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Studies on antioxidant enzymes in *Canna indica* plant under copper stress

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Abstract

Bright red-flowered *Canna indica* L. plants were subjected to grow in nutrient solution supplemented with five different concentrations (0, 5, 10, 30 and 50 µM) of $CuCl_2$ to study antioxidant defense responses of the plant. Accumulation of Cu was dose-dependent and much higher in the roots (108-191µg g^{-1} d. wt.) than in the leaves (23.36 - 40.43 µg g^{-1} d.wt.). Total ascorbate content did not changed in both tissues, but ascorbate redox state decreased (0.570-0.640) in Cu-treated *Canna* roots. In contrast, both total and reduced glutathione contents increased (387-591.9 nmol g^{-1} f. wt.) considerably in roots, accompanied with enhanced activities of dehydroascorbate reductase (153.3-160 nmol mg^{-1} protein) and glutathione reductase (67-87.5 nmol mg^{-1} protein). No significant change, however, was observed for monodehydroascorbate reductase activity in both tissues of the treated plant. The efficient scavenging of hydrogen peroxide was performed by normal (control level) activities of both ascorbate peroxidase and catalase in leaf and increased activity of only catalase in root, preventing its accumulation at toxic concentrations (despite high superoxide dismutase activity) and subsequent damage of membrane lipids by peroxidation. Together, these ensured normal dry weight of leaves and roots, indicating tolerance of *Canna indica* plant to Cu-induced oxidative stress.

Key words

Ascorbate-glutathione cycle, Antioxidant, Copper-stress, Lipid peroxidation, *Canna indica*

Introduction

Environmental pollution by heavy metals has increased dramatically over the last few decades mainly due to various types of anthropogenic activities. Copper (Cu) loading of agricultural soils is one of the major problems of environmental pollution. Cu is an essential redox-active micronutrient for plant growth (Arnon and Stout, 1939). It acts as a co-factor for Cu/Zn superoxide dismutase, cytochrome c oxidase, polyphenol oxidase etc. (Yruela, 2005). However, its high bioavailability in soils may be toxic to plants, causing inhibition of growth and oxidative stress (Baryla *et al*., 2000; Hall, 2002; Yruela, 2005). It is known to damage integrity of cell membrane by binding to sulphydryl groups of membrane proteins and inducing lipid peroxidation through generation of reactive oxygen species (De Vos *et al*., 1992; Mashoudi *et al*., 1997; El-Tayeb *et al*., 2006). Evidences from several plant species reveal that Cu causes oxidative stress by modulating the activities of antioxidant enzymes (Gupta *et al*., 1999; Cuypers *et al*., 2000).

The antioxidant defense in plants constitutes two primary components-ascorbate and glutathione. These two antioxidant molecules interact with numerous other cellular components as efficient redox buffers, provide valuable information on cellular redox state and most importantly, regulate enzymatic processes in order to maximize defense against stresses (Noctor and Foyer, 1998). The balance between oxidized and reduced forms of both ascorbate and glutathione is crucial in modulating the reactive oxygen species antioxidant interactions as it ultimately determine plant growth and development (Noctor *et al*., 2002; De Pinto and De Gara, 2004; Talukdar, 2011c).

Canna indica L. (Cannaceae), commonly called 'Indian shot', is widely grown in India. Rhizome of *Canna*

indica is rich in high quality starch, and has been used in traditional folk medicines (Swarnkar and Katewa, 2008; Padal *et al*., 2010). The bright color of the flower makes it a valuable ornamental plant and a potential source for extraction of natural colorants for dyeing different types of textiles (Milow *et al*., 2010). A growing interest in *Canna* has been mainly attributed to its capacity for phytoremdiation in order to remove toxic heavy metals such as copper and zinc and to remove excess fertilizer and insecticides (Bose *et al*., 2008; Yadav *et al*., 2010; Mahamadi and Chapeyama, 2011). Extracts of *Canna indica* have a great potential as a source of natural antioxidant agent, which contain fairly high amount of flavonoid and phenolic compounds (Vankar and Srivastava, 2010). Much earlier, Debnath and Mukherjee (1982) observed noticeable effects of barium chloride on anthocyanin content of *Canna indica*, while effects of cadmium on photosynthetic efficiency of *Canna indica* were also reported (Cheng *et al*., 2002). These reports, however, are based on preliminary morpho-physiological parameters, and very little is known (Wu *et al*., 2007) about the intrinsic biochemical defense mechanism of this economically important plant against heavy metal-induced oxidative stress. Keeping this point in mind, the objectives of present investigation was to study the redox status of ascorbate and glutathione and the activities of antioxidant enzymes in the roots and leaves of *Canna indica* plants under imposed Cu-stress, and Cu accumulation.

