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ANALYSIS OF PHYSIOLOGICAL AND SPECTRAL PARAMETERS ON DIFFERENT *ORYZA SATIVA* L. VARIETIES UNDER IRON STRESS

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Abstract: Iron toxicity is an abiotic stress that comes with high concentrations of Fe^{2+} in the soil solution, and which is a well-recognized problem of rice (*Oryza sativa* L.) cultivation in the lowlands. 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 was to study the influence of applied high Fe^{2+} concentrations on the growth, chlorophyll content and antioxidant enzyme activities on these varieties of rice. The spectral reflectivity and absorption of different chemical bonding through Fourier-transform infrared spectroscopy (FTIR) of the four rice varieties were also analyzed. The 7-day-old rice seedlings were treated with ferrous sulfate and subjected to 100–750 ppm for a further 14 days. Iron stress was used to analyze the morphological and biochemical responses. At the same time, the root and shoot parts were exposed to the Fourier transform infrared spectral reflection. The results indicated that the shoot growth and chlorophyll content decreased by 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, a high amount of CAT activity in the Sampriti variety, and high amount of SOD activity in the Dhruba variety, led to a higher tolerance of iron stress, in comparison to the other two varieties studied. FTIR revealed steep band stretching of various functional groups of different compounds in both the root and shoot parts of all the varieties. The 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 toxicity, chlorophyll, antioxidant, fourier transform infrared spectroscopy.

Introduction

Iron (Fe) is one of the essential micro-elements, which is involved in several biological processes of plant cells throughout their lives. Due to the redox status change between the two states of iron, i.e. ferrous (Fe-II) and ferric (Fe-III), Fe acts as an electron donor or acceptor, which is vital for both photosynthesis and respiration [35, 58]. Moreover, in the case of plant growth, Fe enhances enzymatic redox reaction [24]. It also serves as a co-factor of several enzymes and is the major ingredient of the cell redox systems such as heme proteins, cytochrome, catalase (CAT), and peroxidase, and also FeS proteins, including ferredoxin, aconitase and superoxide dismutase isoenzyme (Fe-SOD) [41].

Lowland rice often suffers from Fe toxicity, which affects the yield [6] due to the excess amount of water-soluble Fe. Mineral soils, including acid clay, acid sulphate and peat soils [15], contain a high amount of Fe. The interflow of water increases the Fe content in the fields of lowland rice. [50]. Under the flooded conditions, the microbial reduction of insoluble Fe (III) into soluble Fe (II), results in excess Fe in lowland soil [48]. The nutrient imbalance in plants is created due to the indirect effects of excess Fe, through the reduction of uptake and utilization of essential

nutrients (phosphorus, potassium, calcium, magnesium, and zinc) in plants [19].

Excessive Fe absorption by plants generates a symptom called 'bronzing'. The roots of rice plants affected by Fe toxicity become shorter in length and dark brown in colour, resulting in underdeveloped root and shoot growth [17], as well as inhibition of nutrient uptake [59]. The toxicity is generated by donating or accepting an electron from the surrounding biomolecules with excess Fe, which produces reactive oxygen species (ROS), thus causing major damage to the cellular components. In tissue, Fe catalyzes the ROS formation via the Fe catalyzed Haber-Weiss reaction (Fenton reaction), in the presence of ascorbate or hydrogen peroxide (H_2O_2), resulting in the generation of hydroxyl radicals [26]. Plants show different systems of tolerance to Fe toxicity [7], including shoot-based tolerance mechanism, i.e. the scavenging of ROS by antioxidants like ascorbate, phenolics, glutathione, ascorbate peroxidase (APX), SOD [20, 39]. Similarly, CAT also plays a role in plant-tolerance to Fe toxicity [55]. The phenolic compounds are generally capable of scavenging hydroxyl and lipid radicals [12].

The present report emphasizes the effect of Fe toxicity on the rice seedlings, the mechanisms of its tolerance in seedling growth, and biochemical parameters, i.e. chlorophyll content, antioxidant enzyme activities which include SOD, 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 for analyzing spectral reflectivity and absorption of different chemical bondings, as well as for studying the conformational changing of functional groups of carbohydrates, proteins and lipids [31, 42, 52, 53, 56]. Using this fast method, the initial spectral response to Fe toxicity can be examined by comparing the fingerprints of the global cellular features from very small amounts of samples [3, 5, 14].

