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Biotoxic Effects of Copper on Ureide Metabolism of Pigeon Pea

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The economic and environmental costs of heavy use of chemical nitrogen (N) fertilizers in agriculture are of global concern. Fertilizer N in ground water is a threat to health and causes extensive pollution of surface waters and eutrophication of rivers and lakes in intensively cultivated areas. The nitrous oxides derived from nitrogen fertilizers destabilize the ozone layer (Keeney, 1982). Sustainability considerations mandate that alternatives to N fertilizers must be urgently sought. Biological nitrogen fixation (BNF), a microbiological process which converts atmospheric N_2 into plant usable form, offers this alternative (Bohlool, 1992). Legume - Rhizobium symbiosis is the major source of biologically fixed N in diverse environments. This process is affected by number of factors such as climate, soil and nutritional factors of rhizobia. These factors influence nodule development, nitrogenase activity and several biochemical and physiological parameters related to symbiotic N_2 fixation. Very recently we reported the effects of pesticides on the growth and symbiotic properties of rhizobia and symbiotic N_2 fixation of legumes (Madhavi et al., 1993; 1994). In industrial countries the functioning of biological systems in soil is threatened by increasing higher concentration of heavy metals (Tyler, 1972; Mayer, 1985). Urease, an important enzyme in nitrogen metabolism (Bremner and Mulvaney, 1978) has been extensively studied in relation to its inhibition by heavy metals like Cu and Zn in polluted soils (Tyler, 1974). Nor (1982) reported complete inhibition of urea hydrolysis in soils by 120 mg copper Kg^{-1}. Soils near metallurgic industries or mine waste are subjected to prolonged heavy metal contamination and as a result, urease activity has been found to be lower in soils further away from these sites (Doelman and Hanstra, 1986). On the basis of urease inhibition

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certain biochemical activities of rhizobia and legume - Rhizobium symbiosis may be affected by heavy metal pollution. The ureides, allantoin and allantoic acid, are the major nitrogenous products of symbiotic N_2 fixation. We report here our observations on the effect of copper on N_2 fixation, allantoin, allantoic acid and allantoinase activity in non-nodulated (but fertilizer N supplied) and nodulated pigeon pea (Cajanus cajan).

MATERIALS AND METHODS

Bacterial culture used in the study, Rhizobium sp P116 nodulating Cajanus cajan (pigeon pea), was obtained from Indian Agricultural Research Institute, New Delhi. All the chemicals used were of reagent grade. Allantoin was obtained from Sigma Chemical Co., U.S.A., remaining chemicals and reagents were obtained from Merck, Glaxo, Qualigens, Himedia, Loba etc.

The present investigation was carried out in Hyderabad, India at $17^{o}24'$ latitude and $78^{o}31'$ longitude. The pigeon pea plants were grown for 45 days in a net house with natural light (10-12 h of day light). The environmental temperature was $30^{o}C$ in day and $22^{o}C$ in night with humidity of 55 to 72%.

The pigeon pea plants were grown in pots with fine grained sterilized river sand having proper drainage. The seeds were surface sterilized (Vincent, 1970) and about 6-7 seeds were sown in each pot. Thinning was done after complete emergence of seedlings to maintain four plants per pot. The first inoculation of Rhizobium sp P116 was done at the time of sowing and second inoculation was after the emergence of seedlings. Seeds or seedlings were treated with an actively growing rhizobium culture (about 10^{8} cells/ seed or seedling) for symbiotic infection and nodule development as described by Vincent (1970). Treatment with various concentrations of copper i.e., 0, 0.02, 0.2, 1.0 and 2.0 ppm was started after full emergence of seedlings. Sand pots were flushed with nutrient solution (Evans, 1974) containing various concentrations of copper once a week for up to 45 days. A matching set of uninoculated plants supplied with nutrient solution containing 5 mM NH_4NO_3 were also treated with the above concentrations of copper. By using this level of fertilizer N, sterile sand and effective surface sterilization of seeds, the plants remained free of root nodules throughout their growth.

Fresh wet plant material (nodules, roots, stems and leaves) weighing 1 g was ground in ice cold 5% trichloro acetic acid (TCA) with an equal weight of

acid washed sand in a chilled mortar and pestle. The homogenate was centrifuged at 2°C for 20 min at 10,000 rpm. Total ureides, allantoin and allantoic acid, were determined in the supernatant by the phenyl hydrazine method (Nirmala and Sivaramasastry, 1972).

