RESEARCH ARTICLE

Nitric Oxide Alleviates Iron Toxicity by Reducing Oxidative Damage and Growth Inhibition in Wheat (*Triticum aestivum* L.) Seedlings

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Abstract

Nitric oxide (NO) is an important bioactive signaling molecule in plants which modulates a variety of physiological processes and responses to abiotic and biotic stresses. In this study, the effects of exogenous NO supplied as sodium nitroprusside (SNP) in wheat seedlings under iron-induced oxidative damage was investigated. An appropriate concentration of NO was determined by conducting a preliminary experiment. In solution culture, wheat seeds were grown in the control (100 µM Fe), and toxic Fe (400 µM Fe) levels and the toxic Fe supply was treated with various levels of (50, 100, 200 and 500 µM) sodium nitroprusside (SNP). The results indicated that 400 µM Fe significantly decreased percentage germination, tolerance index, root lengths as well as fresh and dry weight compared to control. Exogenous SNP attenuated the inhibition of wheat seed germination. The promoting effect was most pronounced at 100 µM SNP. The accumulated concentration of iron and active Fe was significantly decreased by SNP treated Fe toxic seedlings. Toxicity of Fe caused oxidative stress by elevating hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and proline contents in roots of wheat seedlings. One hundred µM SNP counteracted Fe toxicity by reducing the H_2O_2, MDA and proline contents of toxic Fe exposed seedlings. Meanwhile, application of SNP markedly reduced the activities of superoxide dismutases (SOD), catalases (CAT), peroxidase (POD), ascorbate peroxidases (APX), non protein thiols (NPT) and of glutathione reductase (GR) and increased ascorbate (ASc) compared with Fe toxic treatment alone, thereby indicating the modulation of the antioxidative capacity in the root under Fe stress by NO. The results indicated that the exogenous application of SNP, improved the antioxidant enzymes activity of wheat seedlings against Fe induced oxidative stress.

Keywords: Antioxidative enzymes, Fe stress, Oxidative damage, Sodium nitroprusside, Wheat seedlings.

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Introduction

Iron is an essential trace element with a critical function in all living organisms. It takes part in numerous vital functions such as uptake mechanisms, photosynthesis, respiration, nitrogen fixation and DNA biosynthesis (Hansch and Mendel, 2009; Broadley *et al*., 2012). The toxicity of Fe in plants is related to high Fe^{2+} uptake by roots and its transportation to leaves via transpiration stream. The excess of Fe^{2+} is harmful to living cells, which causes free radical generation that impairs cellular structure irreversibly and damages membranes, DNA, proteins and ultimately kill the cell (Dorlodot *et al*., 2005). On the other hand, it is a constituent of several antioxidative enzymes. Despite the dual role of Fe in induction as well as alleviation of oxidative stress, the balance of Fe should be strictly controlled (Briat *et al*., 2010).

To scavenge most of the generated ROS by the excess Fe plants may involve well organized antioxidative defense system. The first enzyme that plays a defensive role against ROS- induced damages is SOD, which requires Fe Mn, Cu and Zn as metallic cofactors and catalyzes the dismutation of $O_2^{\cdot-}$ to O_2 and H_2O_2 (Sinha and Saxena, 2006). In addition to SOD, CAT and POD also participate in this protective mechanism, these enzymes also contain iron as a cofactor (Verma and Pandey, 2017).

