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Physiological Tolerance Mechanism of selected Rice Germplasm of Northeast India to Iron Toxicity

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ABSTRACT

Rice cultivation in lowland faces the iron toxicity stress due to the accumulation of excess ferrous iron in reduced soil condition, and causes reduction in productivity. Therefore, the present study was undertaken to investigate the strategies used by the rice cultivars from North-East region of India under iron excess condition for further improvement of rice through breeding programme. The investigation was carried out in five rice cultivars from North-East India *viz.* KD-2-6-3, Phougak, Pyzum, Shahsarang, Guwahati and a popular rice hybrid Arize 6444 produced by Bayer Company. Hydroponically grown rice seedlings of the cultivars were exposed to a high Fe concentration (1000 mg L^{-1} = 17.9 mM Fe^{2+}) and harvested after five weeks to examine the variation in symptom expression in the leaves, biomass retention, tissue iron distribution, root oxidising power and lipid peroxidation. Lowest leaf symptom expression was recorded KD-2-6-3, Guwahati and Shahsarang. Conversely, the symptom expression was severe in case of Pyzum and Phougak. KD-2-6-3 and Arize 6444 recorded the lowest and highest iron concentration in shoot and root tissue, respectively. Lipid peroxidation value was lowest in KD-2-6-3, Guwahati and Shahsarang. The rhizosphere oxidation proceeded at much faster pace in KD-2-6-3 and Arize 6444 than Guwahati and Shahsarang. The root oxidation, though, could not be detected in Pyzum and Phougak. Findings of the study corroborate the differential response to iron toxicity of the rice cultivars with the tolerant rice cultivars employing different tolerance strategies either with shoots- and/or root- based mechanisms.

1. Introduction

Iron toxicity is one among the yield limiting factors contributing to low productivity in lowland rice systems (Dobermann and Fairhurst 2000). It is reported to be widely prevalent in several Asian countries including China, India, Indonesia, Thailand, Malaysia and Philippines. Verma (1991) reported the common occurrence of iron toxicity in northwest Himalayan region of wetland rice, sometimes reaching up to 42% that poses a major constraint to rice yield.

Northeast India is no exception to such scenario as the soils are mostly acidic in nature that accompanies many secondary deficiencies and toxicities (Devi et al. 2016). Although, iron is an essential element in plants which plays an important role in many physiological processes such as chloroplast development, chloroplast biosynthesis, ribonucleotide and dinitrogen reduction, as well as energy-yielding electron transfer reactions of respiration and photosynthesis (Hell and Stephan 2003); but excess concentrations of ferrous (Fe^{2+}) ions may lead to its toxicity.

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The excessive uptake of Fe^{2+} by plants leads to the generation of reactive oxygen species (ROS) through the Fenton reaction (Thongbai and Goodman 2000) that can cause an irreversible damage to membrane lipids, proteins and nucleic acid resulting in the breakdown of photosynthetic machinery (Stein et al. 2009a; Nagajyoti et al. 2010; Pereira et al. 2013). Eventually, the yields are affected and sometimes complete crop failure can occur as a result of severe toxicities at seedling stage (Audebert and Fofana 2009). So far, three tolerance mechanisms are known through which rice plants adapt to iron toxic conditions. In the first strategy, the plants can exclude ferrous iron at the root level through enzymatic oxidation and precipitation of ferrous iron on the root surface (Green and Etherington 1977). In the second strategy, the plants can take up ferrous iron but avoids its further action via internal compartmentalization in less reactive form, ferritin being the best example (Briat et al. 2010). In the third strategy, the plants can take up excess iron and are able to tolerate the generated free radicals. Antioxidants and antioxidative enzymes reportedly protect the plants from ROS damage during iron excess condition (Fang et al. 2001; Gallie 2013; Bode et al. 1995; Fang and Kao 2000; Fang et al. 2001). An integrated approach utilising both genetic tolerance and natural resource is a key to iron toxicity reduction (Sahrawat 2003). Therefore, it is of an utter importance to characterise the germplasm for iron toxicity tolerance and its associated mechanism to be able to use as breeding material in crop improvement programmes. The present study is therefore, envisaged to elucidate the underlying physiological mechanism among selected rice cultivars of Northeast region to contribute further in improving genetic tolerance in rice against iron toxicity.

