Bull. Environ. Contam. Toxicol. (2005) 74:745–754

DOI: 10.1007/s00128-005-0645-8

Bulletin of Environmental Contamination and Toxicology

Possible Involvement of Oxidative Stress in Copper Induced Inhibition of Nitrate Reductase Activity in *Vallisneria spiralis* L.

P. Vajpayee, U. N. Rai, M. B. Ali, R. D. Tripathi, A. Kumar, S. N. Singh

Ecotoxicology and Bioremediation Laboratory, National Botanical Research Institute, Rana Pratap Marg, Lucknow 226 001, India

Received: 21 October 2004/Accepted: 12 January 2005

Copper is an essential micronutrient and is a component of several enzymes participating in electron flow, catalyzing redox reactions (Devi and Prasad 1998). However, accumulation of copper by plants in phytotoxic amounts (25-50 mg kg^{-1} dry foliage) induced phytotoxicity in plants (Chaney 1989). Copper induced toxic effects in plants include retardation of growth, disintegration of membrane integrity, increased potassium efflux, lipid peroxidation, chlorophyll breakdown, denaturing and fragmentation of structural and functional proteins (Fang and Kao 2000; Prasad et al 2001). The stimulation of production of active oxygen species by copper results in inactivation of enzymes containing -SH groups through oxidation and cross linking of thiol groups (Vara and Serrano 1982; Smarrelli and Campbell 1983). Its inhibitory effect on nitrate reductase (NR, E.C. 1.6.6.1-2), the rate limiting enzyme in nitrate assimilation, has also been reported in *Silene cucubalus* (Bladder pod campion), *Sesamum indicum* (Til), *Avena sativa* (Oat) and *Triticum aestivum* (Wheat) by Mathys (1975), Singh et al (1995) and Luna et al (1994, 1997) respectively. Smarrelli and Campbell (1983) suggested that this could be due to the modification of the -SH group in the cytrochome b part of the nitrate reductase molecule by copper. Besides, *in vivo* nitrate reductase was sensitive to oxidative environments e.g. light, hyperoxia and treatment with oxyradical propagating compounds (Kenis and Trippi 1986, 1989; Kenis et al 1992). Further, it has been found that loss in nitrate reductase activity (NRA) was restored by -SH protectors such as cysteine and antioxidants like sodium benzoate (Luna et al 1997). Most of the studies conducted on the impact of oxidative stress on nitrate reductase are on terrestrial plants (Singh et al 1995). No attempt has been made earlier to study the impact of copper stimulated lipid peroxidation on nitrate reductase activity of aquatic plants, in spite of their active role in phytoremediation of toxic concentrations of essential and non-essential metals.

Vallisneria spiralis L (Twisted vallis) of family Hydrocharitaceace is a widely occurring submersed aquatic macrophyte which could grow luxuriantly in metal polluted environment and accumulates toxic metals (Vajpayee et al 2001). The purpose of the present investigation was to study the effect of copper on nitrate reductase activity (*in vivo* and *in vitro*) and other physiological parameters of *V. spiralis*. Experiments were also conducted to examine the possible reasons for inhibition of the nitrate reductase activity in the test plant.

MATERIALS AND METHODS

Vallisneria spiralis L., raised from rhizomes of mother plants growing in hydroponic cultures at National Botanical Research Institute, Lucknow have been used in the

Correspondence to: P. Vajpayee

experiments. Approximately 1.5 g plant material was treated with 200 ml solution of 0, 10, 30, 50, 100 and 200 μM copper ($CuSO_4.7H_2O$) in 5% Hoagland Solution for 24 h under a growth chamber (temperature 25±2°C, 115 μ mol m^{-2} s^{-1} illumination provided through fluorescent tube light for 24 h per day). Experimental cultures were aerated for 6 h per day. In Hoagland solution Sodium EDTA was replaced by sodium sulfate. The harvested plants were washed thrice by deionized water and then used in the study. For metal analysis, dried (1 g) plant samples (leaves, roots and rhizome) were ground in a grinder and digested in HNO_3:$HClO_4$ (3:1, v/v) at 80°C and copper concentration was measured by Atomic Absorption Spectrophotometer (Perkin Elmer 2380). Copper concentrations were observed by measuring the copper in the test solutions as described above. The measured test concentrations varied by no more than 5% from the required test concentrations. The mean recoveries of for 10, 30, 50, 100 and 200 μM copper were 97±4%, 96±%, 98±3%, 97±5% and 98±4.5%, respectively. The standard reference materials of copper (E-Merck, Germany) were used to provide calibration and quality assurance for each analytical batch. Mean recovery of copper was 96±4%. The detection limit for Cu was 0.01 μg ml^{-1}. Replicate (n=3) analysis was conducted to assess the precision of the analytical techniques. Triplicate analysis for the test metal varied by no more than 5%.