For the determination of the allantoinase enzyme, the plant parts (nodules, roots, stems and leaves, 1-2 g) were washed thoroughly with cold distilled water, gently blotted dry between folds of filter paper and ground in ice cold diethanolamine-HCl buffer (0.6 M, pH 7.5, 2 to 3 ml/g fresh weight). The extract was centrifuged as indicated above and the supernatant used as a source of allantoinase. Reaction mixtures for the allantoinase assay contained 0.05-0.5 ml of the crude extract and 50 µ moles of allantoin in diethanolamine-HCl buffer in a total volume of 1.5 ml. After incubation for 30 min at room temperature (30±1°C) the reaction was terminated by adding 0.2 ml of 50% TCA. The reaction mixture was then centrifuged (5000 rpm for 10 min) and the supernatant was taken to determine allantoate by the phenyl hydrazine method (Nirmala and Sivaramasastry, 1972). Protein content of the enzyme was determined using Folins reagent (Lowry et al,1951).

The nodules of five plants along with their roots were taken and immediately used for estimation of nitrogenase activity by acetylene reduction assay using flame ionization gas chromatography (Hardy et al.,1968). Nitrogenase activity was expressed as n moles ethylene produced/plant/hr. Plants were harvested after 45 days and the whole plant material was dried at 60°C for 3-4 days and dry wt were determined. Total nitrogen contents of these dried plant materials were deter mined by semi-micro kjeldahl method (Jackson et al., 1976).

RESULTS AND DISCUSSION

The effect of graded concentrations of copper sulphate in nutrient solution supplied to the rooting system of pigeon pea inoculated with Rhizobium sp P116 and uninoculated (fertilizer N supplied) plants was studied for 45 days. The biotoxic effects of copper on ureide metabolism were observed by measuring allantoin, allantoic acid and allantoinase in various parts of the plant. The ureides, allantoin and allantoic acid are the major nitrogenous translocatable and storage compounds of symbiotic nitrogen fixation in some economically important legumes such as soybean, phaseolus and pigeon pea (Schubert, 1986). The influence of environmental factors especially metal ions on ureide metabolism of legumes has not been studied. Our observations indicated that allantoin and allantoic acid levels were slightly increased with 0.02 ppm

concentration of copper. However, a dose dependent inhibiton of allantoin and allantoic acid contents were observed and was significantly different at higher concentrations than 0.2 ppm in all parts of the pigeon pea due to Cu toxicity (Table 1). Allantion synthesis was more inhibited in nodulated plants when compared to non-nodulated (combined N supplied) plants. Allantoic

Table 1 Effect of copper on total ureide levels in different parts of pigeon pea plant

Part of the plant		Concentration of Copper (ppm)					
		0.00	0.02	0.20	1.00	2.00	l.s.d (P=0.05)
NODULES							
UNC		-	-	-	-	-	-
INC	ALA	292	294	270	240	200	9.16
	AA	223	240	234	225	107	15.70
ROOTS							
UNC	ALA	94	96	91	83	78	3.62
	AA	87	99	89	69	41	1.53
INC	ALA	190	196	184	157	142	1.51
	AA	180	199	183	109	96	2.91
STEMS							
UNC	ALA	117	120	118	115	108	1.83
	AA	109	122	86	63	40	2.59
INC	ALA	239	242	236	220	208	1.61
	AA	246	260	230	201	162	9.50
LEAVES							
UNC	ALA	111	117	114	110	101	0.42
	AA	133	150	120	67	34	5.08
INC	ALA	207	218	215	208	196	1.37
	AA	270	284	248	197	160	3.37

UNC = Uninoculated plants but fertilized with NH_4NO_3;
INC = Inoculated with Rhizobium sp.; ALN = Allantoin;
AA = Allantoic acid; - indicates absence of nodules;
Total ureides expressed in µg/g fresh wt of plant part.

acid synthesis was drastically inhibited in symbiotic N_2 fixing as well as fertilizer N dependent pigeon pea plants when compared to controls. Over 25%, 47%, 44% and 40% reduction in allantoic acid was observed in nodules, roots, stems and leaves of symbiotic N_2 fixing pigeon pea plants respectively at 2 ppm. In non-nodulated plants the reduction in allantoic acid was more in leaves (64%) followed by stems (63%) and roots (53%). In contrast to symbiotic N_2 fixing pigeon pea, allantoin and allantoic acid levels were low in non-nodulated (fertilizer N dependent) plants. This may be due to the dependence of ureide synthesis on symbiotic N_2 fixation (Herridge and Peoples, 1990). The synthesis of ureides was reported to be inhibited by supplemented N (Diatloff et al., 1991).