Nitric oxide (NO) is a water and lipid soluble gaseous free radical compound with high diffusion coefficient, having important physiological role in higher plants (Lamattina *et al*., 2003; Corpas *et al*., 2007a,b; Besson-Bard *et al*., 2008). It shows adaptive responses to various stresses (Graziano and Lamattina, 2007). Nitric oxide is a signal molecule in plants involved in plant growth and development (Neil *et al*., 2003; Pagnussat *et al*. 2004), seed germination (Beligni and Lamattina, 2001; Kopyra and Gwózdz, 2003; Zanardo *et al*., 2005), and breaking of seed dormancy (Bethke *et al*., 2006). Nitric oxide is a highly reactive molecule and being a free radical it allows to scavenge other reactive intermediates and end the chain-propagated reactions (Kopyra and Gwózdz, 2003). First, NO functions as an antioxidant by directly scavenging ROS, such as $O_2^{\cdot-}$, to form peroxynitrite ($ONOO^-$) (Laspina *et al*., 2005). Secondly, NO acts as a signaling molecule in the cascade of events leading to changes in gene expression (Lamattina *et al*., 2003; Laspina *et al*., 2005). The rapid reaction between $O_2^{\cdot-}$ and NO to form the powerful oxidant peroxynitrite ($ONOO^-$) is a harmful mechanism (Leshem, 2000) because $ONOO^-$ oxidizes DNA, lipids, protein thiols and Fe clusters, which result in impaired enzyme activities and cellular damage (Beligni and Lamattina, 1999; Van Breusegem *et al*., 2001). NO can easily form complexes with transition metal Fe in aqueous solutions or those present in diverse nucleophilic compounds such as metalloproteins. The FeII NO complex undergo charge transfer reaction to form $Fe^{II}NO^+$. All of these suggested the

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protective role of NO on plants which might be able to reduce the levels of reactive oxygen in plant tissues and therefore its role in the alleviation of Fe toxicity was studied.

Materials and methods

Sterile seeds were shown in Petri dishes containing three layers of Whatman filter paper. The composition of nutrient solution excluding iron used was a dilution of Hoagland nutrient solution which consisted of 4.0 mM KNO_3, 4.0 mM Ca $(NO_3)_2$, 2.0 mM $MgSO_4$, 10 µM $MnSO_4$, 1.0 $CuSO_4$, 1.0 µM $ZnSO_4$, 100 µM NaCl, 0.33mM H_3BO_3, 0.1 µM Na_2MoO_4. Iron was supplied in two levels 100 µM (control), 400 µM Fe in the form of Fe EDTA alone and 400 µM Fe in combination with four concentrations of sodium nitroprusside (50, 100, 200 and 500 µM SNP) in 4 replications per treatment. The Petri dishes were kept in seed germinator under controlled conditions of light (12-hour photoperiod), humidity (88%) and temperature (22°C) and nutrient solutions were changed every alternate day to maintain the desired level of nutrients. At the end of the experiment, several parameters were measured.

Toxicity of iron in seedlings was evaluated by the decrease in percentage germination, tolerance index, seedling length, and biomass production and increased % phytotoxicity and difference from control. To investigate the ameliorative effect of exogenous NO on oxidative damage induced by toxicity of iron, concentration of lipid peroxidation, hydrogen peroxide H_2O_2, and non-protein thiols and activity of antioxidative enzymes- superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) ascorbate peroxidase (APX) and glutathione reductase (GR) were determined in roots of wheat seedlings. All the biochemical parameters were carried out in the roots of wheat seedling which were harvested after supplying variable iron and SNP for 5 and 10 days by methods described earlier by Verma and Pandey (2017).

The data has been analyzed by one-way analysis of variance (ANOVA) and presented in tables and figures. The present results were the mean of three replicates of four Petri dishes each as a replicate.

Results and discussion

Germination Percent and Seedling Growth

The percentage germination of wheat seeds was maximum in nutrient solution containing 100 µM iron supply. The 400 µM Fe alone and in combination with 500 µM SNP in the medium significantly reduced the germination as compared to control. Similar results were also reported by (El Rasafi *et al*., 2016; Verma and Pandey, 2017) in wheat and green gram seedlings which were affected appreciably in the presence of a high concentration of iron. Nagajyoti *et al*. (2010) found that iron toxicity may be due to the high uptake of Fe^{2+} by roots and its transportation to shoots. These ions injure membranes, DNA and proteins owing to free radicals production (Crichton *et al*., 2002; Dorlodot *et al*., 2005). But at 400 µM Fe+100 µM SNP there was a significant increment in germination in wheat seeds (Table 1), probably due to the role of NO in plants involved in seed germination (Beligni and Lamattina, 2000; Kopyra and Gwózdz, 2003; Zanardo *et al*., 2005). Maximum growth was observed in seedlings raised in Hoagland nutrient solution with 100 µM iron. Length of the root was significantly decreased under toxic iron (400 µM) supply. The excessive decrease in root growth is a result of sensitivity of roots to Fe toxicity, due to direct contact with the growth medium. This is in accordance with El Rasafi *et al*. (2016), who observed a decrease in seedling growth of wheat due to toxic Fe. As compared to seedlings grown in 400 µM Fe supply, root length increased with the addition of SNP from 50 to 100 µM but decreased at a concentration of 200 to 500 µM SNP. The concentration of 100 µM SNP was found to be most beneficial for the alleviation of toxic Fe (Table 1).

