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Mitigation of Cu Toxicity by 28-homobrassinolide in *Zea mays* L.

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ABSTRACT

The effects of 28-homobrassinolide (28-homoBL) on lipid peroxidation and antioxidative enzyme activities in the *Zea mays* L. (var. Partap-1) seedlings exposed to copper (Cu) were studied. The surface-sterilized seeds of *Z. mays* were treated with different concentrations of Cu (0.5, 1.0, 1.5 and 2.0 mM) alone or in combination with 28-homoBL (10^{-4}, 10^{-6} and 10^{-8} mM) for 7 days. The activities of antioxidative enzymes (superoxide dismutase (EC 1.15.1.1), catalase (EC 1.11.1.6), ascorbate peroxidase (EC 1.11.1.11), guaiacol peroxidase (EC 1.11.1.7) and glutathione reductase (EC 1.6.4.2)), protein and malondialdehyde (MDA) content were analyzed in 7-days-old seedlings. The activities of superoxide dismutase and guaiacol peroxide were enhanced whereas the activities of other enzymes declined with an increase in the concentration of Cu alone. However the 28-homoBL treatments further stimulated the activities of all antioxidative enzymes. The level of MDA content decreased under brassinoloide treatments but increased under the influence of metal treatments.

Keywords: antioxidative enzymes, copper, lipid peroxidation, maize
Abbreviations: 28-HomoBL, 28-homobrassinolide; **APOX**, ascorbate peroxidase; **BR**, brassinosteroid; **CAT**, catalase; **Cu**, copper; **GR**, glutathione reductase; **MDA**, malondialdehyde; **POD**, guaiacol peroxidase; **ROS**, reactive oxygen species; **SOD**, superoxide dismutase

INTRODUCTION

Transition metals like Cr, Cu, Ni, Mo, Zn and Mn, etc. are essential micronutrients for growth and development of plants. These metals are key components of many proteins and enzymes (Hall and Williams 2003). Copper (Cu) is an integral constituent of plastocyanin, cytochrome *c* oxidase, copper/zinc superoxide dismutase and an ethylene receptor for apoplastic oxidases (Lolkema and Vooijs 1986; Raven *et al.* 1999; Pilon *et al.* 2006). Excess or deficiency of Cu causes abnormalities in the plant system. A number of deficiency symptoms including retarded growth are often observed in plants (Marschner 1995). However, excess Cu causes interveinal foliar chlorosis by a Cu-induced iron deficiency and predisposes photosystem II to photoinhibition (Pätsikkä *et al.* 2002). The toxic levels of Cu in plants also cause oxidative injury by stimulating the formation of reactive oxygen species (ROS) such as superoxide radicals (O_2^-•), singlet oxygen (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH•) (Schützendübel and Polle 2002). The scavenging system controlling ROS comprises both non-enzymatic antioxidants (e.g. glutathione, ascorbate, tocopherols, carotenoids and proline) and an enzymatic antioxidative system (e.g. superoxide dismutase (SOD), guaiacol peroxidase (POD), ascorbate peroxidase (APOX), catalase (CAT) and glutathione reductase (GR)) (Elstner 1982).

Various plant metabolites, including growth regulators, are involved in protection against oxidative stress generated by ROS. Plant hormones like abscisic and salicylic acid, ethylene, auxins and brassinosteroids (BRs) have been reported to play a significant role in protection against stress (Cao *et al.* 2005). BRs, a new class of polyhydroxysteroidal phytohormones, have been found to influence a wide spectrum of physiological processes in plants (Khripach *et al.* 2000). In addition to their role in plant growth and development, BRs have been reported to ameliorate various abiotic/biotic stresses (Krishna 2003; Kagle *et al.* 2007). The exogenous application of BRs improved the antioxidant system in plants subjected to salt stress (Nuñez *et al.* 2003; Özdemir *et al.* 2004) and heavy metal stress (Ali *et al.* 2007; Hayat *et al.* 2007). Our earlier studies also indicated stress-protective properties of BRs (Sharma and Bhardwaj 2007; Sharma *et al.* 2007; Bhardwaj *et al.* 2008) in *Brassica juncea* plants. In continuation of our previous findings, the present work aimed to study the stress-protective properties of 28-homobrassinolide (28-homoBL) in the seedlings of *Zea mays* L. subjected to Cu metal-stress by analyzing their antioxidative enzymatic system, protein content and extent of lipid peroxidation.