In vivo nitrate reductase activity in leaves and roots of treated plants was assayed by the method of Srivastava (1974). NRA is expressed as n mol NO_2^- h^{-1} g^{-1} FW. For extraction of crude nitrate reductase in leaves and roots, 1.0 g plant material was homogenized in a morter and pestle at 4°C with 4 ml 60 mM phosphate buffer (pH 7.5), containing 0.1 mM Dithiothreitol (DTT) and 3% BSA (Bovine serum albumin). The extract was squeezed through double layer of cheese – cloth and centrifuged at 20,000 g for 20 min at 4°C. *In vitro* NRA was assayed in the supernatant by the method of Bar-Akiva and Sagiv (1967). Supernatant obtained as above was used as crude enzyme extract in entire study. Total chlorophyll was extracted in 80% chilled acetone and the content determined spectrophotometrically, following the procedure of Arnon (1949). Protein contents in various plant parts were estimated according to the method of Lowry et al (1951) using BSA as standard. The level of lipid peroxidation in the plant parts (leaves and roots) was determined as malondialdehyde content (Heath and Packers 1968). Potassium leakage was estimated in the treated solution by using flame photometer (Mediflame).

To determine whether the effect of copper on NRA was mediated through oxidation of sulfhydryl groups and/or through generation of active oxygen species, following experiments were conducted. To study the effect of antioxidants on *in vivo* NRA in the presence of Cu, plants were treated with 5 mM cysteine / 10 mM sodium benzoate in the presence of 0, 30 or 200 μM Cu for 24 h in light at 25±1°C. *In vivo* NRA in leaves and roots was measured as mentioned above. While effects of different antioxidants on *in vitro* NRA in the presence of various copper concentrations (0.0, 30.0, 50.0, 100.0 and 200.0 μM) were studied using crude nitrate reductase extract. The enzyme extract of leaves/roots (100 μl) was incubated in one ml of incubation mixture containing 10 mM potassium phosphate buffer pH (7.5), 300 mM KNO_3, 0.1 mM NADH, different copper concentrations and 5 mM cysteine or 20 mM sodium benzoate. The reaction was terminated after 1 h at 25±1°C with the addition of sulfanilamide (1%, w/v) and NED (0.02% w/v) for the development of colour for the nitrite. The absorbance of pink colour was read at 540 nm and NRA was expressed as n mol NO_2^- h^{-1} g^{-1} FW. In recovery experiments 100 μl of crude nitrate reductase (leaves/roots) were incubated with 30 or 150 μM Cu (without cysteine, EDTA), 1 ml potassium phosphate buffer (pH 7.5), 30

mM KNO_3, 0.1 mM NADH for 30 min at 25±1°C. Incubations were followed by the addition of water, cysteine (5 mM) or EDTA (1 mM) and a second incubation for 30 min at 25±1°C. NRA was estimated as mentioned above. To study the effect of active oxygen generating chemicals on *in vitro* NRA, crude extract of nitrate reductase obtained from leaves (100 µl) was incubated with either H_2O_2 or an ·OH generating system composed of 0.8 mM H_2O_2, 37 µM $CuSO_4$ and 0.8 mM sodium ascorbate (in 100 mM potassium phosphate buffer, pH 7.5) for 15 min at 25±1°C. After 15 min, 4 mM sodium benzoate / 1 mM EDTA / 5 mM cysteine was included in the assay medium and NRA was measured as above.

A two way / one way analysis of variance in complete randomized design was performed to assess the variability and validity of the data (Gomez and Gomez 1984). Duncan's multiple range test (DMRT) was used to determined the variation between two concentrations. The significance of all the statistical tests was tested at 5% level.