Allantoinase (EC 3.5.2.5) (which converts allantoin to

Table 2 Effect of copper on allantoinase activity in different parts of pigeon pea plants

Part of the plant	Concentration of copper (ppm)					
	0.00	0.02	0.20	1.00	2.00	l.s.d (P=0.05)
NODULES						
UNC	-	-	-	-	-	-
INC	941.5	1046.0	840.9	738.6	670.5	2.71
ROOTS						
UNC	45.5	54.0	33.5	28.4	24.4	1.14
INC	71.0	81.3	51.1	39.2	23.9	1.60
STEMS						
UNC	55.1	62.5	56.8	50.0	40.9	0.99
INC	91.4	96.5	67.0	52.8	45.4	0.77
LEAVES						
UNC	75.6	94.8	94.0	53.9	45.5	1.20
INC	97.2	104.0	78.9	68.8	54.5	0.80

UNC = Uninoculated plants but fertilized with NH_4NO_3;
INC = Inoculated with Rhizobium sp.;
- indicates absence of nodules;
Allantoinase activity expressed as n moles of allantoate formed/mg protein/30 min.

allantoic acid) activity was markedly inhibited in all parts of inoculated and uninoculated (fertiliser N supplied) pigeon pea plants due to Cu toxicity (Table 2). The inhibition was more in symbiotic N_2 fixing plants when compared to fertilizer N dependent plants. Over 66%, 50%, 40% and 28% of the activity was inhibited in roots, stems, leaves and nodules respectively. While in non-nodulated plants over 46% inhibition was observed in roots followed by leaves (40%) and stems (26%). The reduction in allantoic acid levels in various parts of pigeon pea (Table 1) is attributed to inhibition of allantoinase activity due to Cu toxicity.

We have also recorded the influence of copper on nitrogenase activity, dry weight and total nitrogen contents of pigeon pea (Table 3). These parameters

Table 3 Effect of copper on nitrogenase activity dry weight and total nitrogen contents in pigeon pea

Conc. of Copper (ppm)	Nitrogenase activity (n moles/ plant/hour)	Dry weight content (mg/plant)		Total nitrogen content (mg/gr. dry wt.)	
		UNC	INC	UNC	INC
0	320.5	160.0	308.0	16.8	43.4
0.02	334.5	185.7	327.8	17.4	46.0
0.2	304.0	160.8	300.0	15.8	44.8
1.0	291.7	142.1	288.5	14.7	40.2
2.0	270.4	138.4	260.1	13.6	36.4
l.s.d. (P=0.05)	10.4	1.2	16.0	0.2	0.1

UNC = Uninoculated plants but fertilized with NH_4NO_3;
INC = Inoculated with Rhizobium sp.

were slightly increased with 0.02 ppm of copper. Little elevation in allantoin and allantoic acid levels as observed in Table-1 is due to the increase in nitrogenase activity at 0.02 ppm of Cu. Higher concentrations of Cu i.e., 0.2, 1.0 and 2.0 ppm inhibited the nitrogenase activity, dry weight and total nitrogen contents and was significantly different

at higher concentration than 0.2 ppm. Over 16% inhibition of nitrogenase activity, dry weight and total nitrogen contents were observed at 2 ppm in symbiotic N_2 fixing pigeon pea. While 13% dry weight and 19% total nitrogen contents were inhibited in asymbiotic (fertilizer N dependent) plants. The decline in allantoin synthesis (Table 1) at higher concentration of Cu is attributed to the inhibition of nitrogenase activity due to Cu toxicity. The reason for the reduction in allantoic acid is due to inhibition of allantoinase resulting in blockage of allantoate synthesis. As observed in Table-3, the reduced dry weight and total nitrogen contents at higher concentration of Cu is attributed to the blockage of ureide N assimilation into plant protein due to Cu toxicity.

In the above findings Cu toxicity is observed at 0.2 to 2.0 ppm on ureide metabolism of pigeon pea under laboratory conditions compared to inhibitory effect of Cu on soil enzymes at 40-140 ppm in polluted soils as reported by Tyler (1974).

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