2. Materials and Methods

Nineteen rice cultivars Chahao amubi, Guwahati, Pyzum, Mani khamnu, Safed khasa, Kali khasa, Phougak, KD-2-6-3, Akhanphao, Shahsarang, Kataktara, Hathia, Mamireang, Chakki Badam, Lalgura, Abhinara, Chandina, Signal and Garumaruti collected from different locations of Manipur, Tripura and Meghalaya in addition to a popular rice hybrid Arize 6444 were selected for the present study. These cultivars were screened initially for tolerance to Fe pulse stress during the vegetative growth stage at 1,000 ppm Fe^{2+} using $Fe_2SO_4.7H_2O$ as described by Wu et al. (2014)) at Indian Council of Agricultural Research (ICAR), Research Complex for North Eastern Hill Region, Tripura Centre, Lembucherra, Tripura, India. Experiments were conducted in Plant growth chamber (Conviron model no. PGW 40) facilities of the institute following standard methods (Wu et al. 2014) with the day/night temperature set at 30/25°C, photosynthetically active radiation (PAR) light of 400 µmol m^{-2} sec^{-1}. Rice seeds were soaked in demineralized water and germinated at 30°C in the dark for 72 hours. Subsequently, germinating seeds were floated in 70.5 mg L^{-1} $CaCl_2$ and 1.625 mg L^{-1} $FeCl_3$ solution in light for another 5 days. Homogenous seedlings were selected and transplanted into 40L tanks filled with half strength Yoshida nutrient solution. In all experiments, six replicate plants per rice cultivars were used. The pH value during the experimental period was maintained at 5.5 and solutions were exchanged every 10 days. Five weeks after the plant growth, half of the plants were exposed to excess iron stress of 1000mg L^{-1} for 5 days. To maintain the low redox potential in the solution, N_2 gas was percolated into the culture solutions for 15 minutes every 2 hours. Leaf bronzing scores were measured on the three youngest fully expanded leaves of the main tiller after five days. Plant materials were harvested for Fe distribution, root oxidising power and lipid peroxidation. Plant shoots of contrasting lines were oven-dried at 60°C until the weight was constant and ground to a fine powder. Fe concentrations in shoots were determined after digesting 250 mg of dry samples with 4 ml 65% HNO_3 at 180°C for 8 hours followed by dilution to 25 ml and filtration. Standard and sample solutions were measured using atomic absorption spectroscopy (AAS, GBC).

Malondialdehyde (MDA) measurement

MDA concentration was determined according to Hodges et al. (1999) to assess the level of lipid peroxidation in leaf tissue. 160 mg of leaf sample was used for extraction of MDA with 2.0 mL of trichloroacetic acid (TCA; 0.1 %) by centrifugation at 10,000×*g* for 15 min at 4°C. The clear supernatant (500 µL) was mixed with 1.5 mL solutions of thiobarbituric acid (TBA; 0.5 %) and TCA (20 %). A control for each sample was prepared without the addition of TBA (-TBA). The samples were incubated in a water bath (90 °C) for 20 min, and the reactions were stopped on ice. Absorbance values were read at 440, 532 and 600 nm. The MDA concentration was calculated from the differences in absorbance at 440, 532 and 600 nm using extinction coefficient value of 157 mM^{-1} $cm^{-1,}$ as shown in the following equations:

$A = (Abs_{532+TBA} - Abs_{600+TBA}) - (Abs_{532-TBA} - Abs_{600-TBA})$,

$B = (Abs_{400+TBA} - Abs_{600+TBA}) \times 0.0571$ and

MDA (nmol mL-1) = (A-B/157000) ×106, where MDA values were expressed as relative to fresh matter.