RESULTS AND DISCUSSION

V. spiralis accumulated a significant amount (ANOVA; $P<0.05$) of copper in roots followed by leaves and rhizomes (Table 1). *In vivo* and *in vitro* nitrate reductase activities in *V. spiralis* (leaves/roots) were affected by copper in the growth medium (ANOVA; $P<0.05$). Reduction in NRA (both root and leaves) was first detected at 30 µM Cu (Table 6) and declined in a concentration dependent manner up to 200 µM (Fig. 1A). Further, inhibition in NRA was more pronounced in roots than in leaves. Malondialdehyde is formed in plant cells due to peroxidation of membrane lipids. It was observed that 10-30 µM Cu has no impact on MDA contents (DMRT, $p>0.05$) in leaves and roots of *V. spiralis* (Fig. 1B). Lipid peroxidation was initiated at 50 µM copper level, which was continued to augment with further increase in copper concentration. Maximum level of lipid peroxidation (in terms of MDA content) was measured at 200 µM copper after 24 h exposure. Roots were more severely affected than leaves. Similarly, another marker of oxidative stress i.e., potassium leakage, associated with disintegration of membrane permeability appeared first (Table 6) at 50 µM copper (Fig. 1B). Below this concentration membrane permeability was not affected (DMRT, $p>0.05$). While NRA and the protein content began to decline at 30 µM Cu level, changes in membrane permeability (increase in MDA content and K^+ leakage) and loss of chlorophyll were initiated at 50 µM. Therefore, NRA and protein appeared to be more sensitive than other parameters (Fig.1A, C).

Total chlorophyll content of the leaves was also affected by copper toxicity; a decrease in chlorophyll content was observed at 50 µM copper level which increased further with the increase in Cu concentration up to 200 µM (Fig. 1C). However, significant increase in protein content of the leaves and roots were recorded at 10.0 µM copper level (DMRT, $p < 0.05$). The reduction in protein content started at 30 µM copper which further augmented with the increase in copper concentration (Fig. 1C).

To determine whether effect of copper on *in vivo* NRA was achieved by affecting sulfhydryl groups and/or by increasing the oxidative stress, either cysteine (5 mM) or sodium benzoate (10 mM) was added to nutrient solution containing 30 and 200 µM copper (Table 2). Cysteine not only protected (leaf) nitrate reductase from copper inactivation but also stimulated enzyme activity at 0 and 30 µM Cu level. On the other hand, sodium benzoate (·OH scavenger) did not prevent nitrate reductase (leaf) inactivation caused by 30 µM Cu. However, the inhibitory effect of 200 µM copper was

