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Iron Toxicity in Rice (*Oryza sativa* L.), under Different Potassium Nutrition

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Abstract: In the present study, the effects of iron toxicity in rice and the possible roles of potassium nutrition in the alleviation of iron toxicity are studied. Rice plants (*Oryza sativa* L.) were cultured in a sand substrate and submerged with Yoshida nutrient solution in greenhouse. Two weeks old rice plants were received various treatments for four weeks. The experiments were carried out in a completely randomized design as factorial. Factor one was iron at five levels (10, 50, 100, 250 and 500 mg L^{-1}) in the form of Fe-EDTA and factor two was potassium which was supplied as KCl (control, 200 and 400 mg L^{-1}). Maximal plant growth occurred at iron concentration of 10 and 50 mg L^{-1} and growth reduction due to iron toxicity was observed at iron concentration of 250 and 500 mg L^{-1}. Potassium nutrition could not alleviate effects of iron stress on plant growth. Excess iron caused higher iron and lower potassium concentrations in both roots and shoots as compared with the 10 mg L^{-1} iron-fed plants and iron accumulation in plants was not affected by potassium nutrition. Toxic iron levels increased hydrogen peroxide, phenolica contents, lipid peroxidation and peroxidase activity while it reduced the activity of catalase. Along with the aforementioned changes related to the oxidative stress, total chlorophyll, soluble protein and carbohydrate contents declined and amino acid content increased under toxic iron levels. The results indicate that iron toxicity induced greater oxidative stress in rice plants and supplemental potassium was ineffective in preventing iron accumulation in shoots and consequently did not ameliorate plant growth under iron toxic levels.

Key words: Iron toxicity, potassium, oxidative stress, lipid peroxidation, antioxidant enzyme activity

INTRODUCTION

Rice is one the most important crops in developing countries and it is a main food stuff for about 35% of the whole world population. Rice grain has a high nutritional value and calorie content as compared with the most other crops and consequently has become a strategic commodity across the whole world. Approximately, 128 million hectares of the irrigated and rainfed lands of the world are under rice cultivation and due to either nutrient deficiency or toxicity yield production has been reduced in about 100 million hectares of these lands (Becker and Asch, 2005).

Iron as an essential element for all plants has many important biological roles in processes as diverse as photosynthesis, chloroplast development and chlorophyll biosynthesis. Iron is a major constituent of the cell redox systems such as heme proteins including cytochromes, catalase, peroxidase and leg-hemoglobin and iron-sulfur proteins including ferredoxin, aconitase and superoxide dismutase (SOD) (Marchner, 1995).

Iron toxicity is the most widely distributed nutritional disorder in lowland-rice production (Dobermann and Fairhurst, 2000). Although most mineral soils are rich in iron, the expression of iron toxicity symptoms in leaf tissues occurs only under specific flooded conditions, which involves the microbial reduction of insoluble Fe^{3+} into soluble Fe^{2+} (Becker and Asch, 2005). The appearance of iron toxicity in plants is related to high Fe^{2+} uptake by roots and its transportation to leaves via transpiration stream. The Fe^{2+} excess causes free radical production that impairs cellular structure irreversibly and damages membranes, DNA and proteins (Arora *et al*., 2002; Dorlodot *et al*., 2005). Iron toxicity in tobacco, canola, soybean and *Hydrilla verticillata* are accompanied with the reduction of plant photosynthesis and yield and the increase of oxidative stress and ascorbate peroxidase activity (Kampfenkel and Montagu, 1995; Caro and Puntarulo, 1996; Sinha *et al*., 1997). Increase in the amount of H_2O_2 and phenolica and decrease of chlorophyll and soluble protein content by oxidative stress have been reported by several researchers as

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Vichnevetskaia and Roy (1999), Schutzendubel and Polle (2002), Blokhina *et al.* (2003) and Kuo and Kao (2004). Promotion of lipid peroxidation and changes in antioxidant enzyme activity is also well documented (Chen *et al.*, 2000; Schutzendubel and Polle, 2002). Iron toxicity in rice leads to the accumulation of the element in plant tissues accompanied with ethylene biosynthesis in shoots, drastic reduction of root growth and loses of yield (Yamauchi and Peng, 1995; Becker and Asch, 2005; Dorlodot *et al.*, 2005). Iron toxicity symptoms are various among rice cultivars and the most common ones are spots on the inter-veins and purplish brown of the leaves followed by drying. Roots become scanty, coarse, short and blunted (Peng and Yamauchi, 1993).