Phytotoxicity % and TI

The values of % phytotoxicity in wheat roots supplied 400 µM Fe was 72%. Earlier we observed that the increase of iron levels increased significantly the percent phytotoxicity and percentage difference from control (% DFC) for germination (Verma and Pandey, 2017). Effect of phytotoxicity in roots caused due to iron toxicity was found to be reduced with addition of NO especially at a concentration of 100 µM SNP, but % phytotoxicity increased at 400 µM Fe+500 µM SNP supply compared to that at other concentrations of SNP (Table 1). The tolerance index of the wheat root was significantly reduced in presence of 400 µM Fe, which is in accordance with the finding of El Rasafi *et al*. (2016), where wheat seedlings were affected appreciably in the presence of a high concentration of iron. As concentration of NO increased in nutrient solution containing 400 µM iron, the TI increased but was lowest at a higher dose of SNP (500 µM) supply. Thus 100 µM SNP was found to be optimal for alleviating phytotoxicity due to iron.

The toxic concentration of iron caused growth inhibition and significant decline in fresh and dry matter yield in wheat seedlings at 5 and 10 d. The decrease in dry matter yield in the toxic concentration of iron has been also reported in the pea (Nenova, 2006) in potato (Chatterjee *et al*., 2006) and wheat (Li *et al*., 2012). The maximum decrease in yield was observed in plants supplied with 400 µM Fe at both stages. In the present work we provide evidence for the involvement of NO in iron tolerance in wheat, as the application of 50, 100 and 200 µM NO along with 400 µM Fe resulted in an increase in dry weight of roots. The maximum increase was observed in 400 µM Fe+100 µM SNP, which showed that 100 µM SNP gave better alleviation (Table 2). The inhibited root growth and reduced dry matter in iron deficient and toxic plants were found to be ameliorated by NO donor SNP which significantly reversed the growth retardation and reduction in dry matter yield.

Table 1: Effect of iron and sodium nitroprusside (SNP) on germination percentage, length, phytotoxicity, and tolerance index (TI) in leaves and root of wheat (*Triticum aestivum* L. var. DBW-17) seedlings

	Supply (µM)					
Parameter	*100 Fe*	*400 Fe*	*400 Fe+ 50 SNP*	*400 Fe+ 100 SNP*	*400 Fe+ 200 SNP*	*400 Fe+ 500 SNP*
Percent germination	98.46	56.09	78.00	88.18	82.20	64.07
Percent DFC	–	43.03	28.78	10.44	16.51	34.92
Root length (cm)	1.29	0.36	0.67	0.93	0.75	0.48
Phytotoxicity in root (%)	–	72.09	48.06	27.90	41.86	62.79
TI in root	–	22.67	48.42	56.17	52.02	24.50

Table 2: Effect of iron and sodium nitroprusside (SNP) on the total active Fe content, fresh matter yield, dry matter yield, and tissue iron concentration in roots of wheat (*Triticum aestivum* L. var. DBW-17) seedlings grown in solution culture.