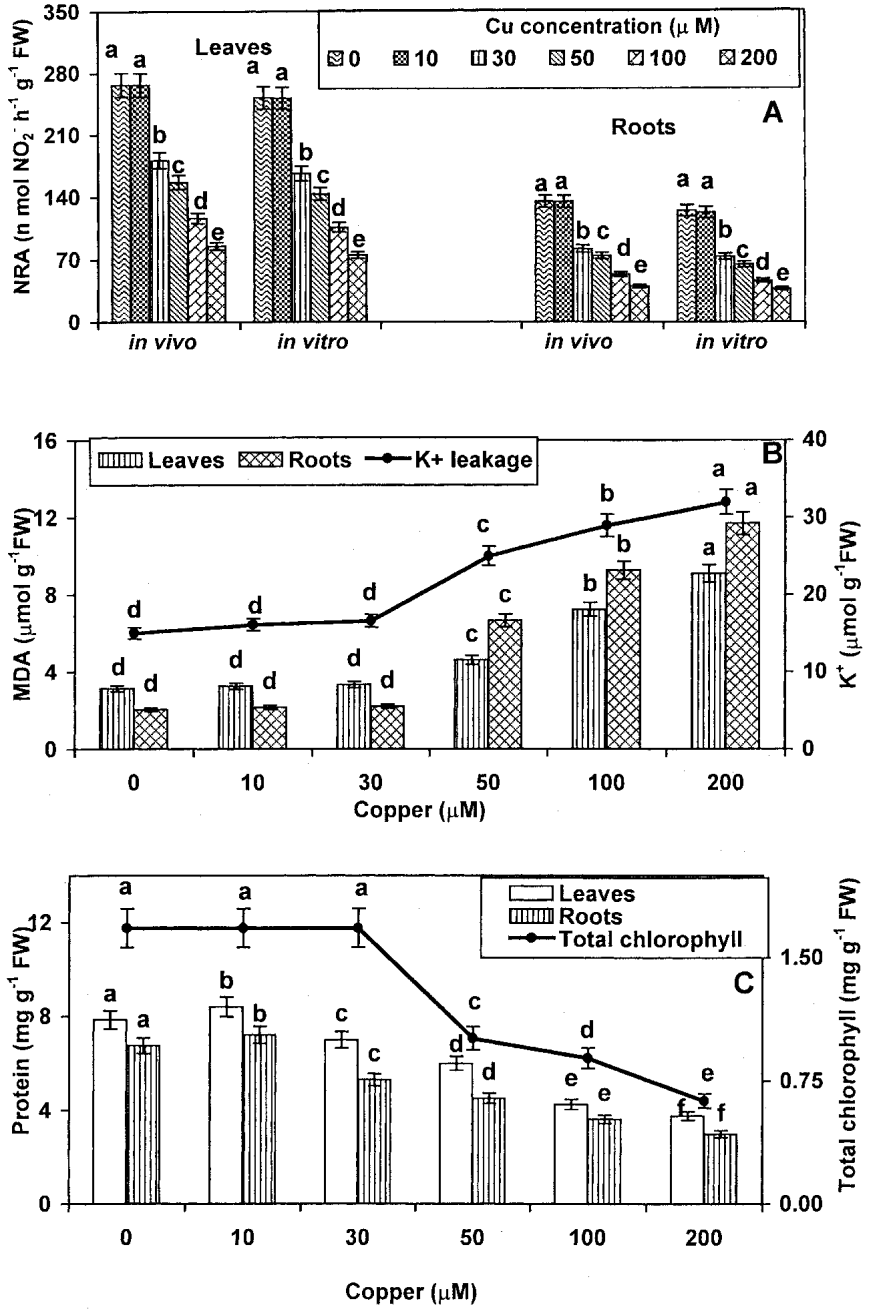

Figure 1. Effect of different concentrations of copper on NRA (*in vivo* and *in vitro*) nitrate reductase activity (A) lipid peroxidation (MDA: malondialdehyde content) and K^+ Leakage (B), total chlorophyll and protein contents (C) in leaves and roots of *V. spiralis* after 24 h of treatment; Mean (n=3) ± SD; ANOVA(one way in complete randomized block design), $P<0.05$ Identical superscripts denote no significant difference ($P< 0.05$) between bars/lines (leaves/roots) according to DMRT.

Table 1. The accumulation of copper by *V. spiralis* after 24 h of treatment.

Cu (μM)	Copper accumulation (μ g g^{-1} DW)		
	Leaves	Roots	Rhizome
0	$9.12^{f} \pm 0.25$	$12.18^{f} \pm 0.50$	$4.30^{f} \pm 0.20$
10	$30.48^{e} \pm 1.32$	$76.20^{e} \pm 2.81$	$18.28^{e} \pm 0.8$
30	$98.32^{d} \pm 3.92$	$236.34^{d} \pm 10.02$	$34.24^{d} \pm 1.50$
50	$150.27^{c} \pm 6.56$	$410.23^{c} \pm 18.50$	$55.32^{c} \pm 2.50$
100	$304.80^{b} \pm 14.23$	$762.10^{b} \pm 36.2$	$180.52^{b} \pm 8.03$
200	$550.30^{a} \pm 25.10$	$1701.80^{a} \pm 85.09$	$234.50^{a} \pm 10.5$

Values are mean ± SD; DW: dry weight; two way ANOVA $p < 0.05$. Different superscripts denote significant difference ($p < 0.05$) between means vertically in a column according to DMRT.

Table 2. The effect of antioxidants on *in vivo* nitrate reductase activity (NRA) in the presence of different concentrations of copper in *Vallisneria spiralis* after 24 h of treatment.

Cu (μM)	*In vivo* NRA (n mol $NO_2^- h^{-1} g^{-1}$ FW)		
	None	Cysteine (5 mM)	Sodium benzoate (10 mM)
		Leaves	
0.0	$267.00^{a} \pm 10.7$ (100)	$332.97^{a} \pm 13.3$ (124.7)	$266.98^{a} \pm 13.3$ (99.9)
30	$181.52^{b} \pm 9.1$ (67.9)	$299.00^{b} \pm 11.9$ (112.0)	$147.28^{b} \pm 5.9$ (55.2)
200	$85.20^{c} \pm 4.3$ (32.0)	$218.94^{c} \pm 12.1$ (82.0)	$187.8^{c} \pm 9.4$ (70.1)
		Roots	
0.0	$135.04^{a} \pm 6.8$ (100)	$164.31^{a} \pm 4.7$(121.7)	$135.04^{a} \pm 6.6$ (100)
30	$82.48^{b} \pm 2.1$ (61.1)	$145.02^{b} \pm 6.4$ (107.4)	$72.64^{c} \pm 3.5$ (53.8)
200	$40.50^{c} \pm 1.6$ (30.21)	$95.85^{c} \pm 10.1$ (70.98)	$92.04^{b} \pm 3.4$ (68.2)