Materials and Methods

Fresh and healthy seeds of bright red-flowered *Canna indica* L. were scarified and allowed to germinate at 25 °C for 16 days in a germinator by using towel paper as a substratum, following the protocol of Joshi and Pant (2010). Germinated seedlings were immediately transferred into 1l plastic pots containing Hoagland's nutrient solution with the addition of 5, 10, 30 and 50 μM $CuCl_2$ in separate sets for 9 d under controlled conditions (25 °C, light intensity of 300 μmol m^{-2} s^{-1} and 16 hr photoperiod). All tests were replicated (5 plants per replication) six times, using Cu-free nutrient solution (0 μM) as control. All the solutions were refreshed at an interval of every 3 days with pH adjusted to 5.5 ± 0.3. Tissues were harvested, thoroughly washed and stored at -20 °C for further analysis. Samples were oven-dried at 70 °C and dry weight of root was taken. For measurement of copper, after oven drying leaves and roots samples were digested on a sand bath with tri-acid mixture (HNO_3:H_2SO_4:$HClO_4$ 10 : 1 : 4 , by volume) to obtain a clear digest. The copper content was measured by the use of AAS (Perkin Elmer AA-400, Norwalk, USA).

The mature fully expanded leaves and roots were collected from six plants after 9 days of exposure. The experiments were performed at 0-4 °C, except mentioned otherwise. Leaf and root samples (1g each) were homogenized in an extraction medium containing 50 mM K-phosphate buffer pH 7.8, 0.1 mM EDTA, 2mM cysteine, 1% w/v PVP and 0.2% v/v Triton X-100. For ascorbate peroxidase activity, 20 mM ascorbate was added to the extraction buffer. The extracts were filtered through two layers of cheesecloth, and the homogenate was centrifuged at 14, 000 *g* for 20 min, at 4 °C. The supernatant fraction was filtered through a column containing 1 mL of Sephadex G-50 equilibrated with the same buffer used in homogenization. The hydrogen peroxide-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm with extinction constant 2.8 mM^{-1} cm^{-1} following the method of Nakano and Asada (1981). Superoxide dismutase activity was determined by the nitro-blue tetrazolium photochemical assay method as described by Beyer and Fridovich (1987). The reaction mixture (3ml) contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 0.1 mM EDTA, 2 μM riboflavin and 0.1 ml of enzyme extract. One unit of SOD was defined as the amount of protein causing a 50% NBT photoreduction. MDHAR activity was determined by following NADH oxidation at 340 nm (ε = 6.22 mM^{-1} cm^{-1}) for 90s (Dalton *et al*., 1986). Dehydroascorbate reductase was extracted with 50 mM K-phosphate buffer (pH 7.8), 1% PVP-10, 0.2mM EDTA and 10 mM β-mercaptoethanol, and its activity was determined following ascorbate formation at 265 nm (ε = 14.1 mM^{-1} cm^{-1}) for 3 min (Nakano and Asada, 1981). Glutathione reductase was extracted with the same medium as for DHAR but without β-mercaptoethanol and with 0.1% Triton X-100, and its activity was measured by monitoring glutathione-dependent oxidation of NADPH at 340 nm (ε = 6.22 mM^{-1} cm^{-1}) for 3 min (Dalton *et al*., 1986). Catalase was extracted in 50 mM K-phosphate buffer (pH 7.0) and 0.5% PVP-10, and its activity was assayed by measuring the reduction of H_2O_2 at 240 nm (ε = 39.4 M^{-1} cm^{-1}) for 1 min (Aebi, 1984). Reduced and oxidized form of ascorbate (AsA and DHA) and glutathione (GSH and GSSG) were measured from leaves and roots following the methods of Law *et al*. (1983) and Griffith (1985), respectively.

