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REGULATION OF ZINC HOMEOSTASIS BY NITRIC OXIDE, GLUTATHIONE AND SULPHYDRYL GROUPS IN PLANTS

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SUMMARY

The present research was aimed to investigate whether Nitric Oxide (NO) is involved in zinc homeostasis in plants. It also aimed to study the involvement of GSH, SH groups and NO in mediating changes in zinc homeostasis. Wheat seeds *(Triticum aestivum* L., cv. giza-167) and bean seeds *(Phaseolus vulgaris,* cv. *Bronco)* were germinated and grown in vermiculite watered with nutrient solution. Zinc was supplied in different concentrations (0.5, 2.7, 5.4 (control), 10.8 and 21.6 µM as $ZnSO_4$). Sodium nitroprusside (SNP) was used as NO donors in a 100 µM concentration and methylene blue (MB), in a 100 µM concentration, was used as NO scavengers. The solutions were supplied to plants by irrigation once a week included in the nutrient solution. 21-d-old plants were harvested. The results revealed that SNP application (NO donor) alleviated the adverse effect of deficient and toxic levels of zinc concentrations by increasing shoot and root biomass allocation, RGI% and protein content. Moreover, SNP application maintains a suitable zinc concentration in both deficient and toxic levels of zinc supplies in both wheat and bean seedlings. Nitric oxide induced significant changes in total and free/total SH, GSH content and SOD activity. The effect of nitric oxide was supported by using MB which scavenges the endogenous nitric oxide. The effect of NO is suggested to be a result of the adjustment of total or free SH levels, glutathione content and superoxide dismutase (SOD) activity.

Keywords: **GR, GSH, nitric oxide, SOD, sulfhydryls, zinc.**

INTRODUCTION

After iron, zinc is the most abundant intracellular metal. Virtually all intracellular zinc is associated with proteins (primarily via complex interactions with cysteines), where it is known to be an integral component of numerous metalloenzymes, structural proteins and transcription factors. Zn is an essential catalytic component of over 300 enzymes, including alkaline phosphatase, alcohol dehydrogenase, Cu-Zn superoxide dismutase and carbonic anhydrase. Zn also plays a critical structural role in many proteins (Bohm *et al.* 1997). The essential role of Zn in cells is based largely on its behavior as a divalent cation that has a strong tendency to form stable tetrahedral complexes (Berg and Shi 1996).

Zn influx into the root symplasm has been hypothesized to occur as the free Zn^{2+} ion as well as in the form of Zn complexes with nonprotein amino acids known as phytosiderophores (Taga *et al.* 1984) or phytometallophores (Welch 1993). Higher plants contain two major types of cysteine-rich, metal-binding peptides, the metallothioneins (MTs) and the phytochelatins.

While protein bound cysteine might be used for redox-signaling and regulation of protein activity, this mechanism as such is insufficient to maintain the cellular

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redox balance as the abundance of many of the proteins might not be sufficiently high for effective redox control. Thus, in order to keep the internal medium in a reduced state, biological evolution invented a rather complex redox buffering system with the tripeptide glutathione (c-L-glutamyl-L-cysteinyl-glycine) as the most prominent ingredient. Glutathione is a redox active molecule that can be present in its reduced form (GSH) or the oxidized disulfide form (GSSG). The extent of disulfide bridge formation in proteins frequently tracks the oxidation state of the glutathione redox buffer (Schafer and Buettner 2001). The level and redox state of the cellular glutathione pool co-ordinates some of the key re-adjustments underlying the physiological and developmental plasticity in response to biotic and abiotic stress (Noctor *et al.* 2002, Mou *et al.* 2003).

Multiple biological functions have been ascribed to NO as a molecule serving signaling or regulating tasks or acting as a cytotoxic molecule, depending on its mode of enzymatic synthesis, its local concentration and its chemical reactions with other molecules. NO may react with molecular oxygen in a reaction mainly depending on the NO concentration to yield higher reactive nitrogen oxides (NO_K such as N_2O_3, etc.), which display a much broader chemical reaction spectrum than NO itself. Among the amino acids present in proteins, preferentially cysteines are modified by NO_K yielding S-nitrosothiols (Wink *et al.* 1994). Prominent targets within cells are proteins containing Fe-S or Zn-S clusters. Zn-S clusters mainly serve as structural elements of proteins mediating specific DNA or RNA binding as well as protein-protein interactions.