MATERIALS AND METHODS

Seeds of *Zea mays* L. (var. Partap-1) were procured from Punjab Agricultural University, Punjab, India. Healthy and uniform-sized seeds of maize were surface sterilized with 0.05% mercuric chloride for 5 min followed by repeated rinses in sterile distilled water. The surface sterilized seeds were germinated on Whatman No. 1 filter paper lined glass Petri dishes (10 cm diameter, 10 seeds per dish) containing different concentrations of Cu (0.5, 1.0, 1.5 and 2.0 mM) only, 28-homoBL (10^{-4}, 10^{-6} and 10^{-8} mM) alone and various concentrations of Cu supplemented with different concentrations of 28-homoBL (Sigma-Aldrich, New Delhi, India). The Cu metal treatment was given in the form of $CuSO_4$•$5H_2O$ (CDH, New Delhi, India). Each Petri dish was supplied with 4 ml of test solution on the first day and 2 ml of test solution on alternate days, up to 7 days. Control seedlings were supplied with distilled water. Each treatment was replicated 5 times. The experiment was conducted under controlled conditions (25 ± 0.5°C, 16 h photoperiod, 110 µmol m^{-2} s^{-1} light intensity). On the 7th day, seedlings were harvested and shoots and roots of seedlings were separated. The experiment was repeated twice.

Biochemical analysis was carried out in the leaves of 7-days-old seedlings. The leaf extracts were prepared to estimate the activities of antioxidative enzymes and the protein content by homogenizing 1 g leaves of 7-days-old seedlings in 3 ml of ice-cold 100 mM potassium phosphate buffer (pH = 7). The homogenate was centrifuged at 15,000 × *g* for 20 min at 4°C. The supernatant was used to estimate the activities of SOD, POD, CAT, GR and APOX

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and protein content. The activity of SOD was determined by monitoring its ability to inhibit photochemical reduction of nitrobluetetrazolium (NBT) (HiMedia, Mumbai, India) at 540 nm (Kono 1978). POD activity was determined according to Putter (1974). CAT activity was determined by following the initial rate of disappearance of H_2O_2 (Qualigens, Mumbai, India) at 240 nm (Aebi 1983). The activities of APOX and GR were measured by the methods of Nakano and Asada (1981) and Carlberg and Mannervik (1975), respectively. Protein content was determined following the method of Lowry *et al.* (1951) using bovine serum albumin (HiMedia) as a standard. The level of lipid peroxidation was determined as the content of malondialdehyde (MDA) using leaf homogenates prepared in 0.1% trichloroacetic acid (TCA) (Thomas Baker, Mumbai, India) and thiobarbituric acid (TBA) (Loba Chemie, Mumbai, India) as described by Heath and Packer (1968).

Data was analyzed statistically by using one-way analysis of variance (ANOVA) and comparisons with p values ≤0.05 were considered significantly different. Data was presented as mean ± SE.

RESULTS

The studies done on biochemical parameters revealed significant effects of BR treatments. Seedlings treated with 28-homoBL alone showed an increase in soluble protein content and a decrease in MDA content in comparison to untreated seedlings. The treatment of seedlings with 10^{-4} mM of 28-HomoBL resulted in maximum protein content (28.74 ± 1.15 mg g^{-1} FW) when compared to the control (16.33 ± 0.50 mg g^{-1} FW) while 10^{-6} mM of 28-homoBL caused the maximum decrease in MDA content (2.79 ± 0.11 μmol g^{-1} FW) of seedlings when compared to untreated seedlings (3.78 ± 0.07 μmol g^{-1} FW). The protein content of seedlings under heavy metal stress was found to increase significantly in all treatments of 28- homoBL. Maximum protein content (35 ± 1.474 mg g^{-1} FW) was observed in seedlings treated with 10^{-6} mM of 28-homoBL supplemented with 1.5 mM of Cu compared to only 1.5 mM Cu-treated seedlings (31.97 ± 1.293 mg g^{-1} FW). The concentration of MDA increased with Cu treatments but decreased when 28-homoBL was

Table 1 Effect of 28-homoBL on protein content, malondialdehyde (MDA) content and specific activities of antioxidative enzymes (superoxide dismutase (SOD), guaiacol peroxidase (POD), catalase (CAT), ascorbate peroxidase (APOX) and glutathione reductase (GR) of 7-days old *Zea mays* seedlings. Mean ± SE.