Macronutrients can affect response of plants to iron toxicity (Sahrawat, 2004). Potassium is a common macronutrient in plants that activates many enzymes involved in photosynthesis and respiration. This element has many important roles such as starch and protein synthesis, cell expansion, stomatal movement and stress alleviation (Marchner, 1995; Hopkins, 2004). In addition, potassium is involved in anion-cation balances, electrical charge regulation and pH maintenance across biological membranes.

Ramirez *et al.* (2002) have reported that the application of N, P, K, S and Zn fertilizers reduced the unfavorable effects of iron toxicity in rice paddy. Also, high concentration of iron in the soil solution decreased the absorption of Ca^{2+}, Mg^{2+} and especially P and K by rice plants. In rice fields, the application of potassium fertilizers has resulted in the decreased iron and increased K^+ content that accompanied with better grain yields (Yamauchi, 1989). It appears that proper K supply may increase iron exclusion from roots and reduce translocation of iron to aerial parts of plant especially to upper leaves (Sahrawat, 2004). Li *et al.* (2001) has reported that supplemental K in the root medium may increase root oxidation potential which resulted in higher K and lower iron uptake and consequently amelioration of iron toxicity effects in the root medium.

In the present study, rice plant cultivated under different iron and potassium treatments and the concentration of K, Fe, chlorophyll, soluble protein, amino acids, soluble sugars and the activities of some ROS-scavenging enzymes were measured. The main objective was to evaluate the effects of iron toxicity in rice plants and possible alleviation of iron toxicity symptoms by potassium nutrition.

MATERIALS AND METHODS

Culture conditions and growth evaluation: The experiments were conducted at greenhouse of the Gorgan University of Agricultural Sciences and Natural Resources during 2004-2005. Seeds of rice (*Oryza sativa* L. var. shafagh) were obtained from Iran Rice Research Institute and after screening for the uniform size and color, sterilized with a 2.5% sodium hypochloride solution. They were germinated in moistened paper towels and incubated in dark at 25±5°C for 48 h. Germinated seeds were subsequently transferred into pots filled with acid washed sand and pots were subsequently placed in containers waterlogged with Yoshida solution (Yoshida *et al.*, 1976). The experiments were carried out in completely randomized design as factorial. Factor one was iron (Fe^{2+}) concentration added to the medium at five levels (10, 50, 100, 250 and 500 mg L^{-1}) as Fe-EDTA and factor two was supplemental potassium added to the medium at three levels (0, 200 and 400 mg L^{-1}) as KCl. After raising rice plants for two weeks in Yoshida nutrient solution, treatments started for the subsequent four weeks. The pH of the nutrient solution was adjusted daily at 6±0.2 and nutrient solutions were changed every week. The averages of daily maximum and minimum temperatures in greenhouse were 32 and 25°C, respectively during the growing period. Plants were harvested after 45 days of seed sowing.

Iron and potassium analysis: Potassium content was measured by Flame Photometer model Jenway PFP7 and iron content was quantified with Inductively Coupled Plasma (ICP) model GBC (SDS-270) in plant dry material.

Enzymes assay: All enzymes were extracted as described by Kar and Mishra (1976). The fresh leaf samples (0.05 g) were homogenized in phosphate buffer (100 mM, pH 6.8) and the obtained crude extract centrifuged at 17000 x g for 15 min. The clear supernatant was taken as an enzyme source. To assay catalase (CAT) activity, 100 μL of the supernatant was added to a reaction mixture in final volume of 3 mL which consisted of 50 mM phosphate buffer (pH 6.8) and 15 mM H_2O_2 (substrate). Catalase activity was estimated by decrease in the absorbance of 240 nm due to H_2O_2 destruction for 2 min using an extinction coefficient of 40 mM^{-1} cm^{-1} for H_2O_2.

To assay peroxidase activity in a final volume of 3 mL reaction mixture consisted of 25 mM phosphate buffer (pH 6.8), 20 mM guaiacol, 40 mM H_2O_2 and 10 μL of the crude enzyme extract. The reaction was initiated by the addition of H_2O_2 and the change in the absorbance at 470 nm was measured for 2 min. Activity was calculated using an extinction coefficient of 26.6 mM^{-1} cm^{-1} for tetraguaiacol formation per min (Kar and Mishra, 1976; Chen *et al.*, 2000)

Polyphenol oxidase activity was determined in a 3 mL reaction mixture consisted of 25 mM phosphate

buffer (pH 6.8), 10 mM pyrogallol and 100 μL of the crude enzyme extract. Changes in the absorbance of the solution at 420 nm was monitored for 2 min. Activity of polyphenol oxidase was expressed as the amount of purpurogallin formed assuming an extinction coefficient of 2.47 mM^{-1} cm^{-1} (Resende *et al*., 2002).