S-nitrosation of zinc sulfur clusters can affect intracellular metal ion homeostasis. The chemical biology underlying this process as well as other redox sensitive aspects of zinc-sulfur complexes have been reviewed (Maret 2004). Kroncke *et al.* (1994) originally showed that NO could S-nitrosate the major intracellular zinc-binding protein, MT and cause the release of zinquin-detectable changes in free zinc (Berendji *et al.* 1997). Chen *et al.* (2002) confirmed these observations and demonstrated that MT was the requisite target for NO resulting in such changes in free zinc. Subsequently, Spahl *et al*. (2003) noted that iNOS-derived NO increased nuclear Zn and that this increase appeared to require the translocation of MT from cytoplasm to nucleus. The metal status of MT was critical for resultant NO-mediated changes in that: (1) NO did not cause release of zinc in a cell in which most of the MT was in its *apo* form (St. Croix *et al.* 2002) and (2) Cu-MT was also nitrosated and depending upon the copper status and the amount of NO exposure, CuMT served as a copper chaperone for *apo*-ZnSOD or a source of Fenton reactive copper. S-nitrosation: (1) requires the presence of molecular oxygen (Aravindakumar *et al.* 1999), (2) is modified by redox status of the environment (Khatai *et al.* 2004) and (3) is more facile for the MT-III than other isoforms of MT (Chen *et al.* 2002). Collectively, it is apparent that S-nitrosation of zinc sulfur clusters is an important component of NO signaling and that metallothionein appears to be a critical link between NO and intracellular zinc homeostasis (Gow and Ischiropoulos 2002).

On the basis of the chemical properties of NO, the present research was aimed to investigate whether NO is involved in zinc homeostasis in two wheat cultivars. It also aimed to study the involvement of GSH, SH groups and NO in mediating changes in zinc homeostasis.

MATERIALS AND METHODS

Wheat seeds (*Triticum aestivum L.* cv. giza-167) and bean seeds (*Phaseolus vulgaris,* cv. *Bronco*) were supplied by National Research Center (NRC), Dokki-Giza, Egypt. Seeds were surface sterilized in 1.8% (v/v) sodium hypochlorite and rinsed several times in distilled water. Seeds were germinated and grown in vermiculite watered with nutrient solution. The nutrient solution had the following composition: 5.25 µM KNO_3, 7.75 µM Ca $(NO_3)_2$, 4.06 µM $MgSO_4$ and 1.0 µM KH_2PO_4; Micronutrients: 46 µM H_3BO_4, 9.18 µM $MnSO_4$, 40 µM Fe-EDTA, 9.0 µM $CuSO_4$ and 2.0 µM Na_2MoO_4.

Zinc ($ZnSO_4$) was supplied in different concentrations (0.5, 2.7 μM considered as zinc deficient concentrations, 5.4 as zinc sufficient concentration (control), Graziano *et al.* (2002), 10.8 and 21.6 µM as zinc toxic concentrations). The nutrient solution was

adjusted to pH 5.5 and renewed once a week. Plants were grown in open air at 14/10 h (25°C/22°C) day/night regime. The experiment was conducted at September 2005 in Botany Department Garden. 21-d-old plants were harvested and prepared for analysis.

Sodium Nitroprusside (SNP) was used as NO donors in a 100 µM concentration (Graziano *et al.,* 2002); 100 µM Methylene Blue (MB) was used as NO scavengers which inhibit NO production and/or action (Cragan 1999). The solutions were supplied to plants by irrigation once a week included in the nutrient solution.

The biomass allocation was estimated by dividing shoot or root fresh weight by total plant fresh weight. RGI % = average dry mass of treated seedling/average dry mass of control seedlings X 100.

Zinc content was determined in oven dried leaves and roots samples mineralized by wet open digestion in $HN0_3$: H_2S0_4: $HC10_4$. Total zinc concentration in the digest was estimated by atomic absorption spectroscopy. Total protein content was estimated spectrophotometrically according to Lowry *et al.* (1951). Nitric oxide concentration was directly measured in both wheat and bean leaves using the method of Ridnour *et al.* (2000). Assay mixture consisted of 100 µl tissue extract mixed with equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthalethylenediamine dihydrochloride). The solution was immediately mixed by inversion incubated at room temperature for 3 min and then A_{496nm} was measured. NO concentration of the solution was calculated using an extinction coefficient of 6600 $m^{-1}cm^{-1}$.