Treatments of 28-homoBL	Protein content (mg g^{-1} FW)	MDA content (μmol g^{-1} FW)	SOD (mol U mg^{-1} protein)	POD (mmol U mg^{-1} protein)	CAT (mol U mg^{-1} protein)	APOX (mmol U mg^{-1} protein)	GR (mmol U mg^{-1} protein)
0	16.33 ± 0.50	3.78 ± 0.07	1.68 ± 0.014	4.03 ± 0.095	1.04 ± 0.07	1.28 ± 0.05	2.850 ± 0.11
10^{-4} mM	28.74 ± 1.15	2.99 ± 0.09	2.03 ± 0.059	4.14 ± 0.15	1.34 ± 0.05	1.41 ± 0.042	1.029 ± 0.006
10^{-6} mM	28.18 ± 1.09	2.79 ± 0.11	1.83 ± 0.017	4.46 ± 0.10	2.10 ± 0.11	1.64 ± 0.032	1.082 ± 0.005
10^{-8} mM	24.92 ± 0.56	3.18 ± 0.01	2.48 ± 0.032	6.59 ± 0.18	4.25 ± 0.20	2.84 ± 0.062	1.158 ± 0.011
F-ratio	42.38 (3.98)*	25.91 (0.37)*	96.13 (0.15)*	35.84 (0.96)*	149.9 (0.53)*	212.4 (0.22)*	237.6 (0.25)*

*Indicate significant at p≤0.05 (Inside bracket is HSD value)

Table 2 Effect of 28-homoBL on protein content, malondialdehyde (MDA) content and specific activities of antioxidative enzymes (superoxide dismutase (SOD), guaiacol peroxidase (POD), catalase (CAT), ascorbate peroxidase (APOX) and glutathione reductase (GR) of 7-days old Cu-stressed *Zea mays* seedlings. Mean ± SE.

Treatments	Protein content (mg g^{-1} FW)	MDA content (μmol g^{-1} FW)	SOD (mol U mg^{-1} protein)	POD (mmol U mg^{-1} protein)	CAT (mol U mg^{-1} protein)	APOX (mmol U mg^{-1} protein)	GR (mmol U mg^{-1} protein)
Cu (0.5 mM)	24.48 ± 1.206	3.19 ± 0.128	1.20 ± 0.018	5.06 ± 0.057	3.02 ± 0.133	1.49 ± 0.045	1.57 ± 0.009
Cu (0.5 mM) + 28-homoBL (10^{-4} mM)	31.68 ± 0.657	2.65 ± 0.074	1.78 ± 0.073	6.96 ± 0.151	3.29 ± 0.116	1.56 ± 0.035	1.66 ± 0.009
Cu (0.5 mM) + 28-homoBL (10^{-6} mM)	26.11 ± 0.761	2.81 ± 0.105	2.34 ± 0.044	6.59 ± 0.28	4.19 ± 0.205	1.73 ± 0.080	2.47 ± 0.041
Cu (0.5 mM) + 28-homoBL (10^{-8} mM)	28.09 ± 0.935	2.81 ± 0.133	1.69 ± 0.006	5.91 ± 0.25	3.21 ± 0.126	1.50 ± 0.075	1.61 ± 0.074
F-ratio	1.52 (4.139)	4.01 (0.509)	114.3 (0.19)*	16.36 (0.93)*	11.62 (0.68)*	3.16 (0.27)	101.53 (0.19)*
Cu (1.0 mM)	29.83 ± 0.727	3.43 ± 0.108	1.43 ± 0.062	5.46 ± 0.095	2.41 ± 0.104	1.26 ± 0.053	1.38 ± 0.04
Cu (1.0 mM) + 28-homoBL (10^{-4} mM)	29.49 ± 0.828	2.46 ± 0.116	1.96 ± 0.092	7.31 ± 0.27	2.63 ± 0.118	1.69 ± 0.076	1.63 ± 0.052
Cu (1.0 mM) + 28-homoBL (10^{-6} mM)	30.39 ± 1.034	2.46 ± 0.054	1.73 ± 0.039	6.05 ± 0.257	2.32 ± 0.063	1.49 ± 0.035	1.41 ± 0.031
Cu (1.0 mM) + 28-homoBL (10^{-8} mM)	29.96 ± 1.23	2.33 ± 0.073	1.94 ± 0.078	5.87 ± 0.093	3.12 ± 0.136	1.55 ± 0.047	1.39 ± 0.011
F-ratio	0.145 (4.412)	31.243 (0.41)*	12.22 (0.32) *	16.02 (0.9) *	10.79 (0.49)*	10.44 (0.24)*	10.09 (0.167)*
Cu (1.5 mM)	31.97 ± 1.293	3.92 ± 0.093	1.66 ± 0.045	6.68 ± 0.081	2.06 ± 0.116	0.94 ± 0.043	1.31 ± 0.031
Cu (1.5 mM) + 28-homoBL (10^{-4} mM)	32.18 ± 1.16	2.16 ± 0.122	1.90 ± 0.046	7.45 ± 0.061	2.55 ± 0.078	1.34 ± 0.025	1.44 ± 0.029
Cu (1.5 mM) + 28-homoBL (10^{-6} mM)	35.00 ± 1.474	2.23 ± 0.043	1.90 ± 0.056	7.04 ± 0.171	2.12 ± 0.067	1.65 ± 0.071	1.41 ± 0.01
Cu (1.5 mM) + 28-homoBL (10^{-8} mM)	32.79 ± 0.278	3.05 ± 0.064	2.07 ± 0.084	7.04 ± 0.122	3.01 ± 0.11	1.26 ± 0.055	1.45 ± 0.056
F-ratio	1.45 (5.205)	93.55 (0.38)*	8.04 (0.27)*	7.27 (0.52)*	21.36 (0.43)*	32.74 (0.23)*	3.18 (0.159)
Cu (2.0 mM)	26.45 ± 0.123	4.29 ± 0.124	1.96 ± 0.05	7.97 ± 0.151	1.53 ± 0.042	0.78 ± 0.04	1.03 ± 0.047
Cu (2.0 mM) + 28-homoBL (10^{-4} mM)	27.87 ± 0.476	2.49 ± 0.103	2.05 ± 0.077	8.05 ± 0.161	3.84 ± 0.094	1.15 ± 0.026	1.33 ± 0.019
Cu (2.0 mM) + 28-homoBL (10^{-6} mM)	29.49 ± 1.17	3.06 ± 0.111	2.11 ± 0.078	7.97 ± 0.134	4.36 ± 0.07	1.86 ± 0.089	1.80 ± 0.029
Cu (2.0 mM) + 28-homoBL (10^{-8} mM)	29.83 ± 0.965	3.05 ± 0.162	2.47 ± 0.023	9.51 ± 0.404	3.69 ± 0.106	1.52 ± 0.082	1.95 ± 0.05
F-ratio	4.28 (3.73)*	35.89 (0.57) *	13.44 (0.27)*	5.63 (1.44)*	233.9 (0.36)*	51.95 (0.29)*	107.35 (0.18)*