	Supply (µM)					
Days of growth	*100 Fe*	*400 Fe*	*400 Fe + 50 SNP*	*400 Fe+ 100 SNP*	*400 Fe+ 200 SNP*	*400 Fe+ 500 SNP*
			Total active Fe (µg g^{-1} fresh weight)			
5	45.21[a]	93.21[e]	69.92[c]	58.33[b]	70.68[c]	86.41[d]
10	51.00[a]	133.5[e]	85.28[c]	68.37[b]	87.43[c]	98.13[d]
			Fresh weight (mg $plant^{-1}$)			
5	56.70[a]	35.41[b]	38.80[c]	54.23[a]	40.23[c]	37.23[c]
10	62.32[a]	44.08[b]	52.16[c]	60.25[a]	53.17[c]	45.03[b]
			Dry weight (mg $plant^{-1}$)			
5	5.201[a]	2.467[b]	3.289[c]	4.980[d]	3.590[c]	2.876[b]
10	6.780[a]	3.197[b]	4.239[c]	5.377[d]	4.653[c]	3.321[b]
			Tissue Fe (µg g^{-1} dry weight)			
5	54.06[a]	273.3[f]	150.5[c]	122.6[b]	213.5[d]	239.8[e]
10	36.62[a]	285.5[f]	162.7[c]	127.7[b]	221.3[d]	244.0[e]

Tissue Concentration of Iron and Total Active Iron

The tissue concentration and active Fe in roots of plants receiving 400 µM Fe showed more than the two-fold increase at 5 and 10 d of supply. Increase in tissue Fe due to toxic Fe is reported (Dorlodot *et al*., 2005; Mehraban *et al.,* 2008; Jucoski *et al*., 2013), but a considerable decrease in tissue Fe was observed in Fe toxic plants supplied SNP being half the value (122.63 µg Fe g^{-1} dry weight) at 400 µM Fe+100 µM SNP as compared to 400 µM Fe (285.5 µg Fe g^{-1} dry weight) (Table 2). Thus it is concluded that the application of adequate (100 µM SNP) could maintain moderate iron levels in plants which are subjected to toxic concentration. On the other hand 200 and 500 µM, SNP did not bring down the Fe levels. Modulation of Fe by NO-deficient plant has been reported in maize (Kumar *et al*., 2010) and peanut (Zhang *et al.,* 2012).

Lipid Peroxidation and H_2O_2

Lipid peroxidation is a significant indicator of oxidative stress due to metal toxicity and is based on the production of malondialdehyde (TBARS) (Azevedo Neto *et al*., 2006). The increased concentration of O_2^- and H_2O_2 lead to lipid peroxidation, causing damage to the cellular membrane (Pandey *et al.,* 2009). The level of lipid peroxidation and H_2O_2 increased in roots of wheat treated with 400 µM Fe at both the stages being significantly pronounced at 10 d. Enhanced lipid peroxidation due to iron toxicity has also been reported for different plant species (Sinha *et al*., 1997; Souza-Santos *et al*., 2001; Sinha and Saxena, 2006; Jucoski *et al.,* 2013) and has been associated with oxidative stress caused by iron toxicity. Earlier we reported an increase in H_2O_2 content by increasing the concentration of iron (Verma and Pandey, 2017). We also observed that accumulation of MDA and H_2O_2 was decreased in iron toxic seedlings supplied in combination of 50, 100, 200 and 500 µM SNP (Fig. 1). This is in accordance with the finding of (Abdel-Kader, 2007) who found a decrease in lipid peroxidation content in the presence of NO. NO can overcome lipid peroxidation possibly by intercepting different lipid radicals forming ROONO adducts, which help in termination of the chain propagation reaction (Wink *et al*., 1995). It might be possible that the interaction of Fe-NO is the most important reactions responsible for NO inhibition of lipid peroxidation after exposure to SNP. This alleviation was found to be more pronounced at 400 µM Fe+100 µM SNP at 5 d of seedlings showing better alleviation over iron toxicity, although MDA and H_2O_2 concentration remained slightly more than control values.