Total sulphydryl groups content in the leaves of wheat and bean seedling were analyzed for reactive sulfhydryl groups by a modification of the methods reported by Bulaj *et al.* (1998) using sulfhydryl-reactive reagent 5,5'-Dithio-bis-2- itrobenzoic acid (DTNB). Reactive sulfhydryl groups in 2.0 ml aliquots of sulfhydryl buffer solution were assayed after collection, without delay, via spectrophotometry for A_{412nm}. Sulfhydryl concentrations were calculated from standard curves prepared from Cys standards made immediately before the assay. Total reduced glutathione content was determined spectrophotometrically following the method described by Griffith (1980). Glutathione reductase (GR) activity was determined as the oxidation of NADPH at 340 nm (extinction coefficient 6.2 $mM^{-1}cm^{-1}$) according to Donahue *et al.* (1997). Leaf samples of both plants (1 g) were homogenized in phosphate buffer (pH 7.6), 2 mM EDTA. The homogenate was centrifuged at 15000 g for 10 min and supernatant was used for analyses. The assay mixture contained 0.1 mM buffer (pH 7.6), 2 mM EDTA, 0.15 mM NADPH, 0.5 mM glutathione oxidised (GSSG) and 500 µl of the extract. The reaction was initiated by addition of NADPH and followed for 5 min at 25°C. Cu/Zn SOD activity was measured by the photochemical method as described by Giannopolitis and Ries (1977). One unit of Cu/Zn SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of p-nitro blue tetrazolium chloride reduction at 560 nm.

Analysis of variance (ANOVA) and student t-test were performed on all data using SPSS program ver. 11.0.

RESULTS

Shoot and Root Biomass Allocation and RGI %: The results in Table 1 showed that zinc toxicity (10.8 and 21.6 µM) significantly decreased both shoot and root biomass allocation and RGI % in wheat and bean seedlings. SNP treatment significantly alleviated the adverse effect of deficient or toxic zinc concentration in both bean and wheat seedlings. On the other hand, application of MB (NO scavenger) increased the effect of zinc deficiency or toxicity by decreasing shoot and root biomass and RGI %. The data of shoot and root biomass allocation and RGI % indicated that zinc concentration (10.8 and 21.6 µM) could be considered toxic to wheat and bean seedlings as they significantly reduced these parameters when compared to control concentration (5.4 µM).

Protein content: The results in Fig. (1a and b) showed that both zinc deficiency and toxicity induced significant decline in protein contents when compared to control (5.4 µM Zn concentration) in both plants under investigation. Application of SNP induced remarkable increase in protein content whereas treatments with MB

Table 1. Shoot and root biomass allocation and RGI %) of wheat and bean cultivars treated with different zinc concentrations alone or with either 100 μM sodium nitroprusside (SNP) as NO donor or 100 μM methylene blue (MB) as NO scavenger. Values are the means of three replicates±SE

Cultivar	Treatments μM Zn^{2+}	Zn Treated groups	+SNP	+MB
		Shoot biomass allocation		
Wheat	0.5	0.32±0.005*	0.52±0.006	0.28±0.012*
	2.7	0.44±0.003	0.54±0.003	0.39±0.017*
	5.4 (Control)	0.48±0.009	0.55±0.015	0.51±0.009
	10.8	0.41±0.014*	0.57±0.005	0.42±0.015*
	21.6	0.38±0.011*	0.53±0.006	0.30±0.015*
	F	114.58	0.887	59.44
Bean	0.5	0.45±0.012*	0.71±0.02	0.37±0.015*
	2.7	0.59±0.018*	0.72±0.006	0.49±0.015
	5.4 (Control)	0.73±0.015	0.67±0.015	0.53±0.015
	10.8	0.68±0.015	0.86±0.012*	0.41±0.012*
	21.6	0.58±0.018*	0.71±0.016	0.37±0.016*
	F	54.65	31.68	92.74
		Root biomass allocation		
Wheat	0.5	0.21±0.015*	0.46±0.021	0.27±0.016*
	2.7	0.36±0.009*	0.49±0.015	0.41±0.012
	5.4 (Control)	0.52±0.015	0.53±0.012	0.43±0.008
	10.8	0.44±0.014*	0.49±0.011	0.47±0.015
	21.6	0.26±0.020*	0.42±0.018*	0.21±0.014*
	F	80.96	7.06	106.9
Bean	0.5	0.27±0.015*	0.29±0.009*	0.23±0.006*
	2.7	0.31±0.009*	0.38±0.016*	0.26±0.015
	5.4 (Control)	0.45±0.012	0.53±0.015	0.31±0.012
	10.8	0.32±0.012*	0.44±0.012*	0.28±0.017
	21.6	0.26±0.013*	0.39±0.016*	0.13±0.012*
	F	35.54	53.39	53.44
		RGI%		
Wheat	0.5	76.7±2.20*	81.2±1.16*	79.4±1.15*
	2.7	80.2±1.04*	96.0±0.57	81.8±1.19*
	5.4 (Control)	100.0±0.57	100.0±0.58	100.0±0.88
	10.8	103.0±0.88	110.0±1.45*	82.6±1.49*
	21.6	86.9±2.02*	135.1±2.60*	57.4±1.45*
	F	63.7	185.8	148.7
Bean	0.5	38.5±1.17*	56.7±1.18*	57.1±1.45*
	2.7	69.2±0.88*	67.1±0.89*	69.2±0.88*
	5.4 (Control)	100.0±0.88	100.0±1.73	100.0±1.15
	10.8	81.7±1.17*	132.7±2.02	59.4±0.88*
	21.6	20.7±2.04*	145.1±3.18*	32.2±1.45*
	F	609.75	391.9	420.6

