Effect of iron stress on selected physiological and spectral parameter on four rice varieties (*Oryza sativa* L.)

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Abstract

Iron toxicity is an abiotic stress coming with high concentrations of Fe^{2+} in the soil solution which is a well-recognized problem of rice (*Oryza sativa* L.) cultivation in lowland. Rice varieties differ widely in their ability to tolerate excess iron. The present study was undertaken with four rice varieties viz. Dhruba, Sampriti, Dhiren, and Puspa. The objective is to study the influence of high applied Fe^{2+} concentrations on the growth, chlorophyll content, and antioxidant enzyme activities. The spectral reflectivity and absorption of different chemical bonding through Fourier transform infrared spectroscopy (FTIR) of four rice varieties was also analyzed. Seven-day-old rice seedlings treated with Ferrous sulphate and subjected to 100 ppm to 750 ppm for further 14-day iron stress were used to analyze their morphological and biochemical responses. Besides, Fourier transform infrared spectral reflection was attributed in root and shoot part. Results indicated shoot growth and chlorophyll content decreased in 750 ppm in all the selected rice varieties of interest. On the contrary, the catalase activity, protein content, and lipid peroxidation increased in these varieties. However, the expression of high amount of CAT activity in Sampriti variety and high amount of SOD activity in Dhruba variety led to tolerance in iron stress in comparison to other two varieties of interest. FTIR revealed steep band stretching of various functional groups of different compounds in both the root and shoot parts of all the varieties. Results revealed that the change of antioxidant expression and FTIR spectra were attributed to the effect of iron toxicity on rice plants.

Keywords: rice, iron stress, chlorophyll, antioxidant, Fourier transform infrared spectroscopy

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Introduction

Iron (Fe) is one of the essential micro elements which involves in several biological processes of the plant cells throughout their life. Due to the redox status change between two states of iron, i.e. the ferrous (Fe-II) and ferric (Fe-III), Fe acts as an electron donor or acceptor, which is vital in photosynthesis and respiration (Kobayashi and Nishizawa, 2012; Zhai et al., 2014). Moreover, in plant growth Fe enhances enzymatic redox reaction (Gill and Tuteja, 2010). It also serves as a co-factor of several enzymes and it is the major ingredient of the cell redox systems like heme

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proteins, cytochrome, catalase (CAT), peroxidase, and FeS proteins like feredoxin, aconitase, and superoxide dismutase (SOD) (Marschner, 1995). Lowland rice often suffers from Fe toxicity that affect the yield (Baruah et al., 2007) due to excess amount of water-soluble Fe. Excessive Fe absorption by plants generates a symptom called "bronzing". The rice roots of plants affected by Fe toxicity become shorter in length, and dark brown in color resulting in underdeveloped root and shoot growth, (Dorlodot et al., 2005) and inhibition of nutrient uptake (Zhong et al., 2010). In tissue Fe catalyzes the reactive oxygen species (ROS) formation via the Fe catalyzed Haber-Weiss reaction (Fenton reaction). Plants show different systems of tolerance to Fe toxicity (Becker and Asch, 2005), including shoot-based tolerance mechanism, i.e. the scavenging of ROS by antioxidants like ascorbate, phenolics, glutathione, or enzymes, ascorbate peroxidase (APX), and SOD (Fang et al., 2001; Majerus et al., 2007). Similarly, CAT also functions in plant tolerance to Fe toxicity (Xing et al., 2010).

The present report emphasizes the effect of Fe toxicity in the rice seedlings, its mechanisms of tolerance in seedling growth, and biochemical parameters, i.e. chlorophyll content, antioxidant enzyme activities which includes SOD and CAT activity, and lipid peroxidation in four rice varieties viz. Dhruba, Sampriti, Dhiren, and Puspa. The spectral analysis of Fourier transform infrared spectroscopy (FTIR) has been performed to analyze spectral reflectivity and absorption of different chemical bonding.

