed in both Chilarai and Khandagiri shoot under Fe excess condition but expression level was relatively higher in Chilarai shoot than the Khandagiri shoot (Fig.1). In 5mM of Fe stress condition, OsIRT2 showed its higher expression level in Khandagiri. It was earlier reported that the expression of OsIRT1 was higher than OsIRT2 (Ishimaru *et al,* 2007). The results therefore, corresponded with earlier expression reports. OsIRT1 expression in both cases was higher than that of OsIRT2 expression. In addition to IRTs, rice exudes the phytosiderophores like mugineic acids which forms Fe(II)-MA complex for the uptake of iron in ferric form where the transporter TOM1 plays a significant role in transporting MAs and its derivatives into the rhizosphere (Masuda *et al,* 2017). In presence of Fe2+ in the medium, the protein may become less functional to avoid excess uptake. Hence a very low level of expression of TOM1 in both the cultivars of Khandagiri and Chilarai was observed under Fe toxic conditions.

Figure 1: Relative expression of *Os*IRT1, *Os*IRT2 and *Os*TOM1 under ironexcess conditions in *Oryzasativa* L. var. Khandagiri and Chilarai. The cDNA was normalised with *Os*ACTIN



The present work clearly distinguished the possible responses of the contrasting rice varieties under excess iron condition. The degree of tolerance against such abiotic stress can vary within cultivars. Understanding the physiology and the genetic responses can be a better approach for extending further screening of the upland rice varieties that can be helpful in developing a new breed with desired tolerance against iron toxicity in the future.

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