Ascorbate

Ascorbic acid is a major, antioxidant that plays many functions in plants. It directly reacts with H_2O_2, $OH^{\bullet}$, $O_2^{\bullet -}$ radicals, and is significant in the regeneration of α-tocopherol and carotenoids (Potters *et al*., 2002), thus defending the membranes from oxidative damage (Harmens *et al*., 2000; Gill and Tuteja, 2010). Ascorbate concentration was decreased in roots of toxic iron supplied seedlings as well as seedlings supplied with SNP along with iron as compared to seedlings receiving control treatment at 5 d. Ascorbate concentration decreased 53.10% in roots of 400 µM Fe supplied seedlings indicating oxidative damage to root tissue. Compared to control seedlings, seedlings receiving 400 µM Fe+500 µM SNP also showed 52.7% decrease in ascorbate content in roots. The maximum reduction in ascorbate content was found in 400 µM Fe+500 µM SNP as compared to 50, 100 and 200 µM SNP with 400 µM Fe. However, at 10 d, the ASc concentration increased in roots of the excess iron seedling. The maximum increase was observed in seedlings receiving 400 µM Fe and 400 µM Fe+500 µM SNP. At both stages the ASc concentration was near to control values in seedlings supplied 400 µM Fe+100 µM SNP (Fig. 1). Thus 100 µM SNP provided protection from oxidative damage to Fe toxic roots.

Non-protein Thiol

Non-protein cellular thiol, have glutathione (GSH/GSSH) as the main constituent which is an electron donor to DHAR (Rouheir *et al*., 2008). The non-protein thiol contents increased in seedlings exposed to high levels of iron in the roots. Compared to control, seedlings receiving 400 µM Fe alone showed an increase in the concentration of non-protein thiol (264%) in roots compared to control seedlings. Our result is in accordance with (Jucoski *et al*., 2013) who found a marked an increase in GSH content in *Euglena uniflora* under iron toxicity. Seedlings supplied 400 µM Fe with 50,100, 200 and 500 µM SNP showed decreased NPT content in roots at 5 and 10 d, but the values were more than the control (Fig. 1).

Proline

Proline protects plants from free-radical induced damage by quenching of singlet oxygen (Matysik *et al*., 2002). As compared

to control, proline was found to be accumulated in iron toxic seedlings. Accumulation of proline was more in roots (375%) of wheat seedlings receiving 400 µM Fe supply as compared to control seedlings. Seedlings of 50, 100, 200 and 500 µM SNP with iron also showed a decrease in proline content than toxic Fe seedlings, but proline content was more than that of control seedlings. The accumulation of proline concentration was found to be more at 5 d, but after 10 d the concentration of proline was reduced in roots (Fig. 1). Zeng *et al.* (2011) also observed a decrease in proline content by the addition of SNP in NaCl stressed *Brassica juncea* seedlings.

Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) is the first line defense enzyme in a plant system that catalyzes the dismutation of superoxide anion (Gill and Tuteja, 2010) and it is one of the most essential enzymes in defense against the oxidative stress (Foyer and Noctor, 2000; Alscher *et al.*, 2002; Apel and Hirt, 2004). The activity of SOD in roots was increased after treatment with a high iron as well as in seedlings supplied with SNP along with iron as compared to seedlings receiving control treatment at both the stages (Fig. 2). Increase in SOD activity under toxicity of iron has been observed in various