*Significant differences as compared to control zinc concentration (5.4 μM) using student t-test ($p<0.05$)

Table 2. Zinc concentration expressed as μg g^{-1} dry weight, in the leaf extract of wheat and bean seedlings treated with different zinc concentrations alone or with either 100 μM SNP (NO donor) or 100 μM MB (NO scavenger). Values are the means of three replicates±SE.

Cultivar	Treatments μM Zn^{2+}	Zn Treated groups	+SNP	+MB
		Zinc conc. (μg g^{-1})		
		Shoot		
Wheat	0.5	22.35±1.16*	26.08±0.88*	16.46±0.88*
	2.7	52.07±1.14	46.26±0.89*	34.44±1.15*
	5.4 (Control)	53.70±1.15	144.5±2.9	43.70±0.89
	10.8	72.53±1.73*	55.81±1.15*	62.34±1.73*
	21.6	83.42±2.03*	38.41±1.16*	75.00±0.87*
	F	244.4	885.8	402.6
Bean	0.5	73.30±1.16	60.91±1.45	11.88±0.87*
	2.7	56.77±2.32*	57.71±1.47	37.16±1.76*
	5.4 (Control)	72.61±1.74	53.05±1.10	53.05±1.15
	10.8	121.60±2.02*	53.23±0.58	54.48±1.14
	21.6	153.81±2.03*	49.27±0.88	56.25±0.88*
	F	453.8	16.37	241.27
		Root		
Wheat	0.5	33.50±1.17*	38.00±1.15*	21.12±1.73*
	2.7	73.50±1.15*	111.20±2.02*	58.48±1.45*
	5.4 (Control)	114.10±2.03	255.80±5.49	94.86±1.30
	10.8	119.60±2.30	111.40±3.76*	89.38±1.15
	21.6	134.80±3.18*	96.30±01.76*	99.21±0.88
	F	382.2	608.7	599.7
Bean	0.5	45.83±2.60*	40.43±1.51*	14.85±1.19*
	2.7	55.81±0.89*	50.54±1.45*	28.00±1.20*
	5.4 (Control)	66.00±1.53	81.09±1.16	46.25±2.31
	10.8	90.59±2.03*	105.50±3.18*	83.52±0.88*
	21.6	110.00±2.60*	133.70±1.44*	80.58±0.89*
	F	170.58	400.83	479.38
		Total		
Wheat	0.5	55.85±1.15*	64.08±2.30*	37.58±2.31*
	2.7	125.57±2.61*	157.46±1.73*	92.92±0.88*
	5.4 (Control)	167.80±1.58	400.30±5.49	138.56±1.45
	10.8	101.13±5.20*	167.21±2.33*	151.72±2.30*
	21.6	218.22±3.48*	134.71±2.6*	174.21±1.73*
	F	377.3	1598.0	895.2
Bean	0.5	119.13±3.18*	101.34±1.45*	26.73±2.08*
	2.7	112.58±2.03*	108.25±1.76*	65.16±1.73*
	5.4 (Control)	138.61±1.42	134.14±3.48	99.30±1.45
	10.8	212.19±2.31*	158.73±2.31*	138.00±2.03*
	21.6	263.81±2.02*	182.97±2.60*	136.83±3.76*
	F	158.9	194.28	408.8

*Significant differences as compared to control zinc concentration (5.4 μM) using student t-test ($p<0.05$)

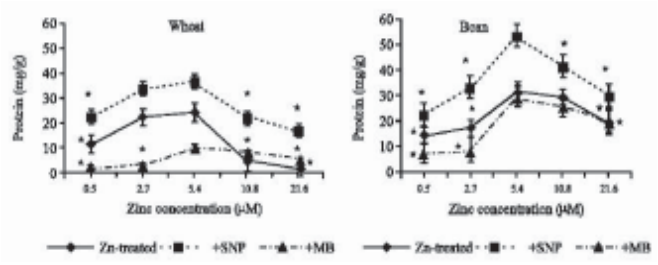

Fig. 1. (a & b). Protein content expressed as μg g^{-1} fresh weight, in the leaf extract of wheat and bean cultivars treated with different zinc concentrations alone or with either 100 μM SNP (NO donor) or 100 μM MB (NO scavenger). Each point corresponds to the mean of two to four independent experiments. Data are the mean of three replica±SE. *significant at p<0.01 when compared to control.

significantly decreased protein contents at all zinc concentrations when compared to Zn-treated groups in both bean and wheat seedlings.

