Pak. J. Bot., 43(1): 515-520, 2011.

NITROGEN METABOLISM AS A BIO INDICATOR OF CU STRESS IN *VIGNA RADIATA*

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Abstract

The possible involvement of Cu on N – metabolism in relation with protease activity, proline, amino acid contents and protein oxidation was observed. These were monitored as bio-indicators in the seedlings of *Vigna radiata* treated with (0–25 ppm) $CuSO_4$ to ascertain the role of Cu in generation of oxidative stress. Copper is an essential nutrient element for the plant growth, may be toxic when in excess by their participation in redox cycles producing hydroxyl radicals ($^{\cdot}OH$) which are extremely toxic to the living cells. Cu produced a significant inhibition of growth as well as a reduction in the water contents in the roots and the leaves of the seedlings. Results showed an increase in the protease activity and decrease in the protein contents, which may be attributed with the plant defense against metal toxicity through hydrolysis of the oxidative proteins. An inverse relation between protease activity and other nitrogen compound showed the effective hydrolyzing role of protease due to which decrease in proline and total amino acid contents were observed, exhibited the highest sensitivity to excess metal, followed by the plant dry weight accumulation, leaf area formation. Decrease in proline contents support the sensitivity of *Vigna radiata* and weak defense of species against Cu stress.

Introduction

The unrestricted developmental activities such as industrialization and urbanization carried out during the past few years have given rise to serious problems of environmental contamination. A general increase in the level of heavy metals poses a pervasive threat to the natural ecosystem (Azmat *et al*., 2005a, 2005b, 2007).

Metal accumulation commonly produced oxidative stress results in oxidized proteins which are selectively degraded by proteases. Changes in protease activity, gelatinase profile and protein oxidation were reported in sunflower cotyledons where proteases might also play a role in the plant adaptation to changing environmental conditions. Proteolysis is also associated to oxidative stress promoted by ROS (O_2^-, H_2O_2, and OH). The protein modification exerted by oxidative stress is characterized for the production of carbonyl groups in the molecules (Palma *et al*., 2002; Vassilv *et al*., 2003). Zhi-Ting *et al*., (2006) observed that Cu-induced nitrogen (N) metabolism damage in the popular vegetable Chinese cabbage (*Brassica pekinensis Rupr*.). The results demonstrated adverse effects of Cu on N metabolism and plant growth. Cu also shortened root length and produced fewer leaves and lower plant biomass. However, Cu exposure increased total free amino acid content in the leaves. N deficiency increased the root/shoot ratio of biomass and the total free amino acid content. The results suggested that Cu toxicity to the plant was at least partly due to an influence on N metabolism.

Proline accumulates heavily in several plants under stress and may be related to a tolerance mechanism for dealing with Cu stress (Wu 1998). Proline accumulation, accepted as an indicator of environmental stress, is also considered to have important protective roles. Proline accumulation in the plant tissues has been suggested to result from (i) a decrease in proline degradation, (ii) an increase in proline biosynthesis, (iii) a decrease in protein synthesis or proline utilization and (iv) hydrolysis of protein providing the plants protection against damage by ROS (Kaul *et al*., 2008). The phyto-toxic effects of Cu are strongly dependant on many factors with different origin. Thus, the sensitivity of the proposed bio-indicators should be tested for the actual species in an experimental design well suited for screening studies (Sharma & Dietz, 2006; Schat *et al*., 1997).

The aim of this work was to study the sensitivity of excess of Cu on seedling growth of *Vigna radiata* and N –bio indicators as a result of oxidative stress based on simple mathematical and biochemical approaches. This article discusses the plant defense system against the metal stress in term of proteolysis in relation with proteins, total amino acids and proline contents.

Materials and Methods

Plant material and growth conditions: Seeds of the *Vigna radiata* were germinated in Petri dish and grown in a natural environment. Two days old seedlings were removed from the petri dish, roots were carefully washed and transferred to 3L containers, 20 plants per container in which $CuSO_4$ was supplemented with different concentrations (0, 10, 20, 30, 40, and 25ppm) (3 L) for hydroponics. The hydroponics medium was half strength Hoagland's nutrient solution (control) and aerated continuously. After 14 days of treatment roots and shoots were used for all further determinations. The experimental set up was completely randomized with three replicates with given concentration of Cu. Proline contents was extracted from 0.5 g of fresh shoot and root into 10ml of 3% sulfosalicylic acid and filtered through Whatman No.2 filter paper. Proline was determined by ninhydrine method as described by Btaes *et al*., (1973) on Schimadzo UV/Visible Spectrophotometer 180 A using pure proline as a standard. Protein contents were estimated using the method of Bradford (1976). Protease activity was determined by the method described by Ainous (1970) using phosphate buffer and absorbance was recorded on Spectrophotometer at 570nm.