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 the lowland areas of West Bengal, with respect to its physiological and FTIR 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. Hence, Fe stresses were given to the different varieties of rice seeds in sterilized Petri-dishes at the germination period, in order to check the tolerance level of Fe stress at different concentrations on the different rice varieties. 50% of the lethal dose was found at 750 ppm. Seeds of the four different varieties of rice (*Oryza sativa* L.), viz. Dhruba, Sampriti, Dhiren and Puspa, were collected from Bankura Rice Research Station, West Bengal. Rice seedlings were cultured under the following conditions: 1000 lux light intensity for 16 hours with the interval of 8 hours, 29 ±2 °C temperature, and 60 ±5 % relative humidity with 'Yoshida solution' [57], for 21 days. Bremen and Moormann (1978) reported that Fe solubility in water-flooded soil and Fe uptake by plants is related to a pH below 5.8. Therefore, the pH of the media was maintained between 5.3 to 5.9.

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

Estimation of Chlorophyll contents

Chlorophyll contents were measured through extraction with 80% acetone, according to Arnon (1949). Since rice is a monocot plant, therefore the whole shoot part was taken for chlorophyll estimation. The following equation was used to calculate total chlorophyll, and the chlorophyll content was expressed on a fresh weight (FW) basis.

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). The whole shoot part and all the rootlets were taken separately for the estimation of protein content. Ice-chilled plant materials were used for the experiment. Plant material was extracted with 0.1 M phosphate buffer pH = 6.8, as mentioned by Kar and Mishra (1976), for the estimation of protein content, CAT activity and SOD activity.

CAT activity was measured in a spectrophotometer at room temperature, by monitoring the decrease in absorbance at 240 nm resulting from H_2O_2 decomposition [1]. One unit (U) of CAT was equivalent to the change of absorbance at the rate of 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 the method based on the performance of the enzyme for the inhibition of pyrogallol autoxidation [38]. One unit (U) of SOD activity is defined as the amount of enzyme required to inhibit 50% of pyrogallol autoxidation [40].

The rate of lipid peroxidation was estimated by the method of Heath and Packer (1968) and calculated as the amount of malondialdehyde produced. The tissue was boiled with thiobarbituric acid reagent (0.25% TBA in 10% TCA). The mixture was at first cooled, and then centrifuged at 10,000X g for 10 min. The absorbance was recorded at 440nm, 532 nm and 600nm [30, 36]. To maintain the accuracy of determining malondialdehyde content the calculation was done in the following manner:

$[(A_{532})-(A_{600})]=A$ (3)

$[(A_{440}-A_{600})0.0571]=B$ (4)

Malondialdehyde content: $(A-B/0.157)$ (5)

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

The FTIR-ATR spectra of rice samples were recorded with an 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-ionized water.

Statistical analysis

The average of five sets of repetitive measurements was taken from each experiment, each one further repeated five times, with randomly chosen samples. All the collected data were analyzed using Analysis of Variance (ANOVA) by SPSS version 20. The statistical analysis was conducted for testing the significance of the stress effect. Duncan Multiple Range test was carried out according to the least significant difference (LSD) values. Standard methods have been used to calculate F-test and critical difference [45]. All the statistical tests were analyzed at 5% of a significant level.

Results

Effect of Fe toxicity on growth parameters of rice seedlings

The shoot-length of all the rice varieties showed different growth in Fe stress. The Dhiren variety showed a marked decrease in shoot-length (31%) at 750 ppm of toxicity, in comparison to control-treatment (Fig.1a). The Puspa, Dhruba and Sampriti varieties showed 11.5%, 9%, and 5% decrease in shoot-growth respectively. The resultant shoot-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. 1b). In contrast, the total root-length increased in the Dhruba and Sampriti varieties by 27% and 12% respectively. The data have a significant mean difference with the P-value = 0.000.