Proteins, amino acids, sugars and chlorophyll measurements: Soluble protein was measured spectrophotometrically at 595 nm by the Bradford (1976) method after extraction of plant material with 0.1 M phosphate buffer pH = 6.8 (Kar and Mishra, 1976). Bovine serum albumin was used as protein standard. The extraction of soluble sugars and amino acids from plant materials were carried out according to the method of Omokolo *et al.* (1996). Soluble sugars were quantified by Anthron as described by McCready *et al.* (1950) and amino acids was measured by Ninhydrin according to the method described by Yemm and Cocking (1955). Chlorophyll content was measured using the method of Arnon (1949).

Hydrogen peroxide, phenolica contents and lipid peroxidation: The hydrogen peroxide content of tissues quantified colorimetrically as described by Jana and Choudhuri (1982). Phenolica was extracted with ethanol as described by Fukoda *et al.* (2003). The extent of tissue lipid peroxidation was determined as described by Heath and Packer (1968) and expressed as the amount of malondialdehyde (MDA) equivalents produced. The plant tissue was homogenized with 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 6000 x g for 15 min. The obtained supernatant (250 μL) was mixed with 2 mL thiobarbituric acid reagent (0.25% TBA in 10% TCA). After heating for 30 min at 95°C in a water bath, the mixture was cooled and centrifuged at 6000 x g for 10 min. The absorbance was recorded at 532 nm and corrected for non-specific absorbance at 600 and 440 nm (Hodges *et al*., 1999).

Statistical analysis: Statistical analyses of data were carried out using SAS statistical software. Comparison of means were performed using LSD test.

RESULTS AND DISCUSSION

Growth parameters and iron and potassium contents: There were no iron toxicity symptoms except at 250 and 500 mg L^{-1} iron treatments, where toxicity symptoms such as lose of root hairs, scanty and coarse roots and bronzing leaves appeared in the treated plants (data not shown). Growth parameters were significantly affected by excess iron. In control K nutrition, the maximum growth was occurred in cultured plant in the presence of 50 mg L^{-1} iron and higher and lower iron resulted in marked decrease in both root and shoot dry weights (Table 1). Potassium nutrition induced marked increase in growth under critical iron concentrations (10 mg L^{-1}), however, at higher iron concentrations growth parameters did not change significantly by the added potassium (Fig. 1). Increase of iron concentration in the root medium led to the gradual decrease in the plant relative water content (Table 1). Also, 400 mg L^{-1} supplemental potassium caused significant decrease in relative content. As iron concentrations increased up to 250 mg L^{-1}. Extreme reduction in root growth occurred under iron treatments and the ratio of shoot:root was increased.

The iron content in roots and shoots of plants increased significantly by increment of iron concentration in the root medium (Fig. 2). Under high iron treatments, supplemental potassium nutrition could not decrease iron content of plants and even at iron concentration of 10 and 50 mg L^{-1} in root medium it led to the increased root iron content. Except in 250 and 500 mg L^{-1} iron treatment in roots, potassium nutrition resulted in the higher potassium concentration in both roots and shoots. In both organs significant decrease in potassium content occurred as iron nutritional levels increased (Fig. 2).

Biochemical factors: As a normal by product of plant metabolism, hydrogen peroxide is generated in cells, however, its accumulation indicates stress condition. Hydrogen peroxide concentration in shoot increased significantly at 250 mg L^{-1} iron treatment as compared with 10 mg L^{-1} iron. Potassium nutrition had no significant effect on hydrogen peroxide concentration of shoots (Table 2).