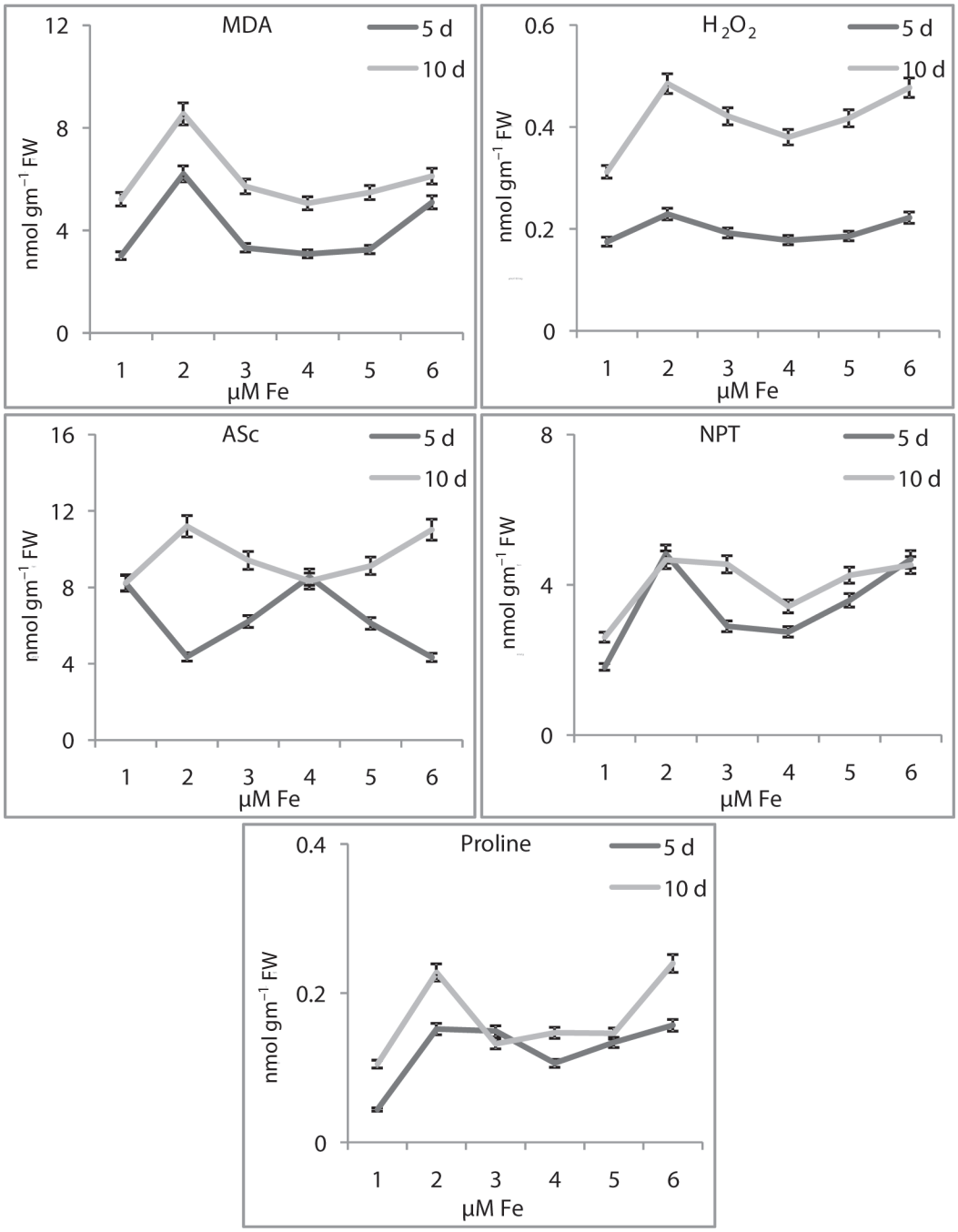

Fig. 1: Effect of iron and sodium nitroprusside (SNP) treatment on concentration of thiobarbituric acid reactive substances (TBARS), hydrogen peroxide (H_2O_2), ascorbate, non-protein thiols and proline in the roots of wheat seedlings grown in solution culture. (Iron and SNP supply: 1–100 µM Fe; 2–400 µM Fe; 3–400 µM Fe + 50 µM SNP; 4–400 µM Fe + 100 µM SNP; 5–400 µM Fe + 200 µM SNP; 6–400 µM Fe + 500 µM SNP). Lines indicate ± SE of four independent values.