The novelty of this study lies in finding the Fe toxicity tolerant variety among the four-rice varieties Dhruba, Sampriti, Dhiren, and Puspa which are locally cultivable in lowland areas of West Bengal, in respect to its physiological and spectral study.

Materials and Methods

Plant material and experimental design

According to Green and Etherington (1977), after flooding, the Fe^{2+} iron concentration of the soil solution increases sharply up to 500 ppm. Accordingly, Fe stresses were given to different varieties of rice seeds in sterilized petri-dishes during the germination period to check the tolerance level of Fe stress at different concentrations on the rice varieties and 50% of lethal dose was found at 750 ppm.

Seeds of four different varieties of rice (*Oryza sativa*) viz. Dhruba, Sampriti, Dhiren, and Puspa were collected from Bankura Rice Research Station, West Bengal. Rice seedlings were cultured under the following conditions: 29±2 ℃ temperature and 60±5 % relative humidity with 'Yoshida solution' (Yoshida et al., 1976) for 21 days.

The treatments comprised of exposure of the seedlings to $FeSO_4.7H_2O$ solution at five different concentrations viz. 0 (control), 100, 250, 500, and 750 ppm after the plants attained the age of 7 days and further imposed for 14-day duration. Then, the length of both the root and shoot of growing rice seedlings were measured.

Estimation of chlorophyll contents

Chlorophyll contents were measured through extraction with 80% acetone according to Arnon (1949). The following equation was used to calculate total chlorophyll:

Chlorophyll A: $12.7(A_{663}) – 2.69(A_{645})$ (1)
Chlorophyll B: $22.9(A_{645}) – 4.68(A_{663})$ (2)

Estimation of soluble protein content and antioxidant activity

Soluble protein was estimated by the method of Bradford (1976). Plant material was extracted with 0.1 M phosphate buffer pH = 6.8 as mentioned by Kar and Mishra (1976).

CAT activity was measured with a spectrophotometer at room temperature by monitoring the decrease in absorbance at 240 nm resulting from H_2O_2 decomposition (Aebi, 1983). One unit (U) of CAT was equivalent to the change of absorbance 0.001 per minute in the presence of CAT. The reaction mixture contained 100 mM sodium phosphate buffer (pH 7.0), 30 mM H_2O_2, and 100 µL of crude extract in a total volume of 3.0 mL.

SOD activity was determined by a method based on the performance of the enzyme in inhibition of the pyrogallol autoxidation (Magnani et al., 2000). One unit (U) of SOD activity is defined as the amount of enzyme required to inhibit 50% of pyrogallol autoxidation. (Marklund and Marklund, 1974).

The rate of lipid peroxidation was estimated as described by the method of Heath and Packer (1968) and was calculated as the amount of malondialdehyde produced. The tissue was boiled with thiobarbituric acid reagent (0.25% TBA in 10% TCA). The mixture was first cooled and then centrifuged at 10,000X g for 10 min. The absorbance was recorded at 545 nm.

Fourier transform infrared spectroscopy-attenuated total reflection (FTIR-ATR)

The FTIR-ATR spectra of rice samples were recorded with a FTIR spectrometer with a germanium coated potassium bromide (KBr) plate and an attached ATR unit in the range of 4000-500 cm^{-1} (Shimadzu Corpn., Japan, IR-Prestige-21). Solutions were prepared using MilliQ de-ionised water.

Statistical Analysis

All collected data were analyzed using Analysis of Variance (ANOVA) by SPSS version 20. The statistical analysis was conducted to test significance of the stress effect. Duncan Multiple Range test was carried out according to the least significance difference (LSD) values. Standard methods were used to calculate F-test and critical difference (Ott and Longnecker, 2008). All the statistical tests were analyzed at 5% level of significance.