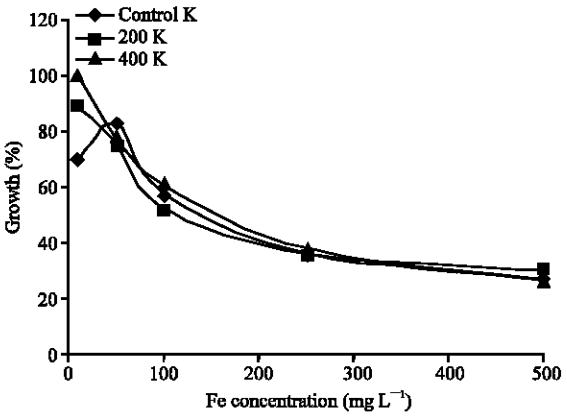

Fig. 1: Effects of iron concentration on relative growth of rice plants grown in sand culture as affected by different potassium concentrations

Table 1: Effects of iron concentration on dry weights of shoot and root, relative water content and shoot: root ratio in rice plants grown in sand culture as affected by different potassium concentrations

		Fe levels (mg L^{-1})					
	K levels (mg L^{-1})	10	50	100	250	500	Mean
DW shoot (g)	Control	1.35±0.09	1.67±0.09	1.27±0.16	0.95±0.05	0.77±0.03	**1.20b**
	200	1.46±0.12	1.57±0.10	1.05±0.12	0.95±0.07	0.86±0.02	**1.18b**
	400	1.81±0.09	1.80±0.11	1.40±0.05	1.04±0.10	0.74±0.06	**1.21a**
	Mean	**1.54a**	**1.68a**	**1.24b**	**0.98c**	**0.79d**	
DW root (g)	Control	0.37±0.02	0.67±0.16	0.28±0.04	0.12±0.02	0.17±0.02	**0.32a**
	200	0.98±0.12	0.51±0.11	0.31±0.04	0.16±0.01	0.13±0.02	**0.42a**
	400	1.26±0.36	0.48±0.04	0.33±0.02	0.10±0.01	0.18±0.02	**0.47a**
	Mean	**0.87a**	**0.55a**	**0.31b**	**0.13c**	**0.16d**	
Relative water content	Control	85.98±0.16	82.84±0.37	83.49±1.41	81.70±0.39	78.29±0.69	**82.46a**
	200	83.10±1.02	82.92±0.49	81.65±0.43	81.02±0.64	79.38±1.18	**81.61ab**
	400	81.04±2.26	82.01±0.46	82.27±0.27	81.39±0.56	78.26±0.75	**81b**
	Mean	**83.4a**	**82.59ab**	**82.47ab**	**81.37b**	**78.64c**	
Shoot:Root	Control	3.17±0.84	3.72±0.30	4.89±0.52	4.57±0.31	3.61±0.30	**3.99a**
	200	2.67±0.17	3.46±0.23	3.93±0.16	4.77±0.26	4.32±0.19	**3.82a**
	400	2.39±0.08	3.97±0.29	4.19±0.21	5.59±0.36	3.16±0.19	**3.86a**
	Mean	**2.74d**	**3.72c**	**4.33b**	**4.97a**	**3.7c**	

Note, Mean±SE of 4 replicates. Means followed by different letters are significantly different by LSD test at 0.05 probability level