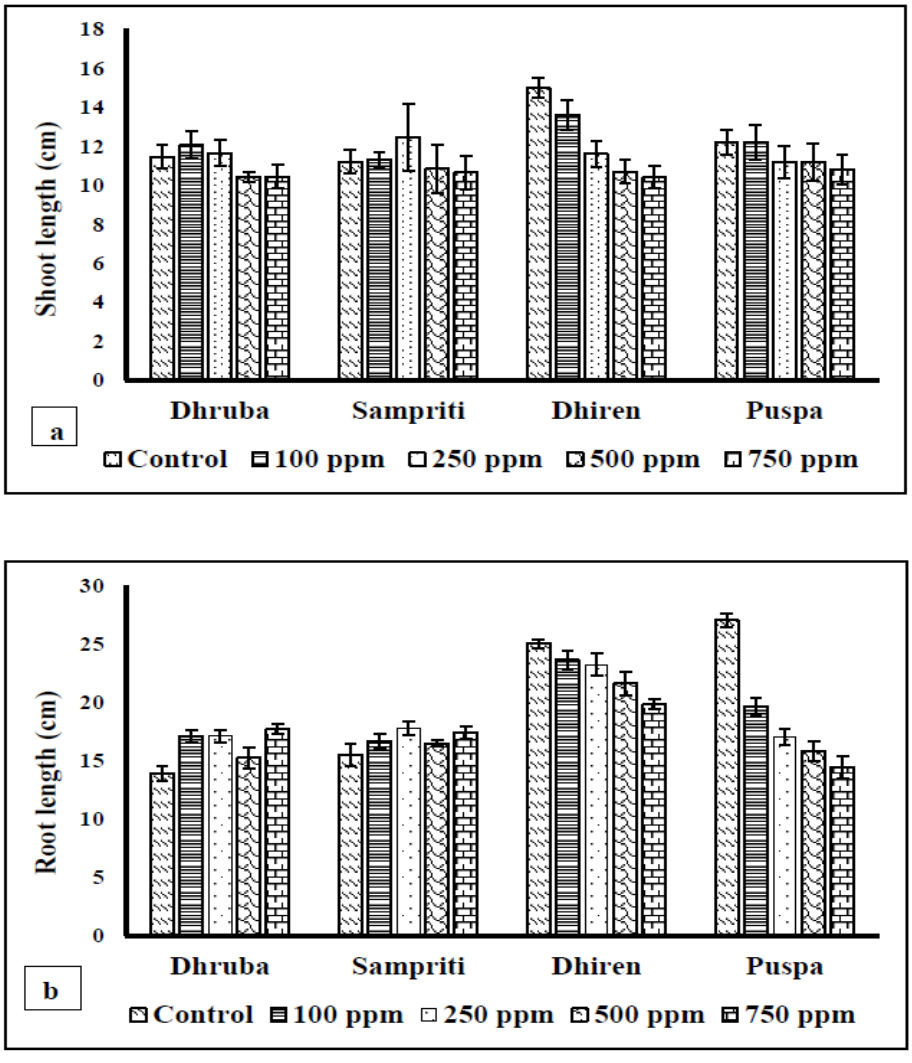


Fig. 1: Effect of iron stress on (a) shoot-length and (b) total root-length of different rice varieties (bars indicate mean values ± (Standard Error) SE, n=5).

Chlorophyll content in rice under Fe toxicity

Chlorophyll contents in rice seedlings showed different responses to Fe stress. The chlorophyll A content decreased significantly in Dhruba by 61%, followed by Puspa, Dhiren, and

Sampriti by 60%, 48%, and 31% at 750 ppm respectively, as compared to the control (Fig.2a). The result correlates significantly with the P-value = 0.05. The chlorophyll B content decreased significantly in Dhruba by 61%, followed by Puspa, Dhiren, and Sampriti by 57% 48%, and 17% at 750 ppm respectively, as compared to the control (Fig. 2b). The resultant data have a significant mean difference with the P-value = 0.000.