Results

Morphological response of rice to Fe toxicity

The shoot length of all the rice varieties showed different growth under Fe stress. Dhiren variety showed a marked decrease in shoot length (31%) at 750 ppm of toxicity in comparison with control treatment (Fig. I. a). Puspa, Dhruba, and Sampriti varieties showed 11.5%, 9%, and 5% decrease in shoot growth, respectively. The resulting shoot

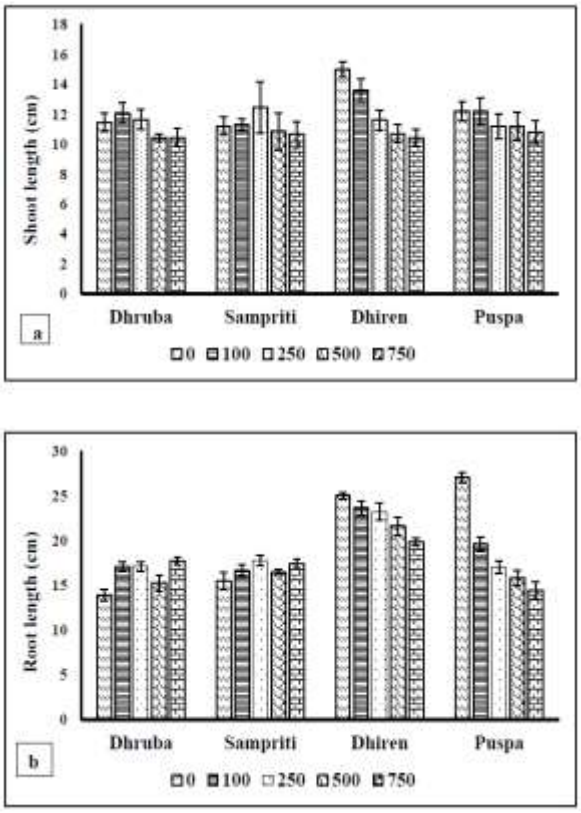

Fig. I. Effect of iron stress on (a) shoot length and (b) total root length of different rice varieties (bars indicate, mean values ± SE (Standard Error), n=5).

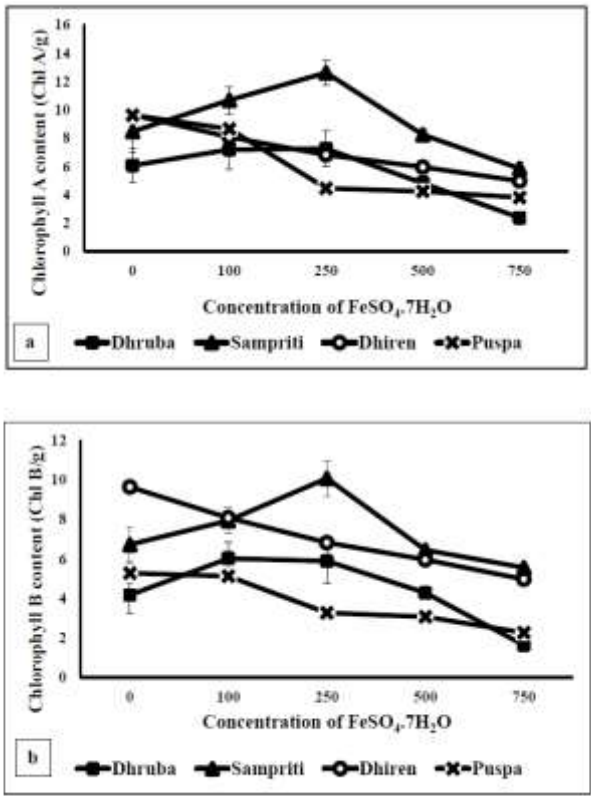

Fig. II. Effect of iron stress on total chlorophyll content in rice seedlings (bars indicate mean values ± SE, n=5).

length was significantly correlated with the P value = 0.002. The root length in Dhiren and Puspa decreased at 750 ppm of Fe toxicity by 20% and 46%, respectively (Fig. I. b). In contrast, the total root length increased in Dhruba and Sampriti varieties by 27% and 12%, respectively. The data showed significant mean difference with the p value= 0.000.