Statistics: Values are mean values ± s.e. Differences among treatments were analyzed by 1-way ANOVA, taking $p<0.05$ as significant according to Tukey's multiple range test.

Results and Discussion

Effect of Cu treatment on plant growth: Results showed that increasing concentrations of Cu in the nutrient solution produced a significant growth inhibition of plants as compared to the non treated plants measured as fresh and dry weight (Fig. 1), the peak undesirable effect being on leaves while root growth was only significantly affected by 25ppm $CuSO_4$ ($p<0.05$). The decrease in the dry weight of leaves was analogous to a reduction in the leaf area (Table 1) but no visible symptoms of toxicity except growth reduction were observed (Fig. 1). The decrease of the root growth with 25ppm Cu was characterized by a reduction of the lateral roots. The effect of higher Cu concentrations ranged between 30-200 ppm was also studied, but plants were considerably damaged with 30ppm therefore for this reason the study was focused on 25ppm as the last highest Cu concentration. Our results are similar as reported earlier that the inhibition of dry weight (DW) accumulation in plants suffering heavy metal stress is widely observed in

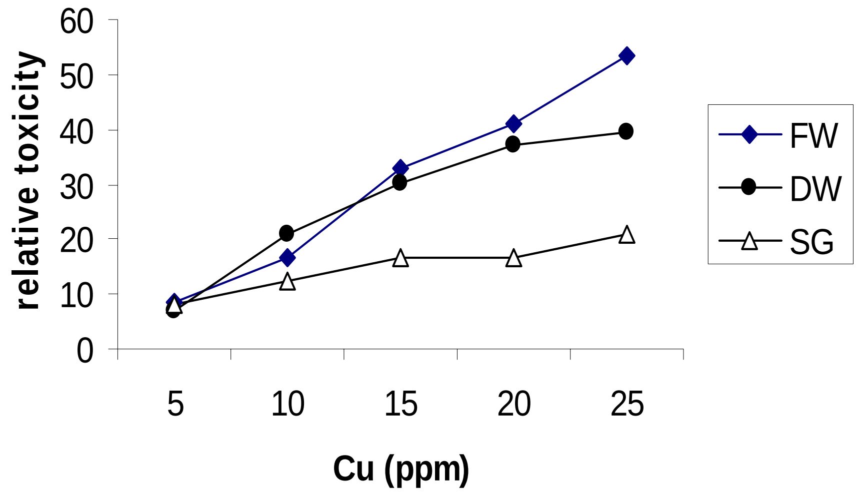

Fig.1. Relative toxicity Cu on growth, fresh weight and dry weight of *Vigna radiata*.
FW= Fresh weight, DW= Dry weight, SG= Shoot growth

Table 1. Effect of Cu on biomass production and relative growth rate of *Vigna radiata*.

[Cu] ppm	DW/FW Root 10^{-2}	DW/FW Shoot 10^{-2}	DW/FW Leaf 10^{-2}	Relative growth rate $(\ln W_2 - \ln W_1/T)$. 10^{-3}
0	1.5 ± 0.03	1.58 ± 0.03	3.2 ± 0.02	-
5	1.7 ± 0.03	1.63 ± 0.05	3.0 ± 0.02	09.0 ± 0.02
10	1.6 ± 0.02	1.28 ± 0.04	3.6 ± 0.01	18.4 ± 0.05
15	1.9 ± 0.01	1.33 ± 0.04	4.3 ± 0.05	39.9 ± 0.04
20	1.9 ± 0.01	1.29 ± 0.03	4.5 ± 0.02	53.0 ± 0.04
25	2.02 ± 0.04	1.73 ± 0.02	5.1 ± 0.03	76.5 ± 0.01