Zinc concentration: Zinc concentration in shoot and root extracts was increased with increasing zinc supply in irrigating nutrient solutions (Table 2). SNP application induced declines in zinc concentration at toxic zinc supply in shoot, root and total plant of wheat seedlings. In bean seedlings, SNP application at toxic zinc supply induced decline in zinc concentration in shoot and increase in zinc concentration in both root and total plant. On the other hand, MB application at zinc toxic supply induced increase in zinc concentration in shoot, root and total plant of both wheat and bean seedlings.

NO Concentration: Application of SNP increased NO concentrations in both plant seedlings while MB induced a significant decline in NO concentrations (Fig. 2a and b).

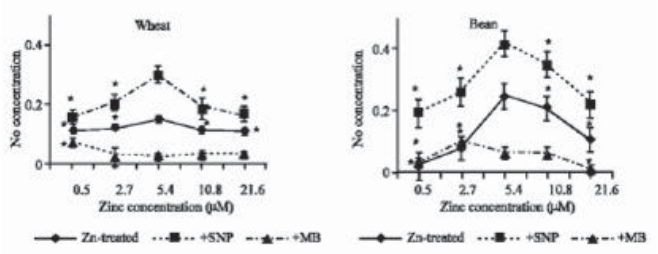

Fig. 2. (a & b). NO concentration expressed as μg g^{-1} fresh weight, in the leaf extract of wheat and bean seedlings treated with different zinc concentrations alone or with either 100 μM SNP (NO donor) or 100 μM MB (NO scavenger).

Total Sulphydryl Groups: The decrease in total SH (Fig. 3a and b) was observed at deficient zinc concentrations with SNP treated wheat and bean

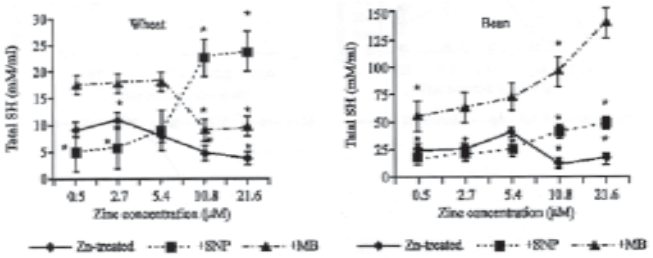

Fig. 3. (a & b). Total SH expressed as mM/g fresh weight in the leaf extract of wheat and bean seedlings treated with different zinc concentrations alone or with either 100 μM SNP (NO donor) or 100 μM MB (NO scavenger). Each point corresponds to the mean of two to four independent experiments. Data are the mean of three replica ±SE. *significant at p<0.01 when compared to control.

seedlings and the same zinc concentration significantly decreased free/total SH ratio (Fig. 5a and b). MB treated wheat seedlings induced a highly significant reduction of total SH with toxic zinc concentration. As zinc concentration increased, free/total SH ratio was increased in wheat seedlings treated with MB. It was also observed that bound SH exhibited the same trend as total SH (Fig. 4a and b). However, free/total SH ratio increased with

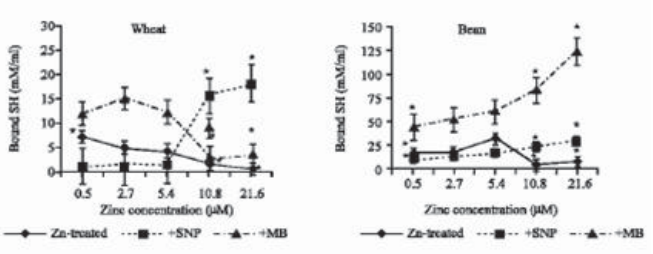

Fig. 4. (a & b). Bound SH expressed as mM/g protein, in the leaf extract of wheat and bean seedlings treated with different zinc concentrations alone or with either 100 μM SNP (NO doner) or 100 μM MB (NO scavenger). Each point corresponds to the mean of two to four independent experiments. Data are the mean of three replica ±SE. *significant at p<0.01 when compared to control.

zinc and MB application in wheat seedlings and declined at toxic zinc concentration in bean seedlings (Fig. 5a and b).