Chlorophyll content in rice under Fe toxicity

Chlorophyll contents in rice seedlings showed different responses to Fe stress. The chlorophyll A content significantly decreased in Dhruba by 61% followed by Puspa, Dhiren, and Sampriti by 60%, 48%, and 31% at 750 ppm as compared with control (Fig. II. a). The result correlates significantly with the P value = 0.05. The chlorophyll B content significantly decreased in Dhruba by 61% followed by Puspa, Dhiren, and Sampriti by 57%, 48%, and 17% at 750 ppm as compared with control (Fig. II. b). The resulting data showed significant mean difference with the P value = 0.000.

Protein content in rice to Fe toxicity

The protein content in the shoot parts of Dhruba, Sampriti, and Puspa varieties of rice seedlings showed slight increase (6%, 9%, and 5.6%, respectively) and Dhiren variety showed slight decrease (2.4%) in 750 ppm of Fe stress with respect to control (Fig. III. a). The protein content in the root parts of Dhruba and Sampriti varieties of rice seedlings showed slight decrease by 4% and 3%, respectively while Dhiren and Puspa varieties showed slight increase by 23% and 8%, respectively in 750 ppm of Fe stress with respect to control (Fig. III. b). (P-values of ANOVA for both shoot and root proteins were 0.000).

Superoxide dismutase activity

The SOD activity in the shoot parts of varieties Sampriti, Dhiren, and Puspa decreased by 22.6%, 16%, and 16%, respectively. On the contrary, there was no significant difference in Dhiren variety (Fig. IV. a). Furthermore, the activity in roots of Dhruba variety increased by 21% while it decreased in Sampriti, Dhiren, and Puspa varieties by 19%, 20%, and 8%, respectively (Fig. IV. b). The result of SOD

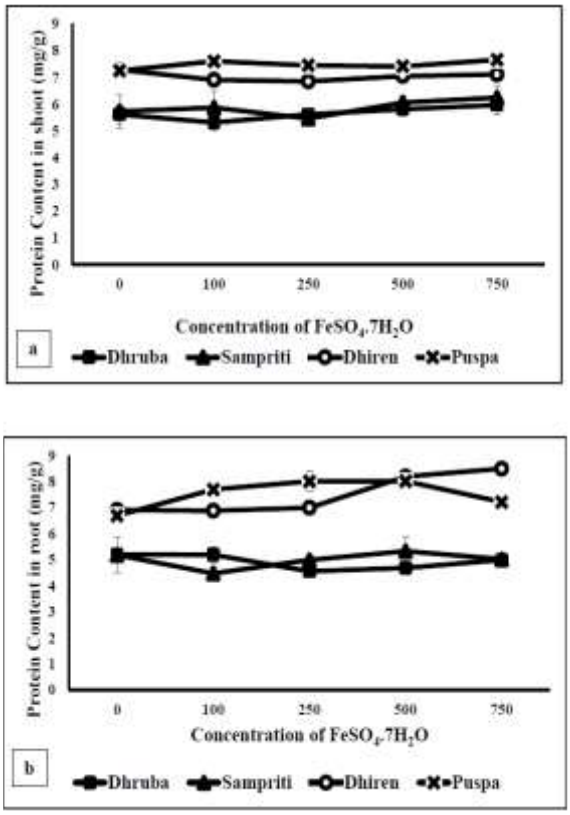

Fig. III. Effect of iron stress on soluble protein content in rice seedlings (bars indicate mean values ± SE, n=5)