Root oxidising power

The root oxidising power was detected using a redox indicator methylene blue following method described by Kotula (2009). Agar solution (0.75%) was prepared by boiling and further allowed to cool down to 60°C with continuous percolation of N_2 gas to completely remove the dissolved oxygen. Methylene blue (2 mg L^{-1}) was added into agar which was cooled further to 35°C followed by addition of 0.75 g L^{-1} sodium dithionite ($Na_2S_2O_4$) to reduce methylene blue that turns the solution colourless. For each cultivar, the roots of three representative plants were carefully placed in 500 ml Erlenmeyer flasks. Gaseous N_2 was percolated to remove air from the flasks. The solution containing reduced methylene blue was poured into the flasks to submerge the whole root system. The open surface of flasks was immediately covered with parafilm to avoid air diffusion. The roots were maintained in the dark by wrapping the flask with aluminium foil. The plants were incubated inside a greenhouse maintained at 30°C for 4 h. Photographs were taken every hour to record the color changes in rhizosphere due to the root oxidation power. All data were analyzed using SPSS 16.0 for windows. One-way analysis of variance (One-way ANOVA) was used to determine whether any significant variation existed between the treatments. When overall differences were found, differences between means were tested by Duncan multiple range test. All differences were considered significant at 5% and the results are presented as mean ± S.E. (standard error).

3. Results and Discussion

Leaf bronzing score is often recorded to screen the plants for tolerance to ferrous-iron toxicity (Devi et al. 2016). During the study, leaf symptom scoring was performed to study the plant's immediate response to excess iron. Result shows that the rice cultivars have varying degrees of leaf bronzing in response to excess iron. Leaf bronzing score ranged from 1.0 to 9.0 in response to iron pulse stress of 1000 mg L^{-1} Fe^{2+} (Figure 1). Among the subjected cultivars, symptoms were more prominent in cultivars Pyzum, Phougak, Chandina, Hathia, Signal, Garumaruti, Abhinara, and Mamireang whereas the cultivars Mani khamnu, Kali khasa, Arize 6444, Chakki Badam, Chinari and Lalgura expressed mild symptoms (Figure 1). Relatively low leaf bronzing scores were observed in cultivar Akhanphao, Chahao amubi, Safed Khasa and Kataktara. On the contrary, leaf symptom expression was not observed in the cultivars KD-2-6-3, Guwahati and Shahsarang. Asch and Becker (2005) in their study found a strong correlation of leaf bronzing with yield reduction in rice. It was estimated that for each increase in symptom score, yield reduction occurs approximately at 400 kg ha^{-1} (Audebert and Fofana 2009). Based on the leaf bronzing score, Guwahati, KD-2-6-3, Shahsarang, Arize 6444 which showed lower symptom expression and Pyzum, Phougak which showed pronounced symptoms (Figure 2) were chosen for relative biomass study, Fe uptake analysis, root oxidation and lipid peroxidation studies. Among the cultivars studied, KD-2-6-3, Guwahati, Shahsarang and Arize 6444 showed significantly higher (*p< 0.05) relative shoot and root growth (Figure 3, 4). The relative root and shoot dry weight of iron tolerant rice lines were found to be higher than the sensitive lines (Wu et al. 2014). Findings of this study suggest a higher biomass retention in KD-2-6-3, Guwahati, Shahsarang and Arize 6444 which can be attributed to their innate tolerance to excess iron. The shoot Fe^{2+} concentration among the cultivars did not vary significantly in control condition. However, in stressed condition significant difference in shoot Fe^{2+} concentration