Lipid peroxidation was determined by measuring the malondialdehyde (MDA) content at 532 nm with extinction coefficient of 155 mM^{-1} cm^{-1} (Saher *et al*., 2004). Hydrogen peroxide (H_2O_2) content of leaves and roots was measured following the methodology described by Cheeseman (2006). Tissue samples were homogenized in the extraction medium 0.1 M K-phosphate (pH 6.4) supplemented with 5mM KCN. The assay mixture contained 250 μM ferrous ammonium sulphate, 100 μM sorbitol, 100 μM xylenol orange, and 1% ethanol in 25 mM H_2SO_4. Changes in absorbance were determined by the difference in absorbance between 550 and 800 nm, and H_2O_2 contents were calculated from a standard curve.

The results are presented as mean ± standard errors of six replicates. Statistical significance ($p<0.05$) between mean values was estimated by *t-test*.

Results and Discussion

Accumulation of Cu followed dose-dependent increase in 9-d-old *Canna* plants, and root accumulated significantly higher content than leaves (Table 1). Compared with control (0 µM Cu), treatment with 5 and 10 µM Cu induced a slight increase in root dry weight. However, root biomass accumulation remained at per control level at 30 µM, but had decreased marginally at 50 µM Cu treatments. In *Brassica juncea*, Cu caused significant inhibition of root growth at 4-16 µM (Wang *et al*., 2004), while Cu-induced necrotic lesions was reported in *Phaseolus vulgaris* leaves (Yurekli and Porgali, 2006). No such type of lesion was formed in the present *Canna indica* foliage, indicating that the proper sequestration of Cu at roots prevented its upward translocation as well as its accumulation at toxic concentration in leaves. The result is in agreement with earlier report on increasing metal accumulation in roots and its lesser translocation in shoots of *Canna indica* plants (Bose *et al*., 2008) and also, biosorption of Cu ions by dried roots of *Canna* (Mahamadi and Chapeyama, 2011). Accumulation of heavy metals in roots and limitations of its transfer to photosynthetic part/s is suggested to explain metal-tolerance in plants (Ozounidou, 1994; Talukdar, 2011a).

Both reduced and oxidized forms of ascorbate (AsA and DHA) and glutathione (GSH and GSSG) changed significantly from their control values in the leaves and roots of *Canna* plants, exposed to Cu treatment. Results in Table 1 indicates significant decrease in root AsA content and accumulation of DHA from 30 µM, resulting in unchanged level of total ascorbate, but considerable reduction in root ascorbate redox (AsA/ (AsA+DHA) pool. No significant change in ascorbate and glutathione content was observed in leaves. By contrast, reduced glutathione (GSH) pool in root had increased by about 2-fold upon imposition of Cu treatment, while its oxidized form, the glutathione disulfide or GSSG, remained unchanged. This led to increase in GSH redox (GSH/(GSH+GSSG) state in Cu-treated *Canna* roots, although both foliar and root GSH-level slipped marginally below control level at 50 µM $CuCl_2$ treatment.

Among the antioxidant enzymes studied here, activities of DHAR and GR increased significantly from 10 µM Cu, whereas levels of SOD, APX and CAT were as per control level in leaves of Cu-treated *Canna* plants (Table 2). In roots, significant increase in SOD, GR and CAT activities were noticed, while APX level was reduced. This was effective from 30 µM Cu (Table 2). Non-significant change, however, was observed in MDHAR activity throughout the treatment regimes. DHAR activity in root remained close to control value even at 50 µM Cu treatment. MDHAR, DHAR and GR are involved in recycling of reduced form of ascorbate and glutathione, respectively, and nearly 1.2-fold increase in DHAR accompanied with normal (control level) activity of MDHAR and >3-fold rise in GR activities in *Canna* leaves helped the plants to maintain AsA and GSH in adequate concentrations, respectively. This recycling capacity was also evidenced in Cu-treated *Canna* roots, where GR level increased by about 3-fold and DHAR activity was quite normal. However, as SOD activity increased at elevated Cu concentrations, there was a possibility of H_2O_2 accumulation in *Canna* tissues. Surprisingly, no over-accumulation of H_2O_2 was observed in Cu-treated *Canna* plants. With at least seven isoforms, APX is the most prolific H_2O_2-scavenging enzymes in plants, but it exclusively requires reduced ascorbate as a co-factor to function efficiently (Asada, 2006). Hossain and Asada (1984) reported that APX activity was greatly reduced in low ascorbate concentration due to rapid inactivation of chloroplast isoforms, particularly in the presence of H_2O_2. Excess H_2O_2 has the capacity to attack and inhibit APX (Cruz de Carvalho, 2008). In the present material, foliar APX activity was normal, but it reduced substantially in roots (Table 2). Despite normal MDHAR and DHAR activity, root ascorbate content gradually declined at elevated Cu-treatments. Presumably, lack of enough ascorbate led to decrease in APX level in *Canna* roots, as also observed in a novel AsA-deficient mutant of the hardy legume, *Lathyrus sativus* L. (Talukdar, 2012). Yet, the H_2O_2-level under Cu-treatment was similar to control in the present *Canna* roots. This utterly contrasting situation can be explained by the fact that CAT activity was enhanced by about 5-fold in the roots. CAT does not require any type of reducing power to catalyze H_2O_2 (Willekens *et al*., 1997), but its activity could be induced by the excess H_2O_2, functioning as a stable and diffusible signal (Polidoros and Scandalios, 1999). Certainly, up-regulation of CAT in the present *Canna* roots compensated the loss of APX function quite efficiently and prevented H_2O_2 accumulation to its toxic level. As soon as, its level decreased slightly at 50 µM Cu treatment, H_2O_2 became accumulated in roots. Normal functioning of both CAT and APX, however, is instrumental in lowering of foliar H_2O_2 level. Compensation of APX function by CAT was also reported in an AsA-deficient mutant of *Lathyrus sativus* L. (Talukdar, 2012).