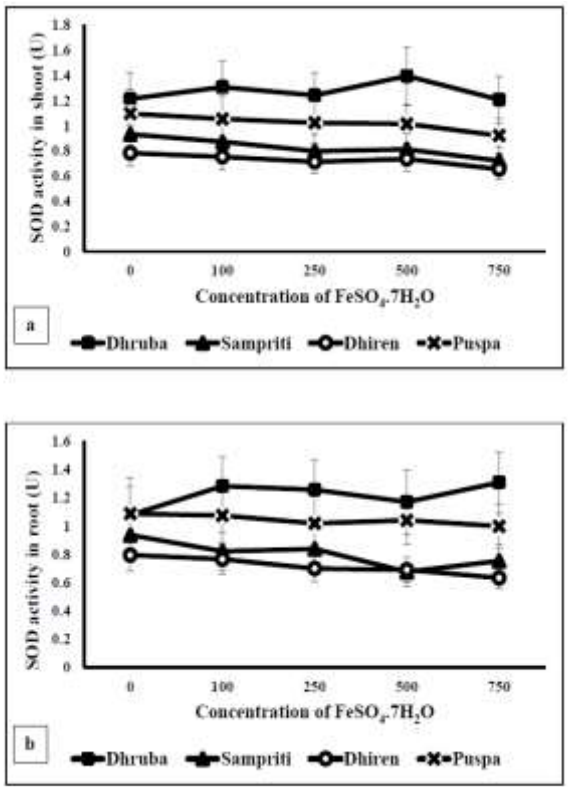

Fig. IV. Effect of iron stress on SOD activity in shoot part (a) and root part (b) (bars indicate mean values ± SE, n=5)

activity in shoot length showed statistically significant differences (P-values of ANOVA for SOD activity in shoot and root were 0.003 and 0.0123).

Catalase activity

The CAT activity in the shoot parts of Dhruba, Sampriti, Dhiren, and Puspa increased by 77%, 46%, 81%, and 17%, respectively (Fig. V. a). Also, the CAT activity in roots parts of Dhruba, Sampriti, Dhiren, and Puspa increased by 95%, 147%, 11%, and 74% respectively, compared with control (Fig. V. b). (P-values of ANOVA for CAT activity both in shoots and roots were 0.000).

Lipid peroxidation

The by-product of lipid peroxidation, malondialdehyde content of shoots in Dhruba, Sampriti, Dhiren, and Puspa increased by 94%, 26%, 0.3%, and 9%, respectively (Fig. VI. a). The activity in roots parts of Dhruba, Sampriti, Dhiren, and Puspa increased to 77%, 8%, 28%, and 35% respectively, compared with control (Fig. VI. b). (P-values of ANOVA for lipid peroxidation in both shoots and roots were 0.000).

FTIR analysis

In FTIR spectrum, the infrared radiation peak values were used to identify the functional group present in the active components based on the regions, observed in the experimental set of all four rice varieties. The strong band changes in all the varieties Dhruba, Sampriti, Dhiren, and Puspa were observed at ~500 cm^{-1} (Fig. VII. a-d) both in the shoot and root parts, attributed to stretching into group C-Br. A dominant band in case of roots of Sampriti, Dhiren, and Puspa (Fig. VII. b, c, and d) was found at ~650 cm^{-1} that indicates stretching of group C-Cl. Band at ~800 cm^{-1} observed for the roots of Sampriti, Dhiren, and Puspa shows C-H bending in aromatic compounds with adjacent H atom followed by band at ~1050-1150 cm^{-1} in all the varieties of the shoot and root parts, was attributed to stretching in halogens group -C-F. The band