FW= Fresh weight, DW= Dry weight

phytotoxicity studies (Vassilev 2003). Roots at 20-25ppm appeared thick and stunted with inhibition of root hairs formation. The growth inhibition of the seedling was accompanied by a significant damage in the nitrogen metabolism (Table 1), which was about 10 times reduced at the highest Cu concentration in comparison with control plants (Azmat *et al*., 2005). The idea of critical or threshold toxicity is often used to establish the point at which metals in the growth substrate caused significant growth or yield decrease (Zhi-Ting *et al*., 2006) as observed in this study because plants need relatively small amount of metals for their growth and soil harbor these by naturally or as a consequence of dumping waste in soil which ultimately disturb the ecology of soil (Zengin & Munzuroglu, 2005). It was observed that treated plant experienced more rapid death as compared to non treated one which may be related with the water deficiency (Fig. 2) and altered N-metabolism. Cu may probably be retard the absorption and translocation of water from root and shoot due to which plant dried and died premature.

N-metabolism as a bio indicator under Cu stress: There was drastic variation in nitrogen metabolism (Table 2). The decrease in protein, proline and amino acids contents (p<0.005) both in root and shoot of seedling may be related with the oxidative stress due to which protease activity was increased that results in the hydrolysis of protein. The soluble protein contents in plant cells are an important indicators of their physiological state (Ainous, 1970). The protein degradation to amino acids is in fact an adaptation of the cells to the carbohydrate deficiency. These results suggest that Cu toxicity to the plants was at least partly *via* influence on N metabolism (Zhi-Ting 2006). The reduced amount of total protein contents and proline contents in the root and shoot of the Cu treated plants was most probably is a result of the reduced biosynthesis or the accelerated protease activity or catabolic processes (Zengin & Munzuroglu, 2005). Proteases are vital for living cells and play a role in the plant cell adaptation to environmental conditions. This suggestion is also confirmed by Vassilev *et al*., (2003 a & b) who reported that the protein degradation to amino acids is in fact an adaptation of the cells to the carbohydrate deficiency. On the other hand, the accelerated catabolism is probably due to the considerable disturbances in the membrane systems, in response to the metal phytotoxicity. Protein carbonylation is an irreversible oxidative process leading to a loss of function of the modified proteins. These oxidized proteins are selectively recognized and degraded by proteolytic enzymes (Palma *et al*., 2002). Extreme environmental conditions that induce oxidative stress have been associated to increased carbonyl groups content and to an induction in protease activity. Decrease in proline contents in seedlings showed that Cu effect the plant defense system which may be attributed with the visual reduced morphological growth symptoms. Our results are similar to those Ali & Saradhi (1991) who reported that proline by absorption of OH play defensive role but decrease in proline contents in present investigation showed that Cu adversely disturb the N-metabolism of specie under study. Marylène (2008) reported that protease activity shows stimulations of up to 2.5 times the activity of the unperturbed control in uncontaminated samples only. Proline accumulation, accepted as an indicator of environmental stress also considered to have important protective roles. Toxic metal stress leads to proline accumulation (Kaul *et al*., 2008, Alí & Saradhi, 1991) may be for absorption of OH radical to protect the plant but toxicity may lead in decrease in a proline degradation, an increase in proline biosynthesis, or a decrease in protein synthesis or proline utilization and hydrolysis of proteins (Charest & Phan, 1990). In *Vigna radiata* the decline in proline contents in this investigation suggests the weak antioxidant system. Because proline commonly involve in absorption of OH radical, protect the plant from stress with an appreciable affinity to forming various complexes with cupric ions, which may be partly attributable to the reduction in Cu toxicity (Wu, 1998). It also acts as an effective singlet oxygen quencher (Ali & Saradhi, 1991, Charest & Phan, 1990). Proline increases the stress tolerance of the plants through such mechanisms as osmoregulation, protection of enzymes against denaturation, and stabilization of protein synthesis (Weckenmann & Martin, 1984). Accumulation of free proline in response to Cu, and metal-tolerant *Silene-vulgaris* (Moench) Garcke; the constitutive proline concentration in leaves was 5 to 6 times higher in the metal-tolerant ecotype than in the non tolerant ecotype (Kaul *et al*., 2008; Schat *et* al., 1997). But the decrease in proline contents in this study showed that Cu effect the plant defense system of *Vigna radiata* (Costa & Morel, 1994). Kaul *et al*., (2008) reported that the elevated tissue proline levels under stressful growth conditions constitute a component of cellular antioxidative network involved in mitigation of stress effects. Chen (2004) reported that proline supplement to Cu-treated rice seedlings not only reduced the Cu absorption in the roots but also the Cu exclusion, suggesting supplement of proline accompanied by Cu exposure induce a barrier of Cu influx and efflux in rice roots. Proline accumulation in plants under Cu stress is induced by a Cu-forced decrease of the plant water potential (Fig. 2) and the functional significance of this accumulation would lie in its contribution to water balance maintenance; proline-mediated alleviation of water deficit stress could substantially contribute to Cu tolerance (Nassar 2004; Costa & Morel 1994; Weckenmann & Martin, 1984).