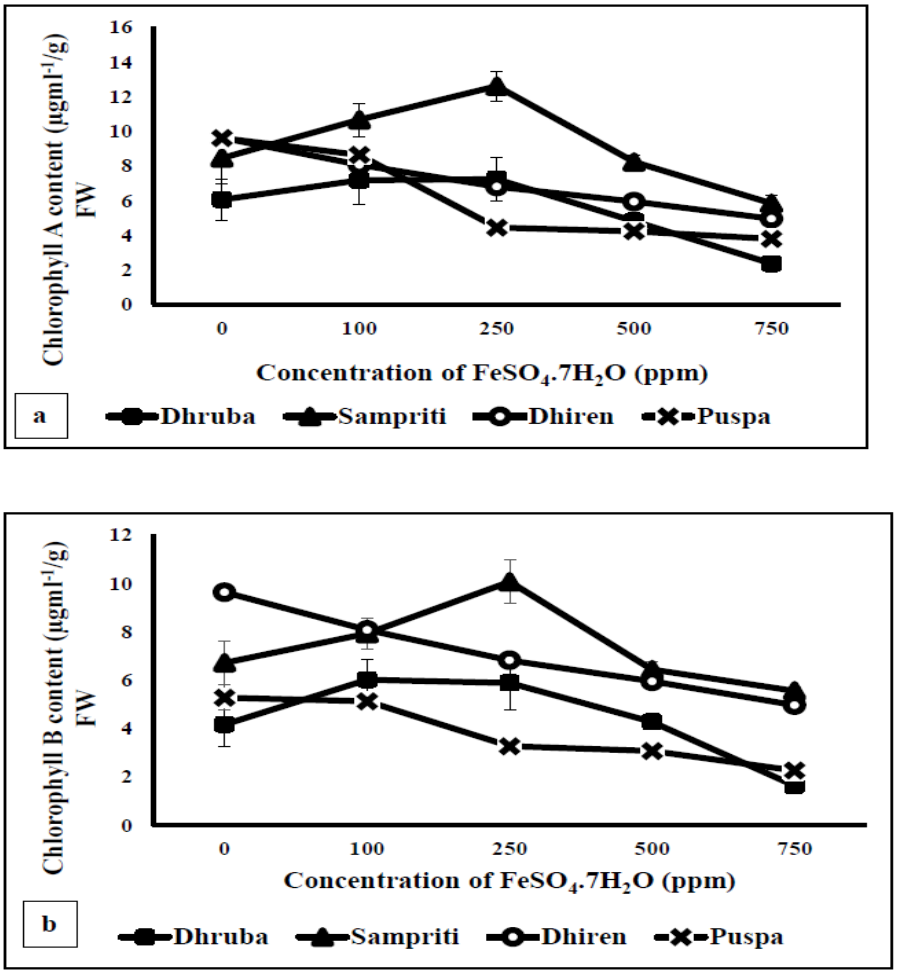


Fig. 2: Effect of iron stress on total chlorophyll content in rice seedlings (bars indicate mean values ± SE, n=5).

Protein content in rice to Fe toxicity

The protein content in the shoot parts of the Dhruba, Sampriti and Puspa varieties of rice seedlings showed a slight increase (6%, 9% and 5.6% respectively), while the Dhiren variety showed a slight decrease (2.4%) in 750 ppm of Fe stress as compared to the control (Fig. 3a). On the other hand, while the protein content in the root parts of the Dhruba and Sampriti varieties of rice seedlings showed a slight decrease (4% and 3% respectively), the Dhiren and Puspa varieties showed a slight increase (23% and 8% respectively) in 750 ppm of Fe stress as compared to the control (Fig. 3b). (P-value of ANOVA for both shoot protein and root protein was 0.000).

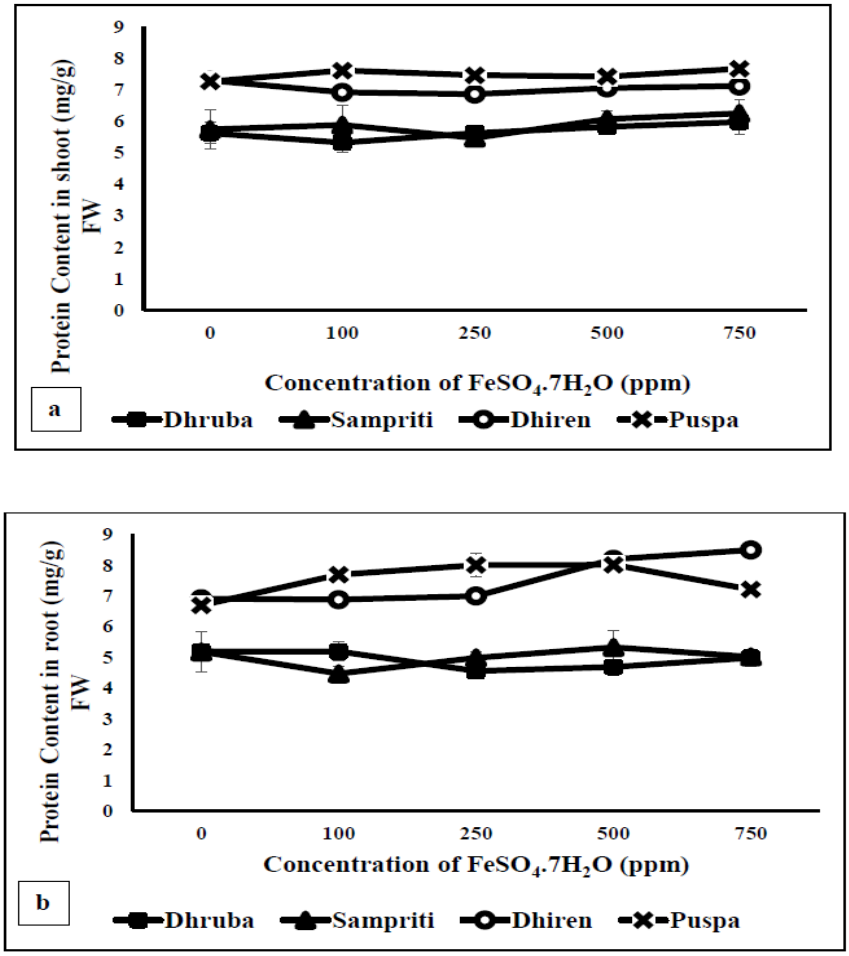


Fig. 3: Effect of iron stress on soluble protein content in rice seedlings (bars indicate, mean values ± SE, n=5).