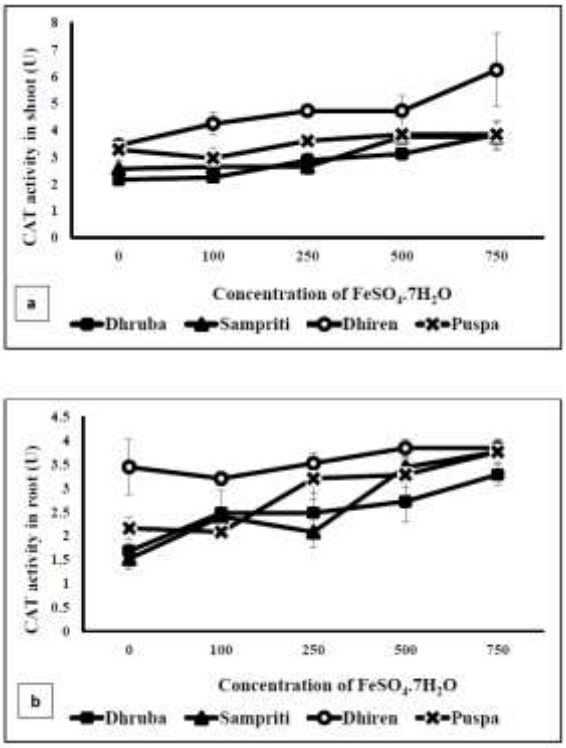

Fig. V. Effect of iron stress on CAT activity in shoot (a) and root (b) parts (bars indicate mean values ± SE, n=5).

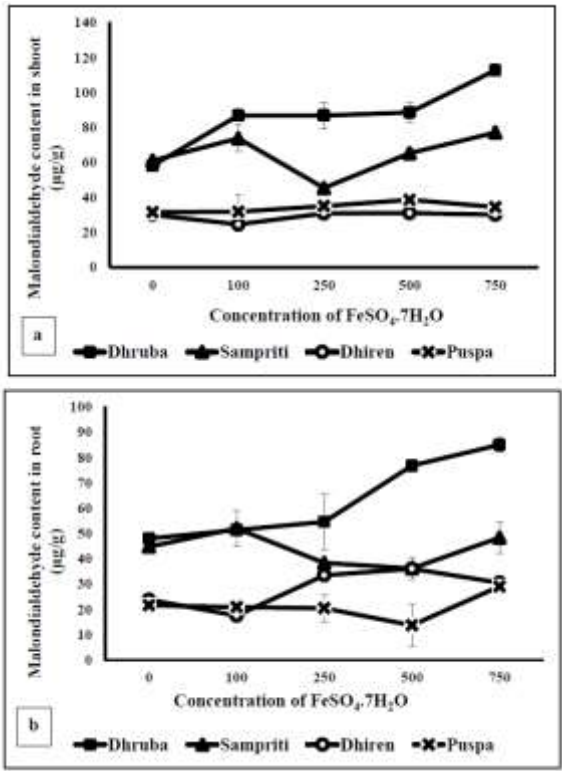

Fig. VI. Effect of iron stress on lipid peroxidation in shoot (a) and root (b) parts (bars indicate, mean values ± SE, n=5).

