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Effect of 24-Epibrassinolide on Polyamine Titers, Antioxidative Enzyme Activities, and Seedling Growth of *Raphanus sativus* L. under Copper Stress

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ABSTRACT

In the present investigation, exogenous application of 24-epibrassinolide (24-epiBL) to *Raphanus sativus* L. cv. 'Pusa chetki' seedlings, under copper (Cu) stress showed the synthesis of various polyamines (PAs). Cu metal treatment alone enhanced production of putrescine (Put), spermidine (Spd) and spermine (Spm) significantly over control values. However when metal treatment was supplemented with different concentrations of 24-epiBL, total PA content showed a significant decrease under Cu stress. Put/Cadaverine (Cad) ratio showed maximum rise when seedlings were treated with 10^{-7} M 24-epiBL alone whereas a maximum increase in the Put/Spd ratio was found in 10^{-11} M 24-epiBL treatment alone and the minimum ratio was recorded in Cu and 10^{-9} M 24-epiBL combination treatment. Besides these, 24-epiBL also altered the activities of peroxidase, catalase and superoxide dismutase. The shoot and root growth reduced by Cu metal treatment was restored to normal values by 24-epiBL.

Keywords: antioxidative enzymes, 24-epibrassinolide (24-epiBL), polyamine (PA)

Abbreviations: BR, brassinosteriods; **Cad**, cadaverine; **24-epiBL**, 24-epibrassinolide; **PA**, polyamine; **Put**, putrescine; **Spd**, spermidine; **Spm**, spermine

INTRODUCTION

Brassinosteriods (BRs) are a recently explored group of phytohormones which occur ubiquitously throughout the plant kingdom. They have been reported to play an essential role in plant growth and development, cell elongation, cell division, vascular differentiation and reproductive development (Sasse 2003; Clouse 2008). They confer resistance to plants against various biotic and abiotic stresses (Haubrick and Assmann 2006; Jagger *et al*. 2008). These steroids were reported to improve plant resistance to various environmental stresses like salinity (Ali *et al*. 2007), thermal stress (Kurepin *et al*. 2008), and heavy metal stress (Almeida *et al*. 2005; Hayat *et al*. 2007). However, the exact mode of BRs' action is yet to be explored, to study whether BRs are implicated in the modulation of plant responses to oxidative stresses by stimulating activities of antioxidative enzymes (Hayat *et al.* 2007) or by interacting with other phytohormones like auxins (Halliday 2004), gibberellins (Jagger *et al.* 2005), cytokinins and ethylene (Arteca and Arteca 2008) and polyamines (PAs) (Pang *et al.* 2007; Tassoni *et al.* 2008).

PAs like Put, Cad, Spd and Spm are low molecular weight organic cations, found in a wide range of organisms from bacteria to plants and animals (Kuznetsov *et al* 2007; Pang *et al.* 2007; Tassoni *et al*. 2008). They have been reported to be actively involved in the regulation of a large number of physiological processes like embryogenesis, cell division, morphogenesis and development (Groppa and Benavides 2008; Kumar *et al*. 2008). They are now accepted as an important component of plant stress responses (Ruben *et al*. 2006; Groppa and Benavides 2008). The changes in the endogenous titers of PAs have been associated with the retardation of senescence, osmosis and salinity (Pirintsos *et al*. 2004; Tang *et al*. 2005; Shevyakova *et al*. 2006; Liu *et al*. 2007; Wen *et al*. 2008) and heavy metal stresses (Shevyakova *et al*. 2006; Liu and Moriguchi 2007; Zapata *et al*. 2008; Kuznetsov *et al.* 2007). They also have a strong capacity for scavenging free radicals and reactive oxygen species generated by various stresses, thus proving their strong antioxidant nature (Ha *et al.* 1998).

Heavy metals are major environmental contaminants in soils and water habitats as their uptake and accumulation in plants might introduce them into the food chain (Chary *et al.* 2008). Among these heavy metals, copper (Cu) is widely distributed in nature and is an indispensable element required for normal plant growth. However, higher concentrations of Cu lead to phytotoxicity due to its pleiotropic implication in numerous physiological activities (Kopittke *et al.* 2007). Cu toxicity is mediated by the formation of free radicals (Luna *et al.* 1994) and by the catalysis of the Haber-Weiss reaction (Halliwel and Guteridge 1984).