Values are mean (n= 3) ± SD; 3x 3 x3 factorial (involving three Cu concentrations, three replications and three treatments) two way ANOVA significant at 5% level for each set of data i.e. leaves and roots. Different superscripts denote significant difference ($p < 0.05$) between means vertically in a column for each set of data i.e. leaves or roots according to DMRT.

partially overcome by the sodium benzoate. At this concentration, cysteine (a -SH group protector) also mitigated the nitrate reductase inhibition to some extent. However, sodium benzoate alone had no effect on NRA (DMRT, insignificant at 5% level). On the contrary, cysteine alone had stimulatory effect (124%) on NRA (Table 2).

Similar observations were found in roots. However, NRA in the roots seemed to be more severely affected by copper treatment than in leaves. When *in vitro* NRA was measured in the presence of 5 mM cysteine or 20 mM sodium benzoate along with Cu (30, 50, 100 and 200 μM), cysteine not only protected *in vitro* NRA but also promoted it at 30 μM copper. However, at higher concentrations cysteine only ameliorated the effect of copper (Table 3). On the other hand, sodium benzoate neither protected nor promoted *in vitro* NRA in *V. spiralis*. Results suggested that copper toxicity to *in vitro* NRA was due to oxidation of –SH groups of enzyme, which was prevented by addition of cysteine in incubation mixture.

Direct inhibition of NRA by copper was reversible at 30 μM; when incubated either with 5 mM cysteine or 1 mM EDTA the enzyme activity was almost recovered fully (Table 4). However, the reversibility was lower when plants were treated with 150 μM copper

Table 3. *In vitro* effects of antioxidants in the presence of different concentration of copper on nitrate reductase activity (NRA).

Cu (μM)	*In vitro* NRA (n mol NO_2^- h^{-1} g^{-1} FW)		
	None	Sodium benzoate(20mM)	Cysteine (5 mM)
Leaves			
0.0	254.92[a] ± 12.50	255.00[a] ± 13.50	258.00[b] ± 13.30
30.0	169.7[b] ± 6.27	160.24[b] ± 7.80	288.96[a] ± 11.50
50.0	144.90[c] ± 6.80	132.16[c] ± 10.50	232.20[c] ± 9.65
100.0	111.39[d] ± 4.68	98.50[d] ± 8.50	211.56[d] ± 10.99
200.0	76.13[e] ± 5.86	61.50[e] ± 6.50	180.86[e] ± 7.48
Root			
0.0	124.50[a] ± 5.80	122.60[a] ± 3.38	126.05[b] ± 4.50
30.0	72.40[b] ± 3.28	73.50[b] ± 3.30	134.87[a] ± 5.74
50.0	64.75[c] ± 3.60	61.25[c] ± 3.98	108.99[c] ± 4.30
100.0	47.30[d] ± 2.16	45.33[d] ± 4.60	98.32[d] ± 3.12
200.0	37.35[e] ± 2.10	28.73[e] ± 4.07	85.95[e] ± 2.98

Mean (n=3) ± SD; NRA = nitrate reductase activity; 5x 3 x3 factorial (involving five Cu concentrations, three replications and three treatments) two way ANOVA significant at 5% level for each set of data i.e. leaves and roots. Different superscripts denote significant difference ($p < 0.05$) between means vertically in a column for each set of data i.e. leaves or roots according to DMRT.

along with 5 mM cysteine or 1 mM EDTA and about 46 and 44% activity was restored, respectively. Results suggested the possible involvement of active oxygen species in inhibition of nitrate reductase activity. To strengthen this observation, crude extract of nitrate reductase was incubated with either H_2O_2 or ·OH generating system composed of H_2O_2, $CuSO_4$ and ascorbic acid (Casano et al 1994). Copper alone at the concentration used in the ·OH generating system, caused 49% inhibition of nitrate reductase in the leaves (Table 5) while H_2O_2 (0.8 and 1 mM) did not affect NRA. The ·OH generating system inhibited NRA to nearly 82% of control which was more than copper alone. No reversal of enzyme activity was observed with 5 mM cysteine / 4 mM sodium benzoate / 1 mM EDTA when enzyme was inhibited by ·OH generating system. Results suggested that ·OH radicals had caused an irreversible oxidative damage to the enzyme.