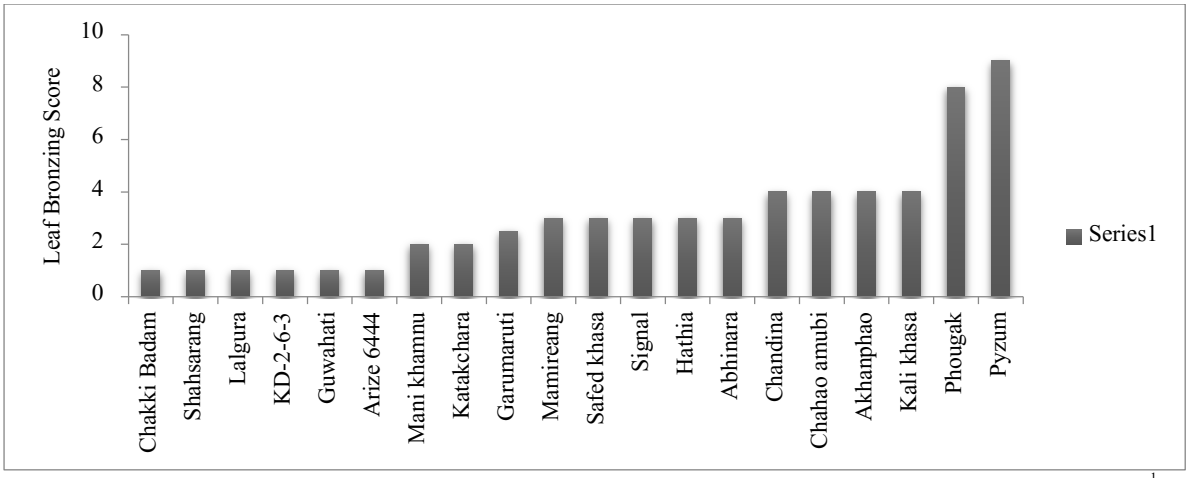

Figure 1. Differential response of rice cultivars (5 weeks old seedlings) to a 5-days exposure to 1000 mg L^{-1} Fe^{2+} ($Fe_2SO_4.7H_2O$) in the culture solution.

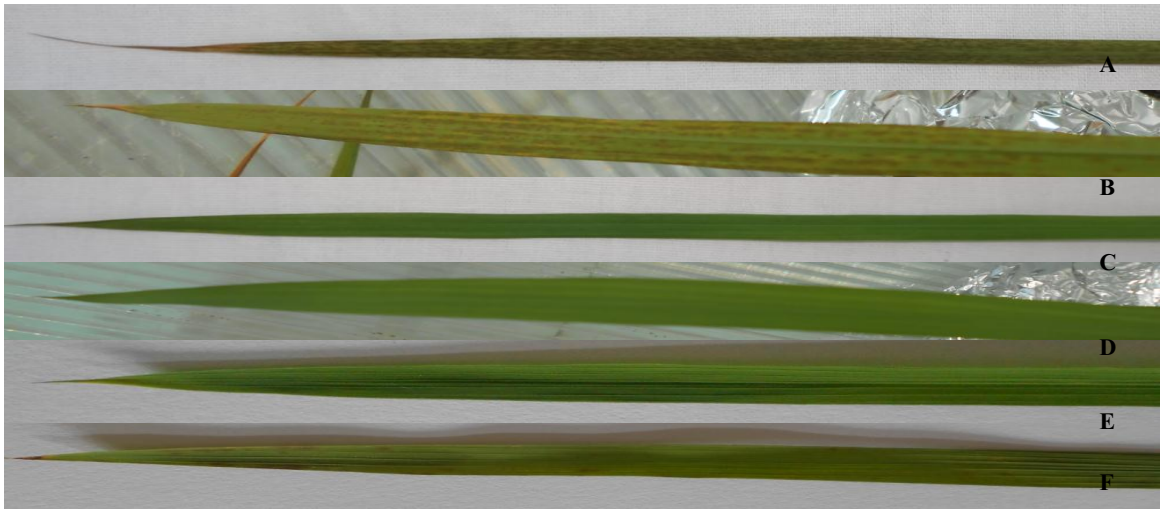

Figure 2. Representative image of leaf bronzing score of Pyzum (A), Phougak (B), Guwahati (C), KD-2-6-3 (D), Shahsarang (E) and Arize (F).