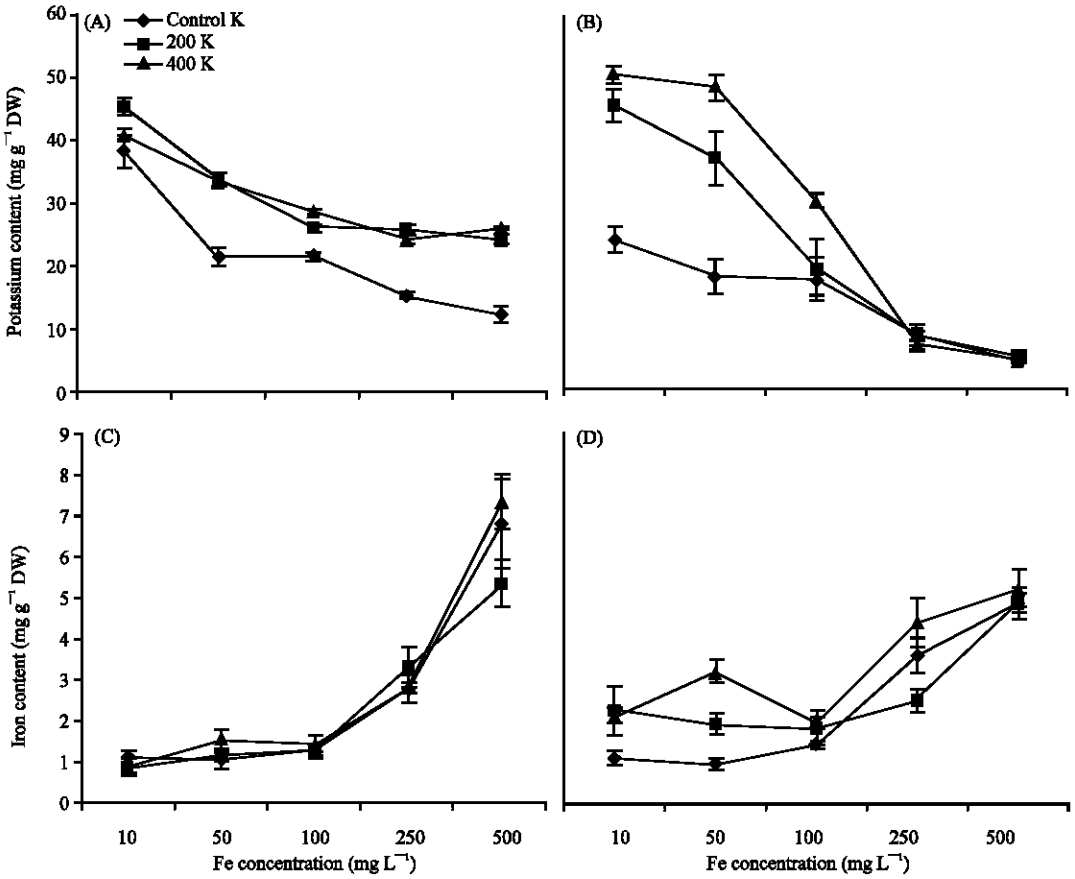

Fig. 2: Effects of iron concentration on potassium content in shoot (A) and root (B) and iron content in shoot (C) and root (D) of rice plants grown in sand culture as affected by different potassium concentrations. Vertical bars represent standard errors

The striking increase in malondialdehyde concentration, as an indicator of lipid peroxidation, occurred as iron concentration was increased in the root medium (Table 2). The increase in malondialdehyde concentration was more drastic in roots than shoots. In 250 mg L^{-1} iron treatment, Malondialdehyde

Table 2: Effects of iron concentration on hydrogen peroxide, malondialdehyde and phenolica content in rice plants grown in sand culture as affected by different potassium concentrations

	K levels (mg L^{-1})	Fe levels (mg L^{-1})		
		10	250	Mean
Hydrogen peroxide in shoot (nmol g^{-1} FW)	Control	35.65±2.80	53.74±4.72	**44.69a**
	200	40.57±1.98	59.61±4.70	**50.09a**
	Mean	**38.11a**	**56.67b**	
Malondialdehyde in shoot (nmol g^{-1} FW)	Control	103.81±2.48	158.53±0.68	**131.17a**
	200	117.08±2.52	185.22±3.60	**151.15a**
	Mean	110.44a	171.87b	
Malondialdehyde in root (nmol g^{-1} FW)	Control	7.99±1.72	42.48±1.86	**25.23a**
	200	10.00±2.22	35.45±3.66	**22.72a**
	Mean	**8.99a**	**38.96b**	
Phenolica in shoot (mg g^{-1} FW)	Control	16.53±0.49	25.27±0.68	**20.9a**
	200	19.71±0.927	26.6±0.64	**23.15a**
	Mean	**18.12a**	**25.93b**	

Note, Mean±SE of 4 replicates. Means followed by different letters are significantly different by LSD test at 0.05 probability level