Superoxide Dismutase activity

The SOD activity in the shoot parts of varieties Sampriti, Dhiren and Puspa was found to have decreased by 22.6%, 16%, and 16% respectively. On the contrary, there was no significant difference in the Dhiren variety (Fig. 4a). But the activity in the roots of the Dhruba variety increased by 21%, whereas in the Sampriti, Dhiren and Puspa varieties it decreased by 19%, 20%, and 8% respectively (Fig. 4b). The result of SOD activity in the shoot-length proved to be statistically significant (P-value of ANOVA for SOD activity in the shoot was 0.003 and 100 ppm to 750 ppm in the root was 0.0123).

Catalase activity

The CAT activity in the shoot parts of Dhruba, Sampriti, Dhiren and Puspa were found to have increased to 77%, 46%, 81% and 17% respectively (Fig. 5a). The CAT activity in the root parts of Dhruba, Sampriti, Dhiren, and Puspa were found to have increased to 95%, 147%, 11% and 74% respectively, as compared to the control (Fig. 5b). (P-value of ANOVA for CAT activity in both shoot and root was 0.000).

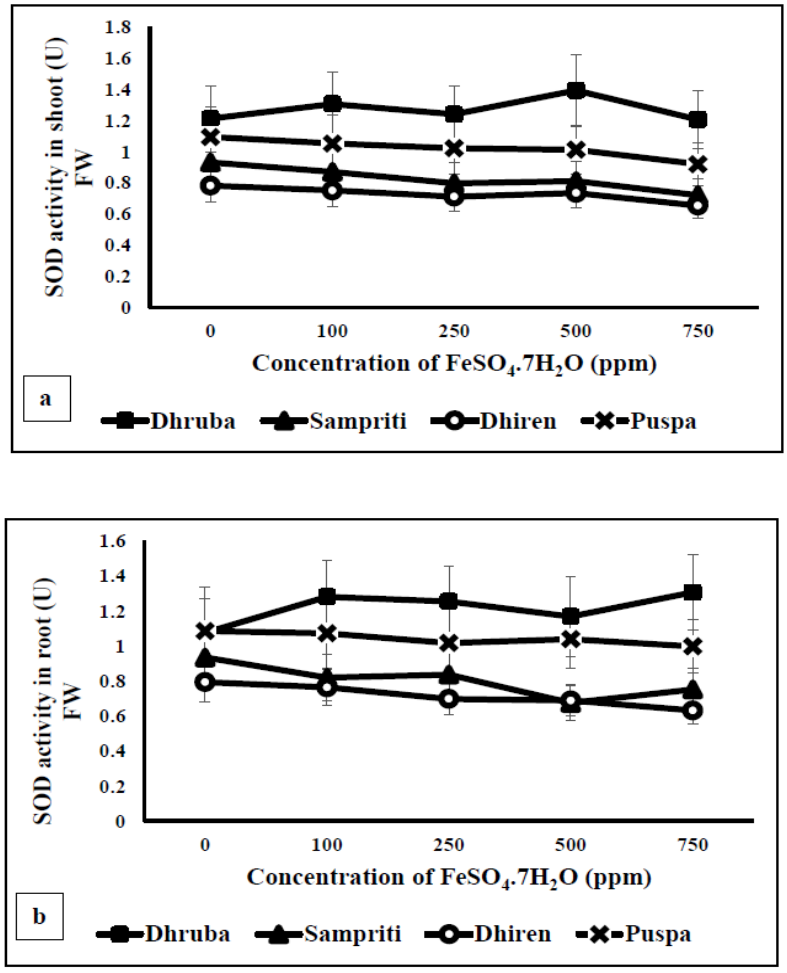


Fig. 4: Effect of iron stress on SOD activity in shoot (a) and root part (b) (mean values ± SE, n=5).

Lipid peroxidation

The content of malondialdehyde (a by-product of lipid peroxidation) in the shoots of Dhruba, Sampriti, Dhiren, and Puspa, increased to 94%, 26%, 0.3%, and 9% respectively (Fig. 6a). The activity in the root-parts of Dhruba, Sampriti, Dhiren, and Puspa was found to have increased to 77%, 8%, 28%, and 35% respectively, as compared to control (Fig. 6b). (P-value of ANOVA for lipid peroxidation in the shoot was 0.000 and for lipid peroxidation in root was 0.000).