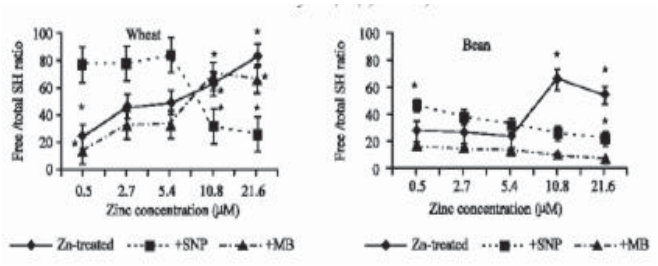

Fig. 5. (a & b). Free/total SH ratio in the leaf extract of wheat and bean seedlings treated with different zinc concentrations alone or with either 100 µM SNP (NO donor) or 100 µM MB (NO scavenger). Each point corresponds to the mean of two to four independent experiments. Data are the mean of three replica ±SE. *significant at p<0.01 when compared to control.

Glutathione Content: In wheat seedlings (Fig. 6a), GSH content reached its lowest level at 0.5 Zn^{2+} supply then increased gradually with increasing Zn^{2+} concentration. SNP application (NO donor) decreased GSH content in Zn^{2+} insufficient concentration when compared to Zn-treated group seedlings while MB application significantly increased GSH content at (0.5, 2.7 and 5.4 µM) Zn^{2+} supplies. In bean seedlings, (Fig. 6b), SNP application increased GSH content at all zinc treatments when compared with Zn-treated group or MB treated seedlings.

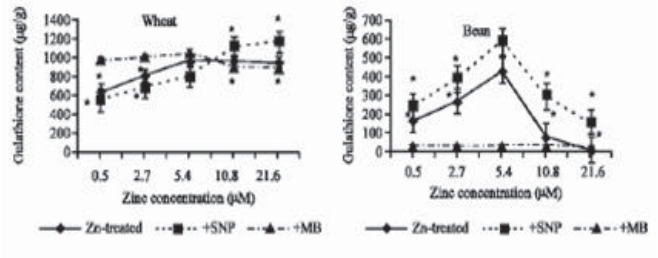

Fig. 6. (a & b). Glutathione content expressed as µgg^{-1} fresh weight, in the leaf extract of wheat and bean seedlings treated with different zinc concentrations alone or with either 100 µM SNP (NO donor) or 100 µM MB (NO scavenger). Each point corresponds to the mean of two to four independent experiments. Data are the mean of three replica ±SE. *significant at p<0.01 when compared to control.

Glutathione Reductase Activity: Different zinc concentrations had a non significant increase on GR activity in wheat seedlings (Fig. 7a) while the increase in GR activity was significantly recorded at toxic zinc concentrations in bean plants (Fig. 7b). On the other hand, SNP application induced a highly significant increase in GR activity at all zinc concentrations in wheat and bean seedlings application of MB (NO scavenger) decreased GR activity with increasing zinc concentration. At toxic concentration of zinc (21.6 µM), GR activity declined with 19.4 and 38.1% in wheat and bean seedlings, respectively when compared to that of control zinc concentration.

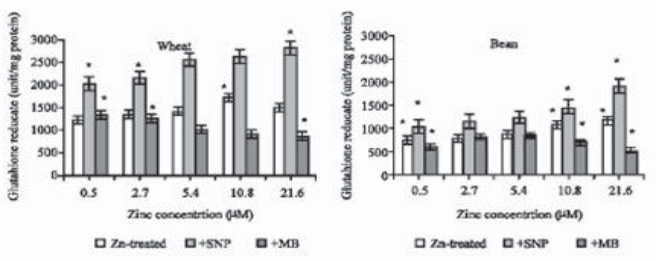

Fig. 7. (a & b). Glutathione reductase activity expressed as unit/mg protein, in the leaf extract of wheat and bean seedlings treated with different zinc concentrations alone or with either 100 µM SNP (NO donor) or 100 µM MB (NO scavenger). Each point corresponds to the mean of two to four independent experiments. Data are the mean of three replica ±SE. *significant at p<0.01 when compared to control.

Cu/Zn SOD Activity: The results in Figure (8 a and b) indicated that Cu/Zn SOD activity significantly decreased at deficient and toxic zinc concentrations in both wheat and bean seedlings when compared to control (5.4 µM Zn) concentration. The decline in endogenous NO concentration (MB application) reduced Cu/Zn SOD activity at all zinc concentration as compared with Zn-

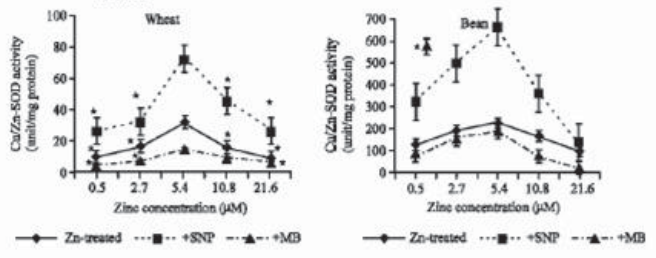

Fig. 8. (a & b). SOD activity expressed as unit/mg protein, in the leaf extract of wheat and bean seedlings treated with different zinc concentrations alone or with either 100 µM SNP (NO donor) or 100 µM MB (NO scavenger). Each point corresponds to the mean of two to four independent experiments. Data are the mean of three replica±SE. *significant at p<0.01 when compared to control

treated seedlings. On the other hand, application of exogenous NO (SNP treatment) enhanced Cu/Zn SOD activity at all zinc concentrations when compared to Zn-treated or MB treated seedlings.