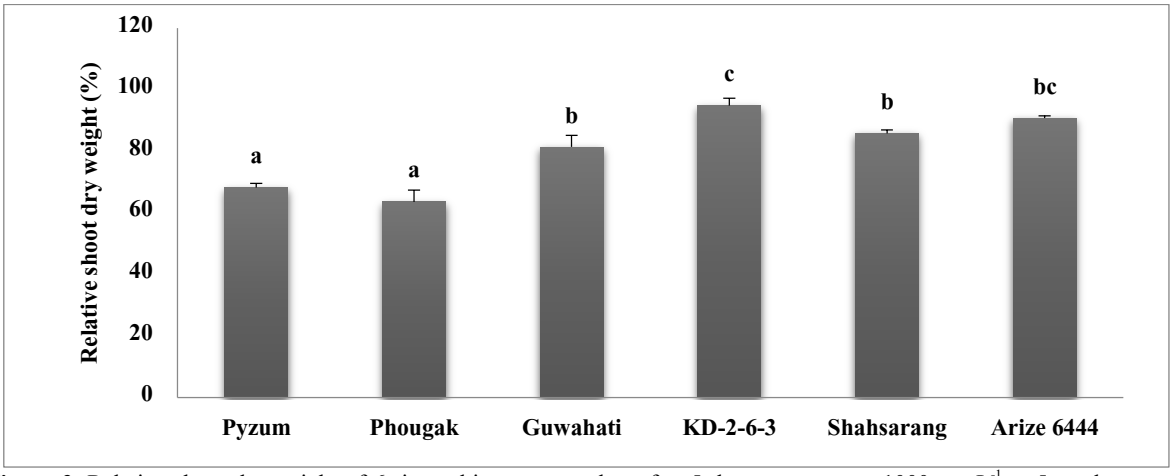

Figure 3. Relative shoot dry weight of 6 rice cultivars exposed to after 5 days exposure to 1000 mg L^{-1} at 5 week stage of growth in hydroponic solution. Bar represent standard errors of the mean (n=6). Different letters indicate significant differences between cultivars by LSD-test (p<0.05).

(*p< 0.05) was noted among the cultivars studied (Table 1). Significantly (*p< 0.05) lower shoot Fe concentration was observed in cultivars KD-2-6-3 and Arize 6444 as compared to Pyzum and Phougak whereas Shahsarang and Guwahati did not differ significantly from these two cultivars (Table 1). It is interesting to note that although Pyzum, Phougak, Guwahati and Shahsarang had higher shoot Fe concentration value, leaf symptom expression was not detected in Shahsarang and Guwahati. Our finding indicates that Guwahati and Shahsarang cultivars are able to tolerate a high level of shoot Fe^{2+}concentration either through compartmentalisation or activation of anti-oxidative tolerance mechanism, and therefore could probably explain the lack of leaf symptom expression in these cultivars. On the contrary, Pyzum and Phougak expressed pronounced leaf symptom through higher translocation of iron in stem, playing no role in inclusion or avoidance mechanism (Onaga et al. 2013; Devi et al. 2016). Samaranayake et al. (2012) suggested that there could be a positive correlation between the leaf symptom expression and the chemical transmitted by the root system, which may be stronger in the susceptible variety than the resistant one. Significant differences were also observed in root tissue Fe^{2+} concentration among the cultivars in control as well as stressed condition. The highest root Fe^{2+} concentration was recorded in KD-2-6-3 and Arize 2444 and the lowest concentration recorded in roots of Guwahati (Table 1) in response to excess iron stress. We further investigated the physiological basis of low shoot Fe^{2+} concentration in KD-2-6-3 and Arize 6444 by assessing the root oxidizing power. Our result clearly indicates rhizosphere oxidation of KD-2-6-3 and Arize 6444 as evidenced by color change of the Methylene-blue indicator proceeded at a faster pace than Guwahati and Shahsarang (Figure 5).