FTIR analysis to monitor the changes in seedlings under iron toxicity

In the FTIR spectrum, the infrared radiation peak values have been used to identify the functional group present in the active components based on the regions. This technique was useful for the study of the conformational changes in carbohydrate, lipid, protein and cell wall components among the experimental set of all the four rice varieties, between the control sample and the sample treated with 750ppm of $FeSO_4.7H_2O$. The changes in the absorption band at 3400 cm^{-1} represent the O-H and N-H stretching vibrations that occur in carbohydrate and protein and that was observed in root and shoot parts of all the four varieties (Fig 7 a-d) [18]. An interesting peak change around 2800 cm^{-1} to 3000 cm^{-1} represents C-H stretching, CH_2 and CH_3 alkyl chains, and olefinic bonds in the lipid region [8, 32]. Again, the band change at 2960 cm^{-1} was due to an antioxidant effect of phenolic acids, observed in the root and shoot parts of Dhruba, Sampriti and

Puspa (Fig.7 a,b,d). Absorption band change at 1745 cm^{-1} was found in the shoot part of the varieties Dhruba, Sampriti and Puspa. It is due to the ketone stretching in ester-containing compounds, diketone, and acid anhydrides and -COOR bonds in the membrane lipid and cell wall pectin [47]. The changes in protein absorption bands ~ 1680 cm^{-1} (amide I band) observed in the shoot parts of all varieties are due to C-O bond bending in proteins [5, 53]. Again, amide II band change in the root and shoot parts of all varieties at ~1550 cm^{-1} is due to N-H bond stretching observed in both the root and shoot part of all the four rice varieties [5]. The amide III band change at ~1250 cm^{-1} was due to C-N stretching in proteins and C–O asymmetric stretching in cyclic polyphenolic compounds observed in the root-parts of Sampriti, Dhiren and Puspa and shoot part of Dhruba, Sampriti. Significant absorbance band change was seen at 1630 cm^{-1}, is corresponded to an antioxidant effect due to H_2O_2-induced protein aggregation [39], which is increased shoot-part of Puspa and Sampriti and root-part of Dhruba. Acid band change at 1420 cm^{-1} is related to the COO- stretching; due to the Fe^{+} absorption observed in the shoot and root parts of all the varieties [33]. Bands around 1000 cm^{-1} to 1100 cm^{-1} in the "fingerprint" region indicate several modes such as C-H bending or C-O or C-C stretching of carbohydrates indicating significant vibration in the shoot parts of all the varieties [5]. Changes in the band at 1050 cm^{-1} in all the root and shoot parts of all the varieties were attributed to iron-oxyhydroxide. The strong band changes in all the varieties were observed at 450 cm^{-1} to 500 cm^{-1} (Fig 7a-d) both in the shoot and root parts, attributed to Fe-O bond stretch [43, 44].

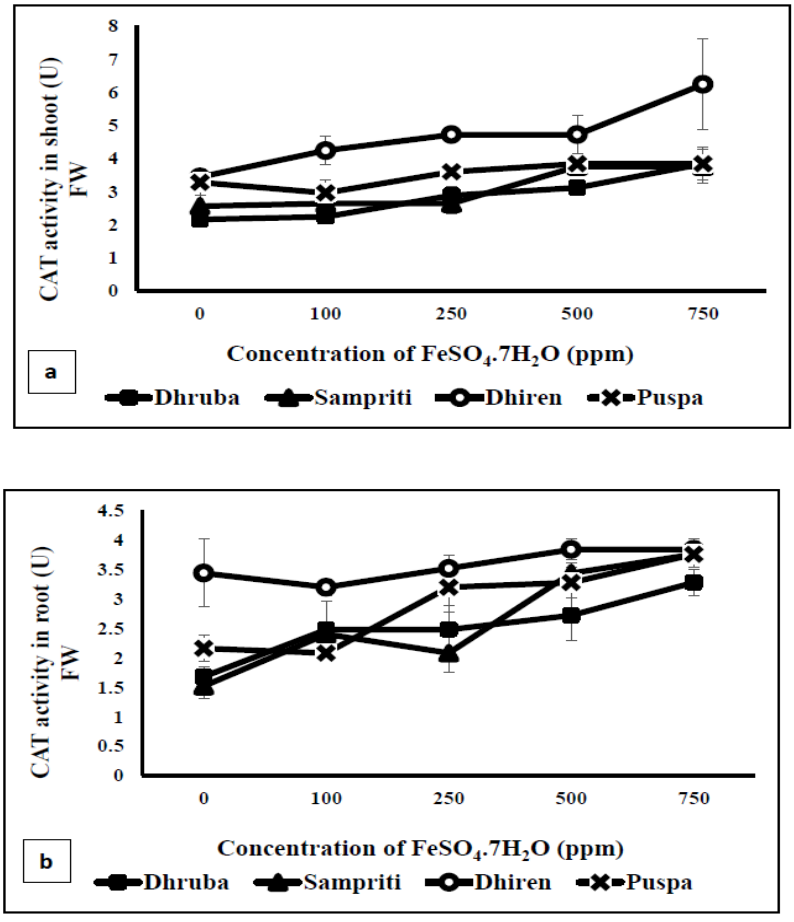


Fig. 5: Effect of iron stress on CAT activity in shoot (a) and root (b) part (mean values ± SE, n=5).