DISCUSSION

Under physiological conditions, the majority of Zn^{+2} within cells is complexed by proteins. To date, MT is the only protein that has been implicated in cellular Zn^{+2} storage and usually a significant percentage of the total cellular zinc is complexed by MT (Kagi and Schaffer 1988).

Morphological results (Table 1) revealed that SNP application (NO donor) alleviated the adverse effect of low or high zinc concentrations by increasing shoot and root biomass allocation and RGI %. NO effect was the same in both wheat and bean seedlings. Growth inhibition is a general phenomenon associated with zinc toxicity (Collins 1981). Zinc-deficiency stress usually involves peroxidative damage of crucial components of biomembranes, enzymes and thylakoids (Bray and Bettger 1990) which are the major targets of toxic O_2 radicals (Elstner 1982). Under aerobic conditions, NO *via* thiolate-nitrosation induces the release of Zn^{2+} from MT *in vitro* (Zangger *et al.* 2001). NO was found to induce an intracellular conformational change of MT, suggesting intracellular Zn^{2+} release from this protein (Pearce *et al.* 2000). In addition, exogenously added NO has been shown to induce S-nitrosation of intracellular MT (Fabisiak *et al.* 1999). Moreover, nitrosative stress induces Zn^{2+} release in various types of cells predominantly within the cytoplasm (St Croix *et al.* 2002).

Application of MB (NO scavenger) proved that NO had a significant effect on increasing protein concentration at all zinc supplies (Fig. la and b). The data in Table (2) showed that SNP application increased total zinc concentration at zinc deficiency in wheat seedlings. This increase was concomitant with a highly increase in free SH and a highly decrease in total SH (Fig. 3a). These changes in free or total SH were also observed when MB was applied. Zangger *et al.* (2001) have found that the exposure of MTs to NO leads to a selective release of all three metals from the N-terminal ct-domain while leaving the four metals in the ct-domain untouched. This finding indicates a possible regulatory role of NO in the specific redistribution of metal from MT. The results in Table (2) revealed that SNP decreased total zinc concentration at toxic zinc concentration in both wheat and bean seedlings. The NO effect was detectable in both seedlings shoots. It was also observed that the MB application (NO scavenger) reverse SNP effect at toxic zinc concentrations. Moreover, decreasing endogenous NO with MB application induced significant decrease of free/total SH ratio and increase in total SH at toxic zinc concentrations.

SNP application (NO donor) increased total SH and decreased free/total SH ratio in wheat (Fig. 3a and 4a) at toxic zinc concentrations and this result was supported by MB application (decreasing endogenous NO). Moreover, the same NO effect was observed at all zinc concentrations in bean seedlings and this effect was reversed by MB application (Fig. 4a and 4b). By binding sulfhydryl groups and phospholipids, Zn^{2+} stabilizes and protects cell membranes and enzyme proteins against oxidative attack of toxic O_2 species (Bray and Bettger 1990). They reported that the plasma membrane was the cell compartment which regulated metal entry into the cell, in addition its proteins, especially the SH groups might be affected in their activity causing damage to membrane stability due to zinc toxicity. Cumming and Taylor (1990) indicated that the mechanism of metal tolerance were due to exclusion and external metal detoxification mechanism, internal metal detoxification mechanisms and multiple mechanisms and co-tolerances. The production of metal-chelating ligands high in thiol groups might also render co-tolerance to Zn^{2+}. Free cysteine with its free amino-, carboxyl- and thiol-groups is an effective chelating agent for metal ions with which it forms five- or six-membered ring structures (Taylor *et al.* 1966). In the presence of H_2O_2 transition metals reduced in this way can undergo a Fenton reaction whereby highly toxic hydroxyl radicals are formed. Thus, homeostatic control of low cysteine levels becomes important for conferring resistance to oxidants. The intracellular concentration of cysteine needs to be maintained in the lower micro-molar range. Due to the resulting diminished ability to reduce transition metal ions, a cell can maintain millimolar concentrations of GSH and thus a concentration 10-50-fold higher than