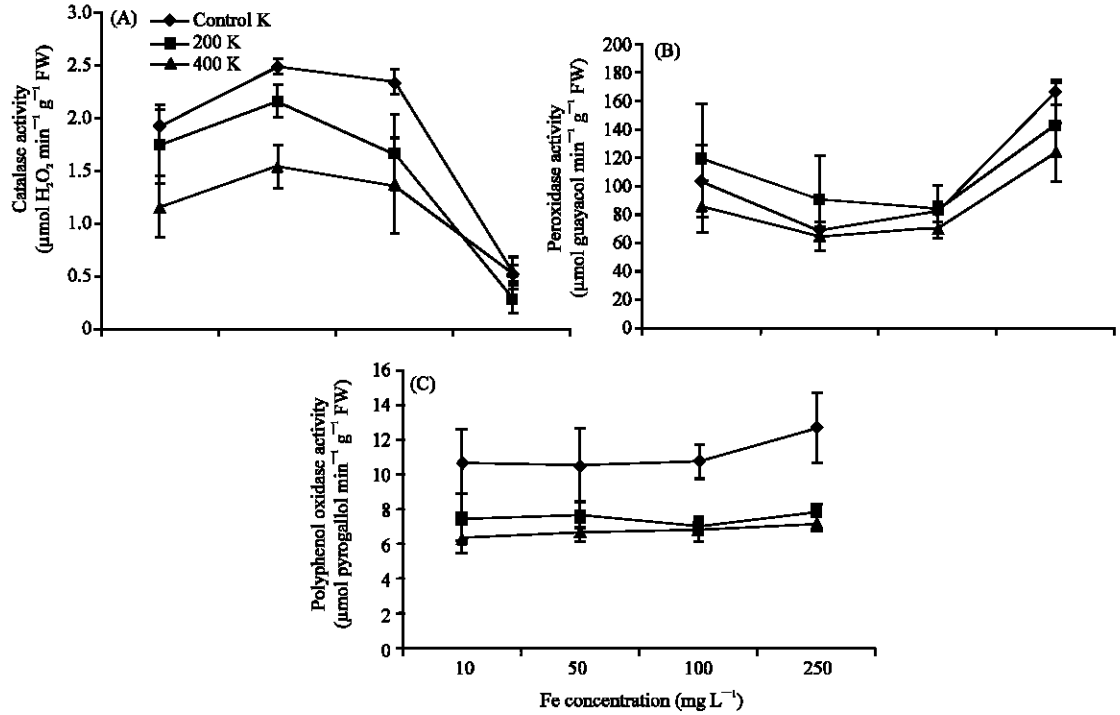

Fig. 3: Effects of iron concentration on the activity of catalase (A), perosidase (B) and polyphenol oxidase (C) in leaves of rice plants grown in sand culture as affected by different potassium concentrations. Vertical bars represent standard errors

concentration increased about 1.5 folds in shoot but more than four folds in roots in compared with 10 mg L^{-1} iron that indicated higher lipid peroxidation in roots. Potassium nutrition had no significant effect on malondialdehyde concentration in plants.

Catalase and peroxidase reduce toxic effects of hydrogen peroxide and in this way control the oxidative stress severity. Catalase activity increased at 50 mg L^{-1} iron treatments as compared to 10 mg L^{-1} iron, however, at 250 mg L^{-1} iron drastic decrease in catalase activity was observed (Fig. 3). At all iron treatments, except 250 mg L^{-1} iron, supplemental potassium nutrition induced marked decrease in catalase activity. Irrespective of potassium concentration in the root medium, maximum peroxidase activity occurred in plants treated with 250 mg L^{-1} iron (Fig. 3). Supplemental potassium did not show significant changes in peroxidase activity.

One of the most important groups of secondary metabolites are phenolic compounds that act as antioxidant. Phenolica content in shoot rose significantly by high iron treatment but did not change by potassium nutrition (Table 2). Polyphenol oxidases are implied in