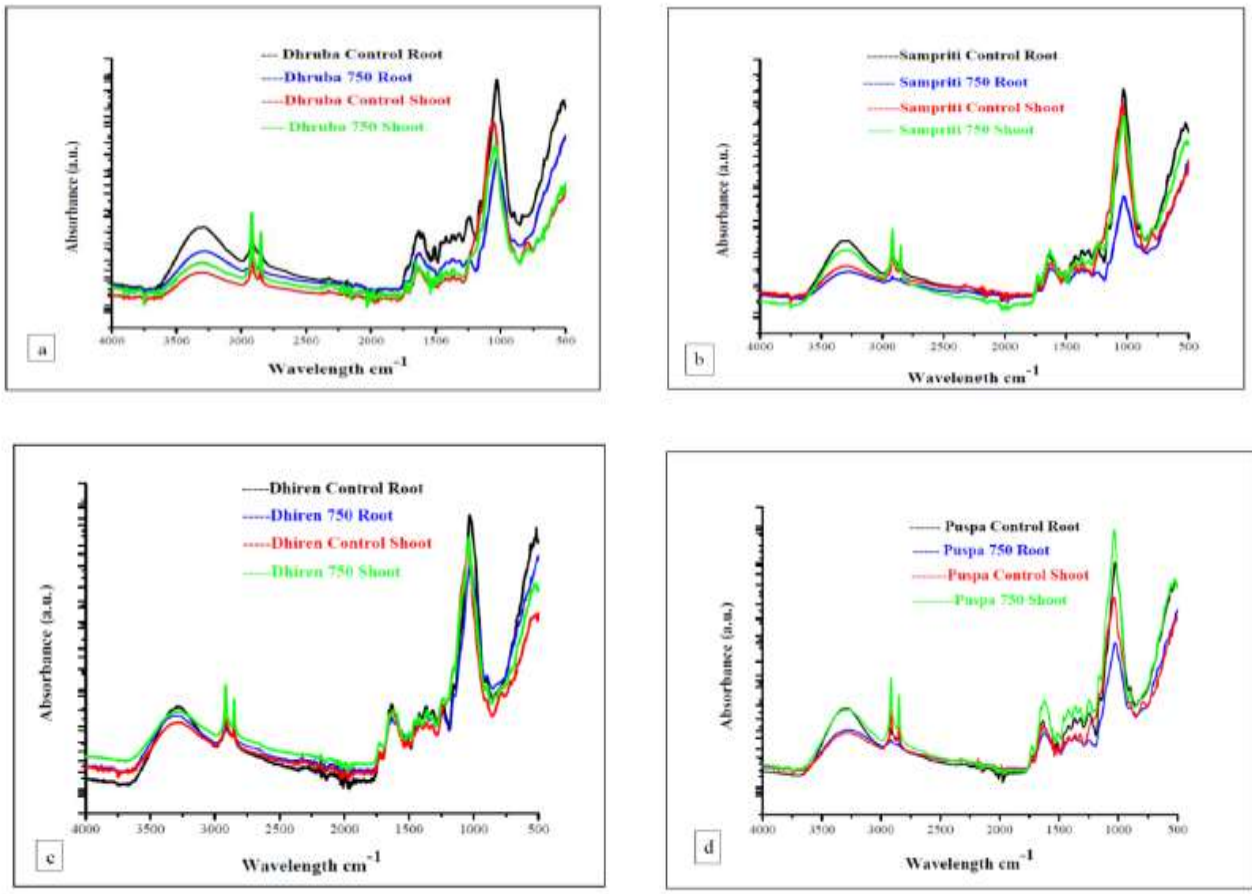

Fig. VII. FTIR spectra curves of shoot and root parts of Dhruba (a), Sampriti (b), Dhiren (c), and Puspa (d) rice variety under 750 ppm against control

at ~1300 cm^{-1} was due to C-H stretching of alkanes and C-N stretching in aromatics in the root parts in all the varieties. Another band changing at ~1400 cm^{-1} was due to the presence of C-H stretching in alkanes in the shoot parts of all the varieties. The change at ~1500 cm^{-1} in root parts of all the varieties was due to nitro compound (N-H and N=O stretching) and C-C, and C-H bond stretching in aromatics. Similarly, peak change at ~1650 cm^{-1} found in the shoot parts of all the varieties was due to N-H bond bending in amines and N=N bond stretching in azo compounds. An interesting peak change at ~1750 cm^{-1} found in shoot parts of the varieties, Dhruba, Sampriti, and Puspa, was due to ketone stretching in diketone and acid anhydrides and -COOR bonds in pectin. This was followed by peak changes at ~2300 cm^{-1}, which showed N-H bond stretched in amino acids. Changes at ~2800 cm^{-1} in the root parts of all the varieties indicated carboxylic acids. The peak changes at ~2900 cm^{-1} in shoot parts of all the varieties were due to the presence of C-H bond stretching in the alkanes. Changes at 3750 cm^{-1} represented the presence of H bond stretching in amides and alcohols (Fig 7 a-d).