Values presented as means ± SE.
*Indicate significant at p≤0.05 (Inside bracket is HSD value)

supplemented. Minimum content of MDA (2.16 ± 0.122 μmol g^{-1} FW) was observed in 10^{-4} mM 28-homoBL supplemented with 1.5 mM of Cu solution compared to 1.5 mM Cu-treated seedlings (3.92 ± 0.093 μmol g^{-1} FW) (**Tables 1, 2**).

The activities of antioxidative enzymes (SOD, POD, CAT and APOX) were enhanced in maize seedlings treated with 28-homoBL alone, 10^{-8} mM being the most effective concentration. The activities of SOD and POD increased as the concentration of Cu increased. Maximum activities of SOD (1.96 ± 0.05 mol U mg^{-1} protein) and POD (7.97 ± 0.151 mmol U mg^{-1} protein) was observed in seedlings treated with 2 mM of Cu. In maize seedlings the activities of SOD and POD were further enhanced by the application of 28-homoBL-supplemented Cu solution to combat the oxidative stress generated by Cu. The maximum enhancement in activities of SOD (2.47 ± 0.023 mol U mg^{-1} protein) and POD (9.507 ± 0.404 mmol U mg^{-1} protein) was observed in seedlings treated with 10^{-8} mM 28-homoBL supplemented with 2.0 mM of Cu solution. Under Cu stress, the activities of CAT, APOX and GR decreased as the concentration of Cu increased. Minimum activities of these enzymes were observed in seedlings treated with the most toxic concentration of Cu (2 mM). But the application of 28-homoBL supplemented Cu solution to seedlings enhanced the activities of these antioxidative enzymes. Maximum enhancement in activities of CAT (4.36 ± 0.07 mol U mg^{-1} protein) and APOX (1.86 ± 0.089 mmol U mg^{-1} protein) was observed in seedlings treated with 10^{-6} mM 28-homoBL supplemented with 2.0 mM of Cu solution. Further, maximum enhancement in activity of GR (2.47 ± 0.041 mmol U mg^{-1} protein) was observed in seedlings treated with 10^{-6} mM 28-homoBL supplemented with 0.5 mM of Cu solution (**Tables 1, 2**).

DISCUSSION

In the present study we observed that the application of 28-homoBL to Cu-stressed maize seedlings significantly enhanced the tolerance to oxidative stress generated by Cu. The treatment of 28-homoBL supplemented Cu solution to maize seedlings resulted in enhanced activities of antioxidative enzymes and protein content. It was further observed that the application of 28-homoBL supplemented Cu solution caused a decline in the MDA content which was otherwise enhanced under the heavy metal stress, thereby protecting maize seedlings from membrane disintegration.