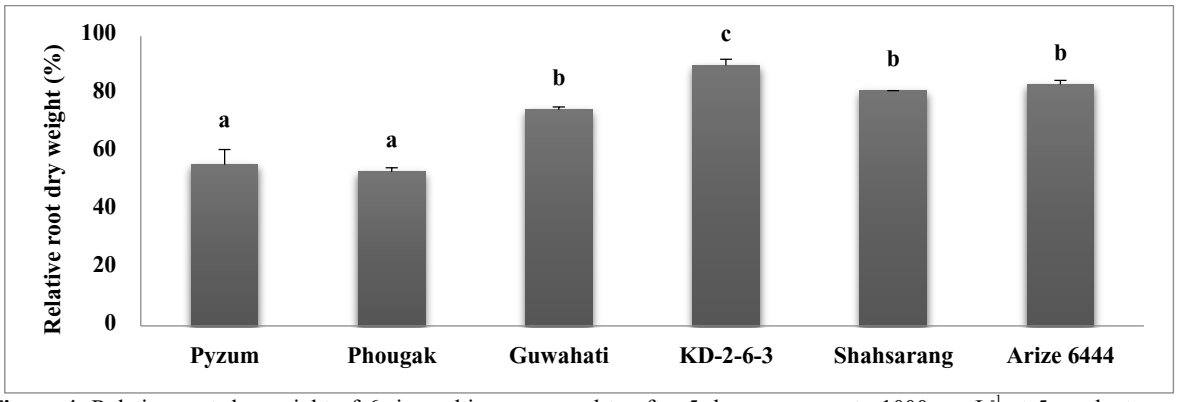

Figure 4. Relative root dry weight of 6 rice cultivars exposed to after 5 days exposure to 1000 mg L^{-1} at 5 week stage of growth in hydroponic solution. Bar represent standard errors of the mean (n=6). Different letters indicate significant differences between cultivars by LSD-test (p<0.05).