Efficient scavenging of H_2O_2 in *Canna* plants was substantially evidenced by the level of MDA, an indicator of membrane lipid peroxidation. Increasing reports suggest that H_2O_2 level is well correlated with MDA content, and therefore, both these compounds are routinely used as the biochemical markers of oxidative stress (Bandeoglu *et al*., 2004; Cheeseman, 2006; Cruz de Carvalho, 2008; Talukdar, 2011b, c). In the present study, MDA content had not

Table 1 : Plant dry weight (g plant^{-1}), Cu (µg g^{-1} d. wt.), ascorbate and glutathione (nmol g^{-1} f. wt.) contents in leaves and roots of 9-day-old *Canna indica* exposed to $CuCl_2$ treatmemts

	$CuCl_2$ treatments (µM)				
Parameters[a]	**0**	**5**	**10**	**30**	**50**
Leaf dry weight	0.37 ± 0.1	0.38 ± 0.2	0.36 ± 0.11	0.33 ± 0.16	0.34 ± 0.15
Root dry weight	0.62 ± 0.1	0.69 ± 0.2	0.71 ± 0.09	0.63 ± 0.11	0.56 ± 0.08
Cu content (Leaf)	23.36± 0.3	27.5 ± 0.1	30.8 ± 0.07	37.93 ± 0.14	40.43 ± 0.21
Cu content (Root)	108± 0.09	115 ± 0.21	146.8± 0.2*	168.9 ± 0.3*	191 ± 0.19*
AsA (Leaf)	193 ± 0.12	187 ± 0.10	189.3 ± 0.12	188.4 ± 0.17	200.3 ± 0.13
AsA (Root)	135 ± 0.10	133 ± 0.17	131.5 ± 0.38	101.6 ± 0.2*	88.7 ± 0.65*
DHA (Leaf)	27.8± 0.7	33 ± 0.10	30.7 ± 0.11	30.0 ± 0.11	22.3 ± 0.07
DHA (Root)	24.7 ± 0.5	25 ± 0.21	27.8 ± 0.18	55.4 ± 0.26*	65.2 ± 0.18*
AsA-redox (Leaf)	0.870± 0.5	0.851± 0.7	0.860± 0.1	0.863± 0.9	0.890± 1.5
AsA-redox (Root)	0.840± 0.9	0.842± 1.0	0.823± 1.3	0.640± 1.0*	0.570± 0.9*
GSH (Leaf)	290 ± 1.2	293 ± 1.0	290.6 ± 1.8	288 ± 2.0	285.3 ± 2.2
GSH (Root)	307 ± 1.7	387 ± 2.0*	590 ± 1.7*	591.9 ± 3.2*	279.8 ± 4.7
GSSG (Leaf)	33.3± 0.08	37 ± 0.10	32.7 ± 0.11	35 ± 0.16	28.3± 0.28
GSSG (Root)	37 ± 0.10	35 ± 0.13	39 ± 0.17	32 ± 0.16	40.2 ± 0.20
GSH redox (Leaf)	0.890± 0.1	0.880± 0.9	0.890± 0.10	0.890± 0.11	0.900± 0.4
GSH redox (Root)	0.891± 0.3	0.940±0.1*	0.930± 0.7*	0.941± 0.5*	0.870± 0.10