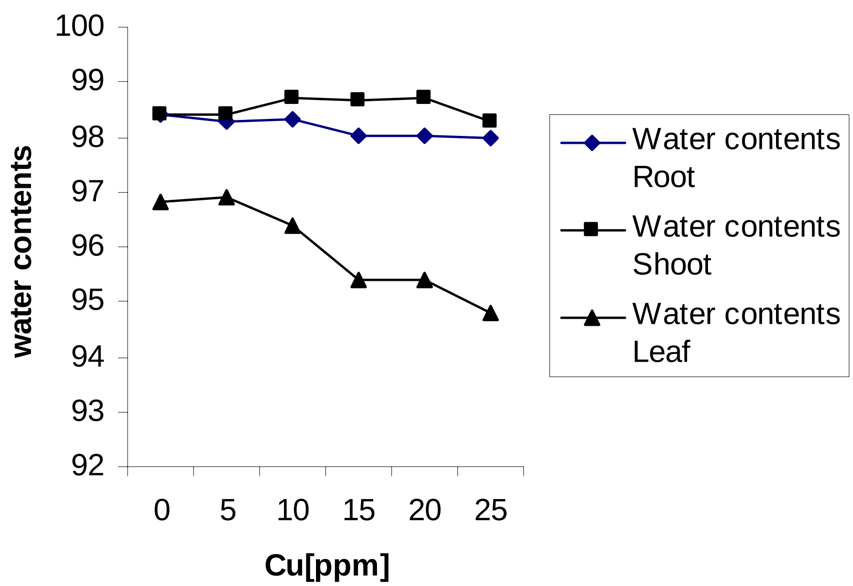

Fig. 2. Effect of Cu on water contents of *Vigna radiata.*

Table 2. Effect of Cu on N- compounds of *Vigna radiata.*

[Cu] ppm	(%) proteins	Protease µgml^{-1}	Proline µgml^{-1}	Total (%) amino acids
		Root		
0	7.70 ± 0.5	72 ± 11.0	5.00 ± 0.9	0.31 ± 0.01
5	7.52 ± 0.6	84 ± 09.0	2.05 ± 0.2	0.25 ± 0.01
10	7.67 ± 0.7	96 ± 12.0	1.50 ± 0.1	0.23 ± 0.01
15	7.59 ± 0.5	99 ± 12.4	1.00 ± 0.2	0.327 ± 0.03
20	7.56 ± 0.6	101 ± 15.2	1.00 ± 0.1	0.15 ± 0.02
25	5.00 ± 0.5	110 ± 12.3	0.9 ± 0.09	0.16 ± 0.02
		Shoot		
0	2.90 ± 0.2	78 ± 12.0	2.0 ± 0.02	3.35±0.02
5	3.15 ± 0.3	92 ± 11.0	1.9 ± 0.01	3.13±0.01
10	3.14 ± 0.1	98 ± 14.0	1.2 ± 0.03	2.62±0.02
15	3.06 ± 0.2	100 ± 11.0	0.9 ± 0.02	3.01±0.01
20	3.01 ± 0.2	102 ± 12.9	0.7 ± 0.01	2.47±0.02
25	1.69 ± 0.3	116 ± 13.2	0.5 ± 0.01	1.68±0.03

Conclusion

The presented investigation highlighted the interaction of Cu metal, induced variety of toxic effects on growth and N-metabolism of seedlings of *Vigna radiata*. Protease, proline, amino acid and proteins are the suitable biochemical indicators of plant under metal stress. Oxidative stress produced by Cu results in the decrease in protein, proline and amino acid contents which may be attributed with weak plant defense system.

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(Received for publication 31 December 2009)