Various researchers (Halliwell and Gutteridge 1984; Panda *et al.* 2003; Yruela 2005) reported that Cu induced the formation of ROS in plants via Haber-Weiss or Fenton type reactions. Hence the presence of excess Cu can cause oxidative damage in plants and subsequently increased the activities of antioxidative enzymes and antioxidants. Some of the important constituents of the antioxidative pathway, which were affected by excess of Cu, include APOX, monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), GR, SOD and POD. The ascorbate and glutathione cycle has been reported to be involved in response to excess of Cu (Devos *et al.* 1991; Wang *et al.* 2004).

Among ROS, superoxide radical ($O_2^{\bullet -}$) is dismutated by SOD into H_2O_2 and is further removed by CAT in the peroxisomes or by APOX of the ascorbate-glutathione antioxidant cycle in the chloroplast or by membrane bounded POD (Foyer *et al.* 1997). GR activity maintains the pool of glutathione in a reduced state, which in turn reduces dehydroscorbate to ascorbate through the ascorbate-glutathione cycle (Noctor and Foyer 1998). It is widely accepted that ROS are responsible for various stress-induced damages to macromolecules and lipid peroxidation (Halliwell and Gutteridge 1999). Consequently, the role of antioxidative enzymes, such as SOD, CAT, POD, GR and APOX becomes very important.

In our study we observed that the application of 28-homoBL to Cu-stressed seedlings improved stress tolerance by further boosting their antioxidative defence system (**Tables 1, 2**). Earlier studies also revealed the stress-ameliorative properties of BRs (24-epibrassinolide and 28-homoBL) against heavy metals, salt, thermal and fungal stress, when applied at nM-μM concentration (Khripach *et al.* 2000; Haubrick and Assmann 2006). Application of 24-epibrassinolide and 28-homoBL (3 μM) reduced the salt stress by enhancing the chlorophyll content, activity of nitrate reductase and by increasing the rate of seed germination (Anuradha and Rao 2001, 2003). Zhang *et al*. (2007) reported the salt stress-ameliorative properties of BR (5 μM/L) in *Medicago sativa* L. by increasing the germination percentage, fresh weight and activities of antioxidative enzymes (POD, SOD and CAT). BRs have been reported to confer tolerance against heavy metals either by reducing their uptake or by activating the antioxidative enzymes in *B. juncea*, *B. campestris* and *Zea mays* (Bhardwaj *et al.* 2007; Sharma and Bhardwaj 2007; Sharma *et al.* 2007). In mungbean seedlings BR (brassinolide, 0.1 ng L^{-1}) ameliorated Al toxicity and promoted the growth of seedlings (Bilkisu *et al.* 2003). 28-HomoBL also ameliorated the cadmium toxicity in *B. juncea* and *Cicer arietinum* plants by increasing the activities of POD, CAT and SOD (Hasan *et al.* 2007; Hayat *et al.* 2007).

28-homoBL also improved seedling growth by increasing the protein content and by decreasing the level of lipid peroxidation (**Tables 1, 2**), which may be due to the regulation of transcription and translation processes of specific genes related to stress tolerance by BRs. Kagale *et al.* (2007) reported that 24-epiBL-treated *Arabidopsis thaliana* seedlings tolerated drought stress by accumulating *rd29A*, *ERD10* and *rd22* mRNAs at a higher level. The increase in the transcript levels of these genes suggests that 24-epiBL-treated *A. thaliana* seedlings tolerate drought stress better than untreated seedlings. Further Sam *et al.* (2001) observed in tomato leaf discs that BB6 (BL analogue with spirostanic structure as active ingredient) increased the rate of production of heat shock proteins, which protected mRNA from stress-induced degradation. As membrane destruction results from ROS-induced oxidative damage (Mittler 2002), the 28-homoBL-treated seedlings might be scavenging ROS more effectively by activated antioxidative enzymes than those treated with Cu (0.5-2 mM) alone. The observations are consistence with those of Özdemir *et al.* (2004), who reported that the level of lipid peroxidation induced by NaCl was significantly lower in rice seedlings when treated with 24-epiBL.

The present study therefore reveals the stress ameliorative properties of 28-homoBL in maize seedlings exposed to heavy metal (Cu) stress by decreasing the extent of lipid peroxidation and by further stimulating the activities of key antioxidative enzymes. So the application of BRs at an appropriate concentration and at an appropriate stage of plant development helps to mitigate oxidative stress generated by heavy metals. This study further strongly recommended the application of BRs to maize plants under heavy metals stress to improve their yield and stress tolerance.

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