V. spiralis accumulated large quantities of copper in roots followed by leaves and rhizomes after 24 h exposure. Similarly, high accumulation of copper by *Ceratophyllum demersum* and *Spirodela polyrrhiza* has been reported (Devi and Prasad 1998; Pandey et al 1999). The high concentration of copper in roots was due to less translocation of Cu in leaves (Ali et al 2003). The 10 μM copper has no toxicity to *V. spiralis*, but 30 μM Cu showed phytotoxicity in terms of reduced NRA and protein. The phytotoxic amount of copper might vary with species. It has been reported that 2 and 50 μM copper showed phytotoxicity in *Ceratophyllum demersum* and *Spirodela polyrrhiza*, respectively (Devi and Prasad 1998; Pandey et al 1999).

The accumulation of copper in aquatic plants is often accompanied by an induction of cellular changes. The reduction in NRA (*in vivo* and *in vitro*) was first noticed at 30 μM copper and was more pronounced in roots than leaves (Fig. 1A). This was probably due to less translocation of copper from roots to shoot. The experiments throw some light on possible action of Cu on nitrate reductase activity. It appears that copper acts through generation of reactive oxygen species (ROS), especially at higher concentrations (50 μM

Table 4. Reversibility of toxic effects of copper to *in vitro* NRA of *V. spiralis* by cysteine and EDTA.

Cu (μM)	NRA (nmol $NO_2^- h^{-1} g^{-1}$ FW)		
	Water	Cysteine (5 mM)	EDTA (1mM)
Leaves			
0.0	$255.20^a \pm 12.5$	$252.80^a \pm 13.5$	$258.00^b \pm 13.3$
30.0	$153.12^b \pm 6.3$	$248.40^b \pm 7.8$	$288.96^a \pm 11.5$
150.0	$88.12^c \pm 4.7$	$122.40^c \pm 8.5$	$211.56^c \pm 10.9$
Root			
0.0	$128.50^a \pm 3.2$	$131.04^a \pm 2.5$	$104.20^b \pm 3.5$
30.0	$75.83^b \pm 3.0$	$127.10^b \pm 3.2$	$116.03^a \pm 4.1$
150.0	$44.60^c \pm 2.2$	$60.28^c \pm 4.1$	$45.84^c \pm 2.0$

Mean (n=3) ± SD; NRA = nitrate reductase activity; 3 x 3 x3 factorial (involving three Cu concentrations, three replications and three reversibility treatments) two way ANOVA significant at 5% level for each set of data i.e. leaves and roots. Different superscripts denote significant difference ($p < 0.05$) between means vertically in a column for each set of data i.e. leaves or roots according to DMRT.

Table 5. Effect of active oxygen generating chemicals on *in vitro* NRA of *V. spiralis* leaves.

Treatment	Nitrate reductase activity (n mol $NO_2^- h^{-1} g^{-1}$ FW)	Percentage of control
None (Control)	$257.47^a \pm 13.10$	100
Cu (37 μM)	$131.31^b \pm 5.55$	51
H_2O_2 (1 mM)	$257.52^a \pm 12.50$	100
H_2O_2 (0.8 mM)	$256.80^a \pm 10.47$	100
·OH	$46.34^c \pm 1.68$	18
·OH followed by 4M benzoate	$51.50^c \pm 2.40$	20
·OH followed by 1mM EDTA	$48.92^c \pm 1.13$	19
·OH followed by5mM cysteine	$48.60^c \pm 1.81$	19

Mean (n=3) ± SD; One way ANOVA in complete randomized block design significant at 5% level. Identical superscripts denote insignificant ($p > 0.05$) difference between means vertically in a column according to DMRT.

Table 6. Summary of the various effects of the copper to *V. spiralis* after 24 h exposure.

Parameters	Concentrations of the copper exhibiting first effects (μM)		
	No effect	Stimulatory	Inhibitory
Total chlorophyll	30	-	50
Protein	-	10	30
MDA	30	50	-
K+ leakage	30	50	-
Nitrate reductase activity (*in vivo* and *in vitro*)	10	-	30

onwards). It is likely that copper could interfere with the electron flux from PSII to PSI, leading to the formation of superoxide anion radical and H_2O_2 (Kato and Simizu 1985). This metal may also stimulate the production of ·OH in a Fenton type reactions (Halliwell 1978). The two important indicators of oxidative stress are lipid peroxidation and membrane disintegration (DeVos et al 1992; Baryla et al 2000). In the present study, MDA content (end product of lipid peroxidation) and K^+ leakage (marker of membrane