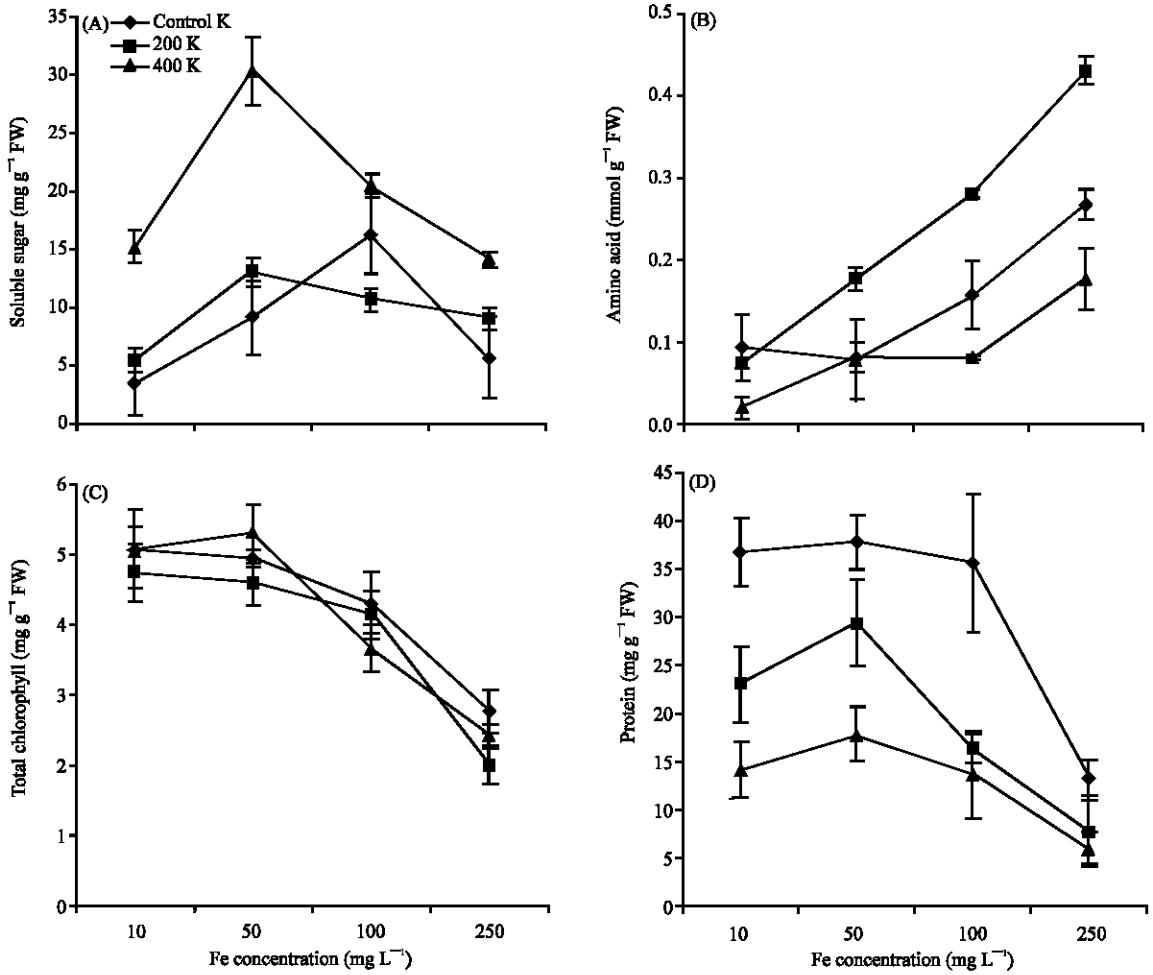

Fig. 4: Effects of iron concentration on total soluble sugar (A), amino acid (B), total chlorophyll content (C) and protein (D) content of leaves of rice plants grown in sand culture as affected by different potassium concentrations. Vertical bars represent standard errors

response of plants to oxidative stress (Mayer, 2006). Polyphenol oxidase activity was decreased significantly by supplemental potassium but iron treatments did not affect any significant changes (Fig. 3).

Leaf soluble proteins were decreased drastically by supplemental potassium nutrition. Also, iron nutrition of 250 mg L^{-1} induced marked decrease in leaf soluble proteins (Fig. 4). Leaf amino acid content increased significantly by increment of iron in the root medium. The maximum increase in the leaf amino acid content occurred at 200 mg L^{-1} potassium treatment, however at higher potassium levels i.e., 400 mg L^{-1}, amino acid content remained at the lowest levels (Fig. 4). Iron nutrition up to 50 mg L^{-1} increased the soluble sugar concentration but at the highest iron treatment marked reduction in soluble sugar concentration occurred. Significant increase in soluble sugar concentration at all iron treatments occurred when plants fed with concentration of 400 mg L^{-1} potassium (Fig. 4). Leaf chlorophyll content remained unchanged when potassium concentration of the medium was increased. However, increasing iron concentration in the root medium imposed significant decrease in chlorophyll a, b and total chlorophyll (Fig. 4). Due to drastic growth reduction and extremely low plant material in iron concentration of 500 mg L^{-1} biochemical parameters were not determined in plants grown under this condition.

The main objective of the present study was the evaluation of iron toxicity levels in rice and investigating the possible role of potassium nutrition on the alleviation of iron toxicity. The optimum iron concentration that lead to the highest plant growth was found to be 10 and 50 mg L^{-1}. Increment of iron in roots medium up to 250 and 500 mg L^{-1} increased iron content in rice plants

and induced iron toxicity accompanied with drastic reduction in plant growth especially in roots (Fig. 1). Growth reduction under iron toxicity has been reported in rice plants by several investigators (Sahrawat, 2004; Dorlodot *et al*., 2005; Becker and Asch, 2005). The shoot:root ratio increased by iron treatment up to 250 mg L^{-1} that indicates iron toxicity mostly affects root growth. The drastic decrease in relative water content of plants as reported by Dorlodot *et al*. (2005) may be related to the extreme reduction of root volume and lose of root hairs. In an attempt to find out the probable mechanisms of iron toxicity in rice plants and its interaction with potassium nutrition some biochemical parameters concerned with oxidative stress was investigated.