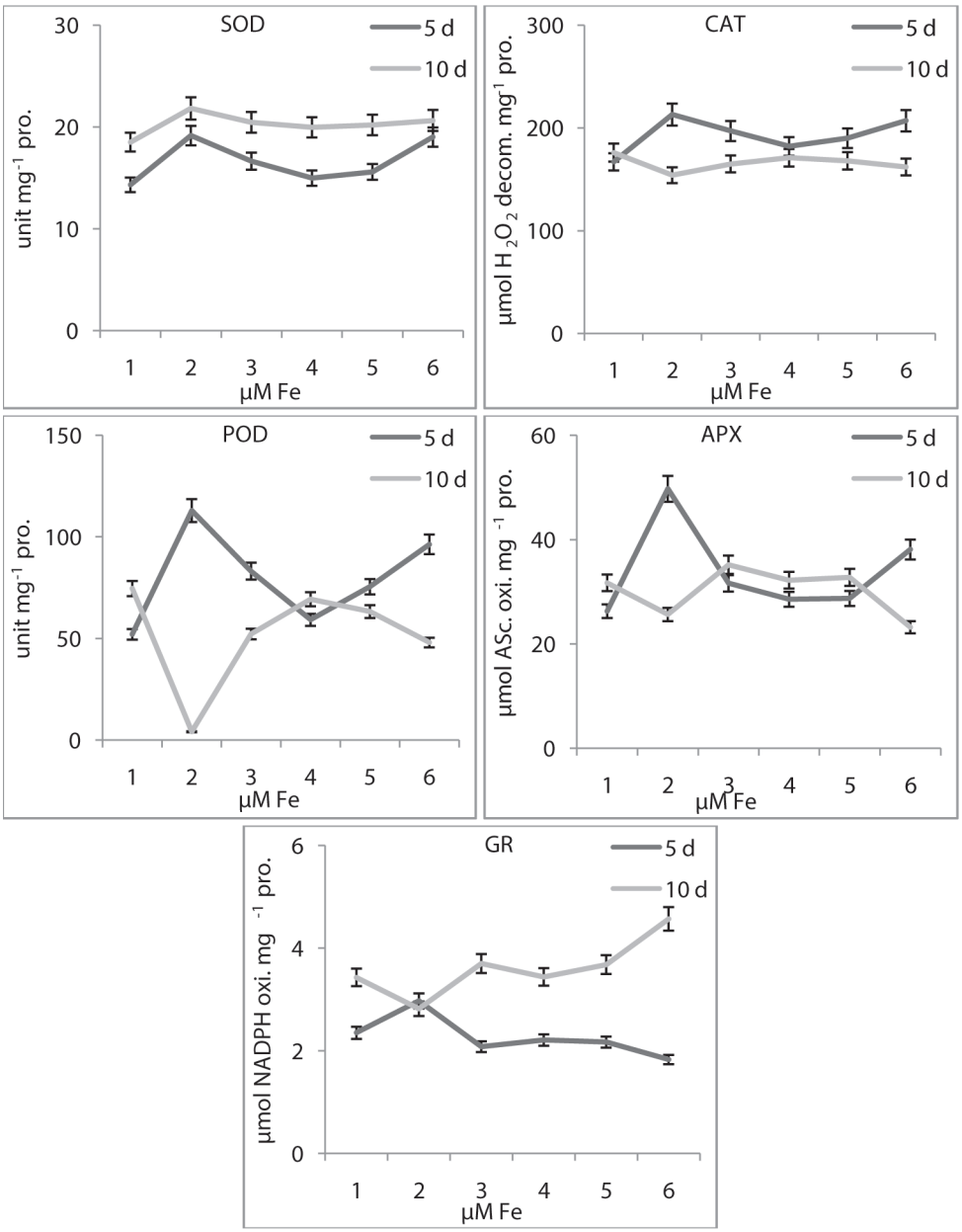

Fig. 2: Effect of iron and sodium nitroprusside (SNP) treatment on activity of SOD (superoxide dismutase), CAT (catalase), POD (peroxidase), APX (ascorbate peroxidase) and GR (glutathione reductase) in the roots of wheat seedlings grown in solution culture. (Iron and SNP supply: 1–100 µM Fe; 2–400 µM Fe; 3–400 µM Fe + 50 µM SNP; 4–400 µM Fe + 100 µM SNP; 5–400 µM Fe + 200 µM SNP; 6–400 µM Fe + 500 µM SNP). Lines indicates ± SE of four independent values.

plant species, such as corn (Kumar *et al*., 2008), rice (Stein *et al*., 2008), *Clusia hilariana* (Pereira *et al*., 2009), *Bacopa monnieri* and *Triticum aestivum* (Li *et al*., 2012) and *Eugenia uniflora* (Jucoski *et al.,* 2013).