concentrations of free cysteine without triggering deleterious Fenton reactions. It could be suggested that NO generated by SNP mediated Zn release *via* nitrosylation of cysteine thiol groups could explain the increase in free/total SH ratio and the decrease in total and bound SH at zinc deficient condition. Oxidation of cysteine-SH by H_2O_2 leads to the sulfenic acid (Cys-SOH). While, this first step of oxidation is reversible and can be explored for redox signaling purposes, further oxidation to cysteine sulfinic acid (Cys-SO_2H) and cysteine sulfonic acid (Cys-SO_3H) is irreversible and renders the protein labile to oxidative stress (Poole *et al.* 2004). The sulfhydryl groups of cysteine sulfinic acids can undergo reactions with other sulfhydryl groups to form disulfides or reactions with glutathione to form mixed disulfides. Concomitant changes in protein structure and function can lead to changes in metabolism and form the basis of cellular signaling events. The data in Figure 6a and b showed that SNP application induced a highly significant increase in GSH concentration at all zinc concentration in bean seedlings while the increase was only observed in toxic zinc supply in wheat seedlings. The change in GSH concentration was correlated with GR activity in wheat plants at all zinc supplies and in bean plant at deficient and normal zinc supplies only (Fig. 7a and b). Di Baccio *et al*. (2005) found that total glutathione (GSH+GSSG) content was reduced with increasing Zn concentration, while the contribution of oxidized to total glutathione increased in *Populus* spp. Glutathione has been described as a 'transport peptide' *in vivo* for NO through the formation of S-NO groups (Jourd'heuil *et al.* 2000). The glutathione redox couple, a cellular redox buffer which maintains the given thiol/disulfide redox potential, has already been implicated in modulating the metal release from metallothionein in the absence of nitric oxide by Vallee and Maret (1993) and coworkers (Jiang *et al.* 1998; Jacob *et al.* 1998). These authors reported increased metal release in the presence of oxidized glutathione (GSSG) and even slightly tighter metal binding under the influence of reduced glutathione (GSH). Khatai *et al.* (2004) showed that metal release from MT2 by nitric oxide and peroxyinitrite suppressed by reduced but not oxidized glutathione. In terms of molecular mechanism, GSH and GSSG could control the amount of free zinc available once it is released from MT, or bind to MT and affect its conformation and zinc binding. GSH binding would protect MT from the loss of Zn^{-2}, thereby inhibiting its transfer, while providing a reactive thiol for the reaction with GSSG and resulting in zinc transfer through a process that is strictly proportional to and dependent on the concentration of GSSG. Hence, a system consisting of MT-GSH-GSSG provides a mechanism to control zinc transfer among a large variety of acceptors.

SNP (NO donor) increased SOD activities at all zinc concentrations when compared to control (Fig. 8a and b). This finding is supported with that of Zhou *et al.* (2005) who stated that NO donor treatment enhanced the activities of SOD, CAT and APX. Increasing SOD activity resulted in H_2O_2 production which may oxidize SH to form disulfide bond. The increase in GSH content in bean seedlings means that NO activate ascorbate-glutathione cycle which utilize the produced H_2O_2 and subsequently reduced SH oxidation. Moreover, increasing GSH at toxic zinc concentration suppresses zinc release from MT and this explains the reduced levels of zinc when SNP was applied. Jiang *et al.* (1998) have identified glutathione disulfide (GSSG) as a cellular ligand that reacts with MT and mobilizes zinc, resulting in the suggestion that the zinc content of MT is linked to the redox state of glutathione in the cell in such a manner that zinc remains bound to MT as long as high thiol reducing power prevails and is released once the redox balance becomes more oxidizing (Maret 1994).

It could be concluded that nitric oxide plays a significant role in zinc homeostasis in wheat and bean seedlings subjected to different zinc concentration. The effect of nitric oxide was supported by using (MB) which scavenged the endogenous nitric oxide and reversed the action of NO in maintaining suitable zinc concentration for seedlings growth. At zinc deficient supplies (0.5, 2.7 μM) nitric oxide (as SNP application) decreased free/total SH and this mean that zinc is released from MT-rich-cysteine which chelate zinc. Moreover, GSH content was lower than that of Zn-treated seedlings. This decrease may be due to utilization of glutathione in detoxification of H_2O_2 at ascorbate-glutathione cycle or it may conjugate with free SH to suppress Fenton reaction. On the other hand, at toxic zinc supplies, the effect of NO was reversed (increasing total SH and GSH). The increase in GSH content suppresses metal release from MT and this is supported by the increase

in total SH and decrease in free/total SH. The effect of nitric oxide was supported by the application of MB which reduces the endogenous nitric oxide.

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