Since PA synthesis is associated with various environmental stresses, including heavy metals, and BRs are also known to protect plants against heavy metal stress,therefore the present study observes the effects of BRs (if any) on the synthesis of PAs in *Raphanus sativus* L. plants (a model plant for studying BR bioactivity) under Cu metal stress.

MATERIALS AND METHODS

Study material

The study material included seeds and seedlings of *Raphanus sativus* L. cv. 'Pusa chetki'. Seeds were procured from Punjab Agriculture University, Ludhiana, India.

Raising seedlings

Seeds were surface sterilized with 0.01% $HgCl_2$ and then rinsed with distilled water 3-4 times. Seeds were grown in autoclaved

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Petri dishes lined with Whatman No. 1 filter paper at 20-25°C with a 16-h photoperiod under fluorescent white light (175 µmol/m^2/s) in a controlled environmental growth chamber.

Metal treatments

Seeds were treated with different concentrations of 24-epiBL (10^{-7}, 10^{-9} and 10^{-11} M) and 0.2 mM of Cu metal alone and in combination with 24-epiBL (10^{-7}, 10^{-9} and 10^{-11} M). Controls were raised in distilled water only.

On the seventh day, seedlings were harvested (3-3.5 cm long hypocotyls). The concentration of Cu used was selected on the basis of Inhibitory concentration (IC_{50}).

Isolation and characterization of polyamines

The isolation and characterization of PAs was carried out by following the method proposed by Fontaniella *et al.* (2001). 2 grams (fw) of seedlings given treatment of 0.2 mM Cu-metal and untreated control seedlings were homogenized in pre-chilled pestle and mortar with 5% perchloric acid (PCA) (v/v) and left to stand at 4°C for 1 hr. The homogenized extracts were centrifuged at 15,000 r.p.m. for 25 min at 4°C. Supernatants were taken as a source of free PAs and these were dried *in vacuo* at 45°C. The residues were redissolved in 5% PCA (v/v) and stirred with insoluble polyvinylpolypyrollidone (PVPP) at a ratio of 50 mg/ml for the removal of polyphenolics and other impurities. The resulting solutions were filtered through Whatman No. 1 filter paper and then subjected to derivatization by dansyl chloride.

For derivatization, vials (5 ml) previously adjusted to pH 8.0 (using NaOH solution) were used for the derivatization reaction. The reaction mixture consisting of 1 ml of sample, 1 ml of saturated Na_2CO_3 and 1 ml of dansyl chloride (75 mM in acetone) were mixed vigorously and kept at room temperature for 16 hrs. Dansylated PAs were extracted with 3 × 3.0 ml of benzene (HPLC grade) after vortexing for 1 min. Benzene phase was dried at 40°C under a stream of air. Once the derivatization process was over, samples were cleaned by adding 1.2 ml of 5 mM KOH in methanol (HPLC grade) as described by Seiler and Knodgen (1979). Mixtures were left to stand for 1 hr at 45°C and then treated with an aqueous mixture of 300 mg of KH_2PO_4 and 300 mg of Na_2HPO_4. PAs were extracted with 3 × 3.0 ml of benzene (HPLC grade) and the benzene phase was dried. The residue was redissolved in 1 ml of methanol (HPLC grade) for the characterization and quantification of PAs (putrescine (Put), cadaverine (Cad), spermidine (Spd) and spermine (Spm), purchased from Sigma Aldrich Ltd.) by reverse phase HPLC.

Analysis of polyamines

PAs (Put, Cad, Spd and Spm) were analyzed by a Waters 515 Chromatograph HPLC equipped with Waters (717) Plus Autosampler and Photodiode Array Detector (2996). PAs were eluted in a RP-C-18 column (15 cm × 4 mm i.d.), (5 µM particle size) reversed phased column at 30°C using a methanol water gradient. Gradient elution was carried out using Methanol-Milli-Q water in a linear gradient from 50: 50 (v/v) to 80: 20 (v/v) for 30 min. The last proportion was maintained for 15 min until the end of the analysis. Flow rate of the mobile phase was maintained at 1 ml/min. The detection of PAs was carried out by measuring the fluorescence intensity of samples and comparisons were made with the peaks and retention times of standard PAs (Put, Cad, Spd and Spm) (**Figs. 1-3**).