Catalase (CAT) and Peroxidase (POD)

The activity of catalase and peroxidase was increased in the levels of the root of wheat seedlings when compared to control. Seedlings receiving 400 µM iron supply increased the activity of CAT and POD as compared to control treatment. The decrease in activity was found in roots of seedlings subjected to SNP (Fig. 2). Same results were also observed by Abdel-Kader (2007) who observed a decrease in catalase activity in all toxic iron seedlings treated with SNP. The increase in activity of peroxidase du to the toxicity of iron is due to the production of intracellular metal binding compounds, alterations in patterns of metal compartmentation, alteration of cellular metabolism as well as the structure of membrane as reported earlier (Van Assche and Clijsters, 1990). Furthermore, Qureshi *et al*. (2007) also found that the increase in catalase activity with increasing concentration of heavy metals in the nutrient solution is due to metabolizing peroxide decomposition in peroxisome after converting glycolate during photorespiration.

Ascorbate Peroxidase (APX)

Ascorbate peroxidase (APX), is an important constituent of the Ascorbate-Glutathione (ASC-GSH) cycle and plays an important role in ROS detoxification in mitochondria, cytosol, and peroxisomes

(Noctor and Foyer, 1998; Mittler *et al*., 2004). APX reduces H_2O_2 to H_2O and DHA, utilizes Ascorbic acid (ASc) as a specific electron donor. In chloroplast, it is the main enzyme that is involved in the regulation of the intracellular level of H_2O_2. The activity of APX was also found to be increased in roots at all Fe levels as compared to that in control treatment. Seedlings receiving 400 µM Fe showed increased APX activity. Supply of SNP brought down the activity of APX to near normal values especially 100 µM SNP (Fig. 2). Abdel-Kader (2007) also observed that high iron concentration of iron with SNP treatment exhibited a decline in APX activity. This suggests that at the initial stage APX could scavenge H_2O_2 efficiently but at later stages when the stress increased, its efficiency to scavenge H_2O_2 decreased, leading to decrease in APX activity and higher accumulation of H_2O_2. NO increase the APX activity and thus maintains both their intercellular antioxidative capacity and reduces oxidative damage (Kopyra *et al*., 2006; Xiong *et al*., 2009; Xu *et al*., 2010; Panda *et al*., 2011).

Glutathione Reductase (GR)

Glutathione reductase (GR) is another important antioxidant enzyme concerned with the reduction of oxidized glutathione in the chloroplast and cytosol, and regeneration of ascorbic acid. It is a key enzyme of ASC/ GSH cycle which is responsible for the reduction of oxidized glutathione for the chain reaction of scavenging H_2O_2 by APX and GPX to be completed and continued (Apel and Hirt, 2004). Glutathione protects thiol groups in enzymes and reacts with singlet oxygen and hydroxyl radical. Increased activity of GR was observed in toxic iron in response to oxidative damage. Supply of SNP bring the values of GR to near control values, especially 100 µM SNP supply. The activity of GR at 10 d in Fe toxic roots was decreased but this is increased by SNP supply. Seedlings supplied 400 µM Fe+100 µM SNP showed the activity was almost near control values (Fig. 2). Jucoski, *et al*. (2013) in *Eugenia uniflora* reported an increased GR activity at high concentration of iron. Thus the increase was observed as a result of up-regulation of enzyme activity in response to oxidative stress.

Conclusion

This present work demonstrated that iron toxicity affected the antioxidant system in roots of wheat. A suitable level of NO was found advantageous for attenuation of inhibition of seedling growth. Low concentration of NO alleviated iron toxicity effect on growth and scavenged the ROS by promoting seed germination, tolerance index and accelerating the growth rate, inducing a better antioxidant system in plants, and alleviating oxidative damage induced by iron toxicity.

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