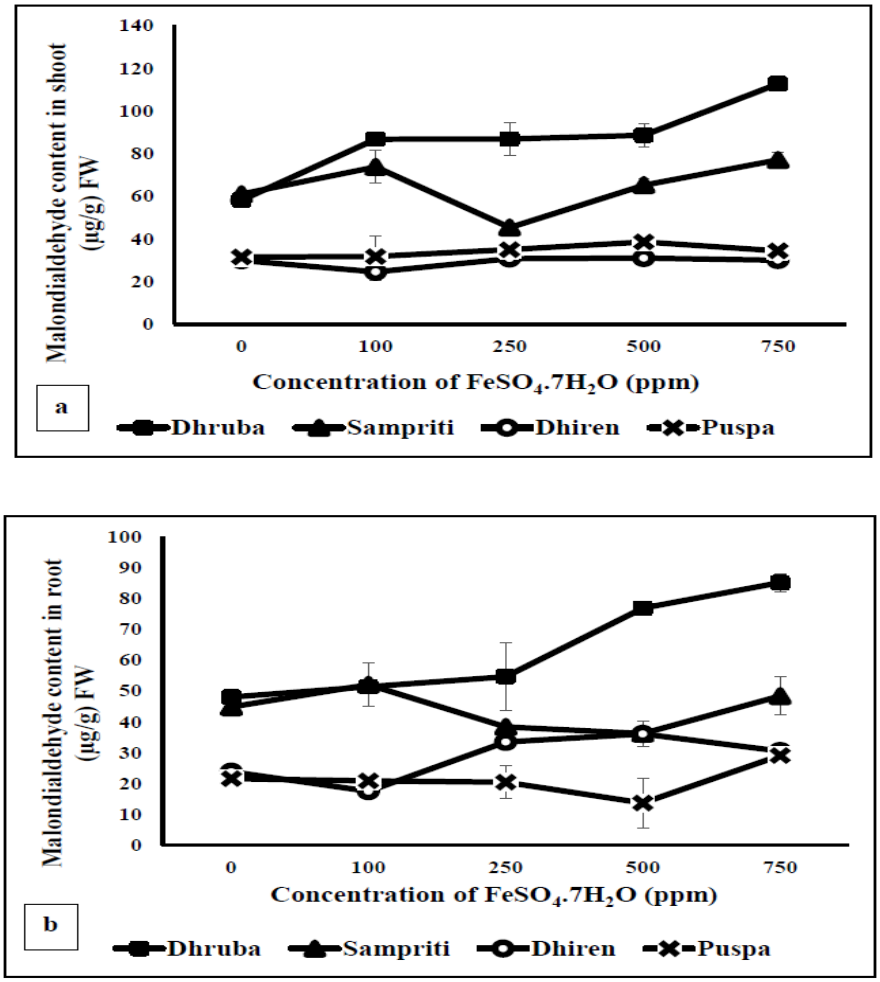
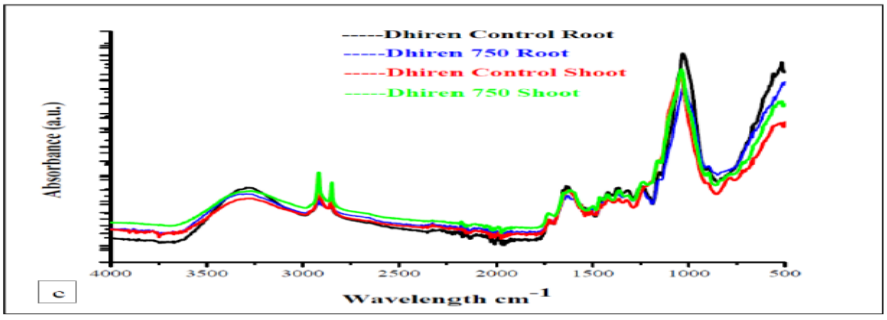


Fig. 6: Effect of iron stress on lipid peroxidation in shoot part (a) (bars indicate, mean values **and root part (b)** (bars indicate, mean values ± SE, n=5).

Discussion

Growth of all the rice varieties decreased under high concentrations of Fe; Dhiren and Puspa varieties showed significant growth reduction among the four rice varieties. A similar result is reported by Gangarani et al. (2018) and Pereira et al. (2013). Excess of water-soluble Fe present in the flooded situation leads to translocation into plant cells, causing oxidative damage within the cells [49]. Plant pigments like chlorophyll are susceptible to ROS and found to be affected by oxidative stress induced by Fe [37, 51, 54], 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 increased and decreased from 500 ppm, whereas in other varieties chlorophyll (A and B) content decreased from 250 ppm. Excess induced Fe stress reduced ROS resulting in breakage of chlorophyll pigment. The reduced chlorophyll content is one of the reasons that reduces shoot growth through a reduction in the photosynthetic carbon assimilation rate [21].