Exposure of plants to excess of Fe ions shift the balance of free radical metabolism toward production of reactive oxygen species such as hydrogen peroxide (Table 2) due to decreased activity of ROS scavenging enzymes like catalase (Fig. 3). Similarly, loss of ROS scavenging capacity and H_2O_2 accumulation has been reported under cadmium stress (Schutzendubel and Polle, 2002). In parallel to our observation, depletion of catalase activity under environmental stresses such as salinity, heat shock or cold have been reported by several investigators (Feierabend *et al*., 1992; Hertwig *et al*., 1992). Peroxidase activity increased under iron toxicity (Fig. 3) but it seems not efficient in confronting oxidative stress caused by high iron concentrations, since toxicity symptoms appeared in the plants. Similar observation has been reported by others (Fang and Kao, 2000; Sinha and Saxena, 2006).

High iron concentration led to drastic increase in lipid peroxidation especially in roots (Table 2) accompanied with growth retardation. Similarly, lipid peroxidation increased by excess copper in rice (Chen *et al*., 2000), high levels of mercury in tomato (Cho and Park, 2000) and NaCl salinity in rice (Vaidyanathan *et al*., 2003). The reduction in leaf soluble proteins along with amino acid accumulation and the depletion of chlorophyll and soluble sugars in plants grown at high iron concentration (250 mg L^{-1}) can be attributed to oxidative stress generated by iron toxicity (Fig. 4). Several investigators have reported oxidative attack on proteins, fragmentation of the peptides and increased susceptibility of proteins to proteolysis by ROS (Blokhina *et al*., 2003; Bhattacharjee, 2005). Chlorophyll depletion under oxidative stress has also been reported by other investigators (Vichnevetskaia and Roy, 1999; Kuo and Kao, 2004; Gaewska and Sklodowska, 2006).

Phenolica content increased in plants. This probably is due to H_2O_2 production (Table 2) which is suppose to change plant secondary metabolism (Schutzendubel and Polle, 2002). It could reduce oxidative stress due to their high tendency to chelate metals such as Fe^{2+}, decreasing membrane fluidity and restricting peroxidative reaction and directly scavenge molecular species of active oxygen (Michalak, 2006). There have been many reports of induced accumulation of phenolic compounds and peroxidase activity as observed in the present study (Table 2, Fig. 3) in plants treated with high concentrations of metals. The induction of phenolic compound biosynthesis was observed in wheat in response to nickel toxicity (Michalak, 2006) and in maize in response to aluminium (Winkel-Shirley, 2002).

Although supplemental potassium resulted in marked growth increase under non-toxic iron concentration (10 mg L^{-1}), it did not affect plant growth under toxic iron treatment (Fig. 1). Li *et al*. (2001) has reported improvement of root growth of rice by potassium nutrition with toxic iron concentration equal to 50.4 mg L^{-1} in the root medium. Ramirez *et al*. (2002) has reported improvement of rice yield under iron toxicity by application of N, P, K, S and Zn fertilizers in soil. Also, the reduction of severity of bronzing under excess iron and increase of the dry matter production by application of potassium sulfate has been reported in rice (Yamauchi, 1989). In the present study, it was found that supplemental potassium could not prevent iron accumulation in plant tissues under iron toxicity (Fig. 2). This is probably why supplemental potassium was not efficient to affect significantly H_2O_2 and phenolica concentration, lipid peroxidation and catalase and peroxidase activity in rice plants (Table 2, Fig. 3). Sahrawat (2004) has reported exclusion of iron and its translocation from roots to shoots by potassium fertilizer application under iron toxicity. Such an effect was not observed in our experiments. Apparently, iron toxicity might be intensified in rice fields by potassium deficiency and accordingly use of K fertilizers increases K^+ concentration in plants and ameliorate iron toxicity. On the other hands, it maybe said that the concentration at which iron toxicity symptoms is appeared in plants, depends on available potassium levels.

The results of the present study indicate that iron toxicity induced drastic decrease in plant growth accompanied with changes in some parameters relevant to oxidative stress for example ROS generation, lipid peroxidation and ROS scavenging activity that in turn imposed harmful effects. Potassium nutrition was found to be ineffective in the amelioration of iron toxicity symptoms in rice plants.

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