disintegration) increased substantially in response to copper (50 μM and onwards). Further, sodium benzoate, a scavenger of ·OH, prevented the copper induced inhibition of NRA *in vivo*. Inhibition of *in vivo* NRA under oxidative stress and its restoration by antioxidants like sodium benzoate has also been reported in other plants (Kenis and Trippi 1986; Kenis et al 1992). In our study, *in vitro* supply of ·OH generating chemicals (i.e. 0.8 mM H_2O_2, 37 μM $CuSO_4$ and 0.8 mM sodium ascorbate) caused a drastic and irreversible inactivation of the crude nitrate reductase (leaves/roots). It is possible that the Cu induced generation of ·OH had a direct effect on nitrate reductase. Casano et al (1994), suggested that nitrate reductase exposed to ·OH might undergo changes in its secondary and/or tertiary structure, probably as a consequence of free radical reactions with tryptophan, tyrosine, histidine and/or cysteine residues. The experiments also demonstrated that the NRA was more responsive to Cu (30 μM) than chlorophyll and protein (50 μM). This is due to labile nature of the enzyme while chlorophyll and proteins are products of a few biochemical reactions. Decline in total chlorophyll and protein, might be attributed to the phytotoxic consequences of the ROS mediated effects in plants (Davies 1987; Somashekaraiah et al 1992).The inhibition of NRA by lower concentrations of Cu (<30 μM) was negated to some extent by cysteine and EDTA, indicating the possible involvement of –SH group oxidation by the copper. Prevention of inactivation of NRA by cysteine and EDTA is also reported by other workers (Smarrelli and Campbell 1983; Luna et al 1997).The results suggested two possible mode of action by copper on -SH groups of nitrate reductase: (a) reduction of Cu^{2+} with the concomitant oxidation of the -SH groups (Wong et al 1984; DeVos et al 1991), which, in turn, could be re-induced by exogenous supply of cysteine; and (b) a 'weak' binding or chelation, in which copper did not cause significant –SH oxidation and could be displaced by chelating agents. The latter appears to be the mechanism mediating copper inhibition of nitrate reductase, since 1 mM EDTA overcame reduction by copper to a similar extent to that obtained with cysteine. Our results are in agreement with Luna et al (1997) who have also proposed two ways of copper toxicity to nitrate assimilatory enzyme.

It may be concluded that *V. spiralis* accumulated Cu in roots>leaves>rhizome. When *V. spiralis* were exposed to various concentrations of copper, nitrate assimilation in leaves and roots might be affected due to decreased nitrate reductase activity. NRA and protein content of *V. spiralis* were very sensitive to Cu. Further, copper reduced nitrate reductase activity involving direct modification of - SH groups and an increased generation of active oxygen species. This study was conducted in laboratory conditions, which eliminates natural variables. In nature, Cu exists with several other metals, which bind more strongly to thiols. Therefore, competing metal ions might have some impact on nitrate reductase activity of *V. spiralis* in natural environment.

Acknowledgments. Thanks are due to Director N.B.R.I., Lucknow for providing necessary facilities. Dr. P. Vajpayee and Mr M.B. Ali are grateful to G. B. Pant Institute of Himalayan Environment Development (Kosi, Almora, U.P.), Ministry of Environment and Forests, Govt.of India, New Delhi for financial assistance. Financial assistance to Mr. A. Kumar by Directorate of Environment, U.P., India is acknowledged.

REFERENCES

Ali MB, Vajpayee P, Tripathi RD, Rai UN, Singh SN, Singh SP (2003) Phytoremediation of lead, nickel and copper by *Salix acmophylla* Boiss.: role of antioxidant enzymes and antioxidant substances. Bull Environ Contam Toxicol 70: 462-469

Arnon DI (1949) Copper enzyme in isolated chloroplasts. Polyphenol/oxidase in *Beta vulgaris*. Plant Physiol 130: 267-272

Bar-Akiva A, Sagiv J (1967) Nitarte reductase in citrus plants properties, assay conditions and distribution within plants. Physiol Plant 20: 500-506

Baryla A, Laborde C, JL Montilet Triantaphylides C, Chagvadieff P (2000) Evaluation of lipid peroxidation as a toxicity bioassay for plants exposd to copper. Environ Pollut 109: 131-135.