[a]As A-reduced ascorbate, DHA-oxidized ascorbate, GSH-reduced glutathione, GSSG-oxidized glutathione; FW-fresh weight, DW-dry weight; Values are mean of six replicates ± SE* significantly different from control value

Table 2 : Antioxidant activities of SOD (Unit mg^{-1} protein) , APX, MDHAR, DHAR, GR and CAT (nmol mg^{-1} protein), H_20_2 accumulation (µ mol g^{-1} f. wt.) and MDA (nmol g^{-1} f. wt.) in leaves and roots of 9-day-old *Canna indica* exposed to $CuCl_2$ treatments

	$CuCl_2$ treatments (µM)				
Parameters[a]	**0**	**5**	**10**	**30**	**50**
SOD (Leaf)	109 ± 9.2	111 ± 7.3	110.2 ±9.0	104 ± 6.2	100.4 ± 10
SOD (Root)	129 ± 9.2	133 ± 9.2	130.3 ±9.2	159.8 ± 9.2*	169 ± 9.2*
APX (Leaf)	168 ± 7.7	170 ± 3.9	171 ± 3.8	173.8 ± 5.0	163.7 ± 11
APX (Root)	193.3± 6.1	182 ± 7.0	173 ± 9.3*	103 ± 8.5*	93 ± 5.5*
MDHAR (Leaf)	83.4 ± 2.0	80.3 ± 2.2	85.4 ± 2.8	83.8 ± 1.9	79.6 ± 3.1
MDHAR (Root)	77.7 ± 2.0	80.6 ± 2.8	76.9 ± 3.0	78.2 ± 1.7	78.0 ± 2.2
DHAR(Leaf)	138.1 ± 1.3	141 ± 1.7	160 ± 1.4*	158.6 ± 2.2*	153.3 ± 2.8*
DHAR (Root)	155.3 ± 1.5	153 ± 1.9	150 ± 2.6	158.2 ± 1.9	150.3 ± 1.7
GR (Leaf)	20.5 ± 0.3	29 ± 0.1	60.5± 0.9*	67.7 ± 1.3*	70.5 ± 1.3*
GR (Root)	27.5 ± 0.9	67 ± 1.1*	84.2± 1.9*	87.5 ± 2.2*	80.8 ± 3.0*
CAT (Leaf)	30.7 ± 0.9	29.3 ± 0.1	22 ± 0.5	29.6 ± 1.1	28.3 ± 1.3
CAT(Root)	41.3 ± 2.5	44.8 ± 1.9	206 ± 10*	210 ± 8.9*	176 ± 7.3*
H_2O_2 (Leaf)	2.43 ± 0.9	2.39 ± 1.1	2.37 ± 1.0	2.40 ± 0.9	2.49± 0.9
H_2O_2 (Root)	3.07 ± 0.8	3.10 ± 1.6	3.19 ± 1.7	3.18 ± 1.1	3.37 ± 1.5
MDA (Leaf)	2.83 ± 0.5	2.93 ± 1.5	3.01 ± 0.3	2.43 ± 0.8	2.69 ± 0.5
MDA (Root)	2.99± 1.2	3.17 ± 0.9	2.67 ± 0.9	3.35 ± 1.5	3.47 ± 1.9

[a] SOD-superoxide dismutase, APX-ascorbate peroxidase, MDHAR- monodehydroascorbate reductase, DHAR-dehydroascorbate reductase, GR-glutathione reductase, CAT-catalase, MDA-malondialdehyde. Values are mean of six replicates ± SE. * significantly different from control

increased even at high Cu-level (Table 2), permitting *Canna* plants to overcome Cu-induced membrane damage by H_2O_2-mediated oxidative stress.

In conclusion, the above results suggested that *Canna indica* L. has the ability to accumulate Cu at high concentration in its roots, preventing its accumulation above ground. In response to Cu-accumulation, it altered its antioxidant defense for efficient scavenging of Cu-induced reactive oxygen species through the processes of regeneration, compensation and over-expression of defense components. All these helped *Canna* plants to overcome Cu-induced oxidative damage and consequently, led to maintain normal growth, as suggested by quite normal dry weight in both tissues.

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