The SOD activity of the Sampriti, Dhruba, and Puspa varieties decreased very nominally under Fe stress. However, in all the rice varieties CAT activity increased significantly. A similar result observed in the findings of Gao et al. (2014) was that under 250 mg/L Fe stress, the SOD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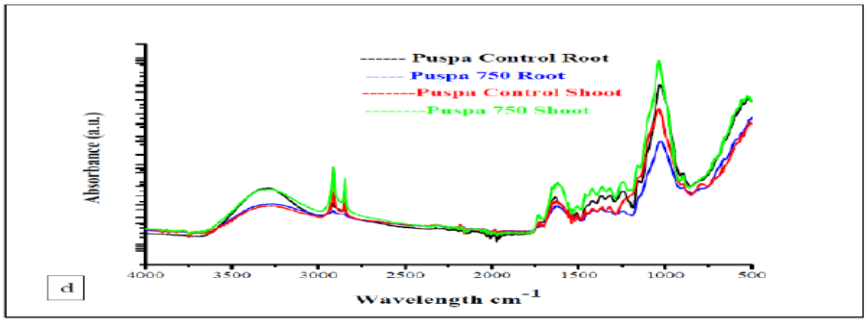
------ Puspa Control Root
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--------Puspa 750 Shoot
Absorbance (a.u.)
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This work concludes that an increase in lipid peroxidation is the primary response of Fe toxicity, and the decrease in chlorophyll content is a part of the overall expression of Fe toxicity. Analysis of FTIR spectra for Fe toxicity in four rice varieties shows the steep band stretching of various functional groups, which is an evidence of structural modification of lipid, protein, and carbohydrates, in both root part and shoot part of the all the four rice varieties. A high amount of CAT activity in Sampriti may lead to reduce lipid peroxidation in the root part, resulting in increased root-length in the stress condition. The decrease in amide I and amide II band peaks are related to the sensitivity of the Dhiren variety, and the increase in those bands corresponds to the tolerance of the Sampriti variety, through the formation of antioxidant enzymes and phenolic compounds. Therefore, the effects of Fe toxicity on the growth as well as other physiological expressions of rice plants are influenced by both variety and the concentration of Fe to which the plant is exposed.

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Conflict of interest: None declared.

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ANALIZA PARAMETRILOR FIZIOLOGICI ŞI SPECTRALI AI DIFERITELOR VARIETĂŢI DE *ORYZA SATIVA* L. ÎN CONDIŢII DE STRES CAUZAT DE SURPLUS DE FIER

(Rezumat)

Toxicitatea feroasă este un stres abiotic ce se manifestă în condiţii de concentraţii crescute de Fe^{2+} în soluţia solului şi este o problemă bine cunoscută în cultivarea orezului (*Oryza sativa* L.) în zonele joase. Soiurile de orez diferă foarte mult în ceea ce priveşte toleranţa la excesul de fier. Acest studiu a luat în calcul patru varietăţi de orez, respectiv Dhruba, Sampriti, Dhiren şi Puspa. Obiectivul studiului a fost observarea influenţei aplicării unor concentraţii diferite de Fe^{2+} asupra creşterii, conţinutului de clorofilă şi activităţii enzimatice antioxidante ale acestor soirui de orez. De asemenea, s-a analizat şi reflectivitatea spectrală şi absorbţia diferitelor legături chimice prin spectroscopia în infrarosu transformată Fourier (FTIR) a celor patru varietăţi de orez. Răsadurile de orez de 7 zile au fost tratate cu sulfat feros şi supuse la 100-750 ppm pentru înca 14 zile. Stresul feros a fost folosit pentru a analiza răspunsurile morfologice şi biochimice. În acelaşi timp, părţile rădăcinii şi tulpinii au fost expuse la reflecţia spectrală infrarosie transformată Fourier. Rezultatele au arătat o scădere a creşterii tulpinilor şi conţinutului în clorofilă, la 750 ppm, în cazul tuturor varietăţilor de orez selectate. Dimpotrivă, activitatea catalazei, conţinutul în proteine şi peroxidarea lipidelor a crescut la aceste varietăţi. Totuși, o creștere a activității CAT în soiul Sampriti și a activității SOD în soiul Dhruba a determinat o toleranţă mai mare la stersul feros faţă de celelalte două soiuri studiate. FTIR a evidenţiat benzi abrupte pentru diferitele grupe funcţionale ale compuşilor diferiţi, atât în părţile rădăcinii, cât şi a tulpinii, în cazul tuturor soiurilor studiate. Rezultatele obţinute au evidenţiat că schimbarea expresiei antioxidante şi spectrele FTIR s-au datorat efectului toxic feros în plantele de orez.

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