Casano LM, Lascano HR, Trippi VS (1994) Hydroxyl radicals and a thylakoid-bound endopeptidase are involved in light and oxygen-induced proteolysis in oat chloroplasts. Plant Cell Physiol 35: 145-152

Chaney RL (1989) Toxic Element Accumulation in Soils and Crops : Protecting Soil Fertility and Agricultural Food Chains, In :Bar-Yosef B, Barrow NJ, Goldshmid J (eds) Inorganic Contaminants in the Vadose Zone. Berlin, Springer- Verlag, p.140

Davies KJA (1987) Protein damage and degradation by oxygen radicals. I. General aspects. J Biol Chem 262: 9895-9901

DeVos CHR, Schat H, DeWaal MAM, Vooijs R, Ernst WHO (1991) Increased resistance to copper-induced damage of the root cell plasmalemma in tolerant *Silene cucubalus*. Physiol Plant 82: 523-528

DeVos CHR,Vonk MJ, Vooijs R, Schat H (1992) Glutathione depletion due to copper induced phytochelatin synthesis causes oxidative stress in *Silene cucubalus*. Plant Physiol 98: 853-858

Fang WC, Kao CH (2000) Enhanced peroxidase activity in rice leaves in response to excess iron, copper and zinc. Plant Sci 158:71-76

Gomez KA, Gomez AA (1984) Statistical procedures for agricultural research. John Willey and Sons, NewYork

Halliwell B (1978) Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts. FEBS Lett 96: 238-242

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplast. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125: 189-198

Kato M, Simizu S (1985) Chlorophyll metabolism in higher plants. VI. Involvement of peroxidase in chlorophyll degradation. Plant Cell Physiol 26: 291-1301

Kenis JD, Trippi VS (1986) Regulation of nitrate reductase in detached oat leaves by light and oxygen. Physiol Plant 68: 387-390

Kenis JD, Morlans JD, Trippi VS (1989) Effect of hydrogen peroxide on nitrate reductase activity in detached oat leaves in darkness. Physiol Plant 76: 216-220

Kenis JD, Silvente ST, Morlans JD, Luna CM (1992) Glycolate, xanthine- and paraquat-mediated inhibition of nitrate reductase in detached oat leaves. Plant Cell Physiol 33: 315-320

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurement with folin phenol reagent. J Biol Chem 193: 265-275

Luna CM, Gonzalez C, Gonzalez VS (1994) Oxidative damage caused by an excess of copper in oat leaves. Plant Cell Physiol 35: 11-15

Luna CM, Casano LM, Trippi VS (1997) Nitrate reductase is inhibited in leaves of *Triticum aestivum* treated with high levels of copper. Physiol Plant 101: 103-108

Mathys TM (1975) Enzymes of heavy-metal-resistant and non-resistant population of *Silene cucubalus* and their interaction with some heavy metals *in vitro* and *in vivo*. Physiol Plant 33: 161-165

Pandey S, Adthana RK, Kayastha AM, Singh N, Singh SP (1999) Metal uptake and Thiokol productin in Spirodela polyrrhiza (L.) SP20. J. Plant Physiol 154: 634-640

Prasad MNV, Malec P, Waloszek A, Bojko M, Strzalka K (2001) Physiological responses of *Lemna trisulca* L. (duck weed) to cadmium and copper bioaccumulation. Plant Sci 161: 881-889

Singh RP, Bharti N, Kumar G (1995) Differential toxicity of heavy metals to growth and nitrate reductase of *Sesamum indicum* seedling. Phytochem 35: 1153-1156

Smarrelli JJr, Campbell WH (1983) Heavy metal inactivation and chelator stimulation of higher plant nitrate reductase. Biochem Biophy Acta 742: 435-445

Somashekaraiah BV, Padmaja K, Prasad ARK (1992) Phytotoxicity of cadmium ions on germinating seedlings of mung bean (*Phaseolus vulgaris*) : Involvement of lipid peroxides in chlorophyll degradation. Physiol Plant 85: 85-89

Srivastava HS (1974) *In vivo* nitrate reductase activity in maize seedlings. Ind J Biochem Biophys 11: 230-232

Vajpayee P, Rai UN, Ali MB, Tripathi RD, Yadav V, Sinha S, Singh SN (2001) Chromium induced physiologic changes in *Vallisneria spiralis* L and its role in phytoremediation of tannery effluent. Bull Environ Contam Toxicol 67: 246-256

Vara F, Serrano R (1982) Partial purification of the proton translocating ATPase of plant plasmamembranes. J Biol Chem 257: 12826-12830.

Wong KH, Boh EE, Steele RH (1984) Copper facilitated chemiluminescence from the sulfhydryl proteins: Yeast alcohol dehydrogenase, spinach ferredoxin and metallothionein. Biochem Biophys Res Comm 125: 425-430