Discussion

Growth of all the rice varieties decreased under high concentrations of Fe. Dhiren and Puspa variety showed significant growth reduction among the four rice varieties. Similar result was reported by Gangarani et al. (2018) and Pereira et al. (2013). Excess of water-soluble Fe present in flooded situation, leads to translocation into plant cells causing oxidative damage within the cells (Sahrawat, 2010). Plant pigments like chlorophyll are susceptible to ROS and found to be affected by oxidative stress induced by Fe (Gallego et al.,

1996; Sinha et al., 1997; Vansuyt et al., 1997; Li et al., 2012) resulting in chlorophyll degradation and non-stomatal limitation of photosynthesis.

The decrease in chlorophyll A and chlorophyll B content varied differently between tolerant and non-tolerant varieties. In Sampriti and Dhruba at 250 ppm, chlorophyll (A and B) content increases and decreases from 500 ppm, whereas in other varieties chlorophyll (A and B) content decreases from 250 ppm. Excess induced Fe stress reduces ROS resulting in breakage of chlorophyll pigment. Again, reduced chlorophyll content leads to retarded plant growth resulting in decrease in shoot length.

The SOD activity of Sampriti, Dhruba, and Puspa varieties decreased under Fe stress. At the same time in all the rice varieties CAT activity increased. Similar result was observed in the findings of Gao et al. (2014) where under 250 mg/L Fe stress, the SOD, and CAT activities of the rice genotypes decreased. Xing et al. (2010), and Hendry and Brocklebank (1985) have revealed that CAT activity is important in the plant resistance to Fe toxicity. Gill and Tuteja (2010) indicated increased SOD activity helps in controlling ROS and repairs oxidative damage caused by Fe^{2+}. There is a significant increase in lipid peroxidation rate in both root and shoot parts of all the rice varieties under Fe toxicity. Production of ROS might be increased parallel with increased lipid peroxidation under this stress. This may result in the formation of Malondialdehyde, one of the two final products of peroxidation of unsaturated fatty acids in phospholipids and that happened due to cell membrane damage as indicated by Halliwell and Gutteridge (1989).

FTIR is one of the efficient techniques for the analysis of structural and composition changes (Griffiths, 1975). By acquiring IR spectra from plant samples, it could even check the minor changes of macromolecule compounds, like carbohydrate, protein, lipid, and also pectin of cell wall (Surewicz et al., 1993; McCann et al., 1992). FTIR spectra for Fe toxicity in four rice varieties showed the presence of steep band stretching of various functional groups of different compounds in the samples. Potential protein structural and chemical compositional changes that occur during abiotic stress like salt stress where IR spectra reveals two absorption regions could be marked as key indicators of stress levels and metabolic status, amide I at 1580-1700cm^{-1}, and pectin accumulation at 1745 cm^{-1} as shown in the work of Griffiths (1975), Barth (2000), and Yang and Yen (2002). The present work indicated similar spectral range in the stressed sets peaks at 1160, 1105, 1060, and 1040 cm^{-1}, characteristic of cellulose as shown by Tsuboi (1957) and Liang and Marchessault (1959). A structural variation of cell wall component like lignin was found that might be due to the increased oxidative stress in wall-modifying plant tissues (Moura et al., 2010). This work revealed that Fe stress had negative effects on seedling growth in rice varieties.

The findings of this work showed Sampriti as tolerant variety under Fe stress whereas Dhiren variety was found susceptible to excess amount of Fe concentration. Among the four selected rice varieties of interest, the lowest reduction in both chlorophylls A and chlorophyll B contents was recorded in Sampriti. High amount of SOD activity in Dhruba and high amount of CAT activity in Sampriti may lead to reduced lipid peroxidation in the root part resulting in increased root length in the stress condition. This work showed that an increase in lipid peroxidation is the primary response of Fe toxicity and the decrease in chlorophyll content was a part of the overall expression of Fe toxicity. Analysis of FTIR spectra for Fe toxicity in four rice varieties showed steep band stretching of various functional groups of different compounds in the samples in amide, pectin, and lignin, which marked as key indicators of stress levels. Therefore, the effects of Fe toxicity on the growth as well as other physiological expressions of rice plants was influenced by both variety and concentration of Fe the plant was exposed to.

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