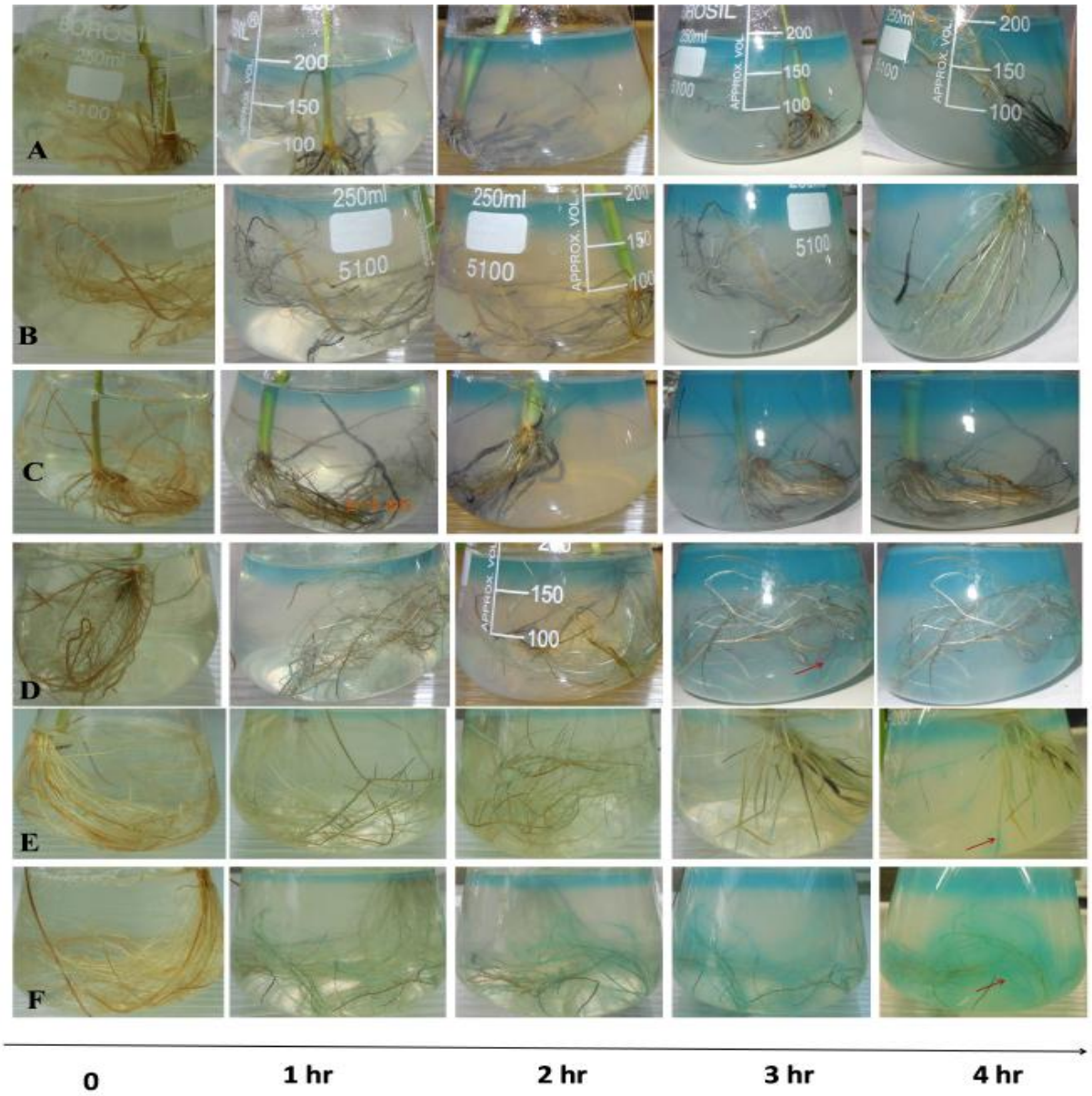

Figure 5. Time course of root oxidizing power of Pyzum (A), Phougak (B), Guwahati (C), KD-2-6-3 (D), Shahsarang (E) and Arize (F) were indicated by color change in Methylene-blue agar solution. Representative photos of 4 replicates per cultivars are presented Horizontal axis represents the time of duration (0-4 hours). Blue colour indicates the site of oxygen release from roots.

However, the root oxidation in Pyzum and Phougak were not detected. These results also affirm that the retention power of rice roots plays a major role in inclusion or avoidance mechanism (Nozoe et al. 2008). The MDA level was highly increased in response to iron stress in Pyzum and Phougak as compared to Shahsarang, Guwahati, KD-2-6-3 and Arize (Table 2). The lowest MDA level was observed in Shahsarang and highest in Pyzum. As discussed above, Guwahati and Shahsarang are able to tolerate high iron levels in shoot without showing leaf toxicity as observed in other reports (Asch et al. 2005; Stein et al. 2009a; Engel et al. 2012). Muller et al. (2015) explained that in such genotypes the plants are capable of oxidizing large amounts of iron translocated to shoots. This process of oxidation starts after the iron is released from the chelator molecule in apoplast, which prevents its entry to symplast, as an exclusion mechanism (Majerus et al. 2007a; Engel 2009). Silveira et al. (2009) suggested that the iron may also be incorporated into organic compounds, such as ferritin protein as an alternative mechanism of exclusion.

Conclusion

Overall findings of the study suggest that tolerant rice cultivars employ different tolerance mechanism against iron toxicity which may be shoots- and root- based mechanisms. While the dominant tolerance mechanism of KD-2-6-3 and Arize 6444 was determined to be exclusion with its root architecture being able to oxidize Fe^{2+} through air transport in rhizosphere, the iron tolerance in Guwahati and Shahsarang was attributed mainly to shoot-based mechanisms either by inclusion or avoidance. In this line, future effort should be made towards genetical dissection of these traits (root and shoot adaptive traits) through novel QTL identification for iron toxicity tolerance, followed by pyramiding the traits through modern breeding approaches.

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Table 1. Fe^{2+} distribution (x 10^3 mg kg^{-1}) in the tissues of 6 rice cultivars exposed to 0 and 1000 mg L^{-1} at 5 week stage of growth in hydroponic solution.

Cultivars	**Shoots**		**Roots**	
	0	**1000**	**0**	**1000**
Pyzum	0.27	0.80	0.57	2.87
Phougak	0.28	0.84	1.04	2.78
Guwahati	0.23	0.78	1.99	2.68
KD-2-6-3	0.26	0.63	0.59	5.75
Shahsarang	0.27	0.74	1.56	2.79
Arize 6444	0.21	0.58	0.41	4.87
SEM	0.12	0.31	0.19	0.35
Prob (diff. in cultivar mean)	NS	S	S	S

Table 2. Malondialdehyde (MDA) levels in leaves of 6 rice cultivars exposed to after 5 days exposure to 1000 mg L^{-1} at 5 week stage of growth in hydroponic solution. Different letters indicate significant differences between cultivars by LSD-test (p<0.05)

Cultivars	**0**	**1000 mg L^{-1}**
Pyzum	10.80±0.61c	28.04±0.14d
Phougak	10.34±0.23c	22.60±0.20c
Guwahati	8.34±0.14a	5.25±0.71a
KD-2-6-3	8.54±0.23a	5.94±0.28a
Shahsarang	7.54±0.34b	5.50±0.47a
Arize 6444	8.90±0.14a	9.58±0.42b

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