Determination of protein contents and antioxidative enzyme activities

1. Preparation of plant extracts

For the estimation of protein content and activities of guaiacol peroxidase (GPOX), catalase (CAT) and superoxide dismutase (SOD), 2 g of plant tissue was homogenized in pre-chilled pestle and mortar with 6 ml of 100 mM potassium phosphate buffer (pH 7.0) under ice-cold conditions. The homogenate was centrifuged at 15,000 × *g* at 5°C for 20 min and the supernatants were used for determining protein content and enzyme activities.

2. Protein estimation

The protein content was determined by following Lowry's method (1951).

3. Guaiacol peroxidase (GPOX) (EC1.11.1.7) activity

Guaiacol peroxidase activity was determined by the method of Putter (1974). The reaction mixture consisted of 3 ml of phosphate buffer, 50 µl guaiacol solution, 100 µl of enzyme sample and 30 µl of H_2O_2 solution. The rate of formation of guaiacol dehydrogenation product (GDHP) was determined spectrophometrically at 436 nm.

4. Catalase (CAT) (EC 1.11.1.6) activity

Catalase activity was determined as per the method of Aebi (1983). The reaction mixture consisted 300 µl of enzyme extract, 1.5 ml of phosphate buffer and 1.2 ml of H_2O_2. Decrease in absorbance per min at 240 nm was recorded.

5. Superoxide dismutase (SOD) (EC 1.15.1.1) activity

Superoxide dismuatse activity was determined by the method of Kono (1978). In this method, a reaction mixture containing 1.9 ml of sodium carbonate buffer, 750 µl NBT and 150 µl Triton X-100 were placed in a test cuvette and the reaction was started by adding 150 µl hydroxylamine hydrochloride. After 2 min, 70 µl of enzyme extract was added. The percentage inhibition in the reduction rate of nitroblue tetrazolium (NBT) was recorded with increase in absorbance at 540 nm.

Seedling growth

The effects of Cu on 7-days old *Raphanus* seedlings were determined. The growth was assessed in the terms of shoot length and root length. Percent germination was calculated for the treated and untreated seeds.

Experimental data

All the experiments were performed in triplicates. The data shown are the means of three replicate experiments along with standard error (n=3).

RESULTS

Endogenous titers of polyamines

The investigation recorded the presence of Put (38.25 µg g^{-1} fw), Cad (57.24 µg g^{-1} fw), and Spd (184.92 µg g^{-1} fw) in control treatments, whereas Spm was not detected in the controls. However, the presence of Put (52.84 µg g^{-1} fw), Cad (5.96 µg g^{-1} fw), Spd (404.99 µg g^{-1} fw) and Spm (1288.75 µg g^{-1} fw) was reported in *Raphanus* seedlings given treatment of 0.2 mM Cu metal. Seedlings, given treatments of different concentrations of 24-epiBL (10^{-7}, 10^{-9} and 10^{-11} M) alone showed varied pattern in the endogenous contents of PAs, whereas Put quantity was significantly enhanced under 10^{-7} M epiBL treatment (105.26µg g^{-1} fw) as compared to control (38.25 µg g^{-1} fw). Similarly 10^{-9} (73.70 µg g^{-1} fw) and 10^{-11} (47.56 µg g^{-1} fw) M epiBL also showed increase in Put level but to a lesser extent than 10^{-7} M epiBL treatment. Combinations of 0.2 mM Cu-metal and 24-epiBL (10^{-7}, 10^{-9} and 10^{-11} M) showed decrease in the synthesis of Put level. Maximum decrease was observed with 0.2mM of Cu-metal and 10^{-11} M 24-epiBL (14.01 µg g^{-1} fw) over combinations of 10^{-9} M (44.72 µg g^{-1} fw) and 10^{-7} M (60.37 µg g^{-1} fw) 24-epiBL and Cu-metal respectively (**Table 1, Fig. 1**).

Cad level found under metal stress was 5.96 µg g^{-1} fw which was much lower than 57.24 µg g^{-1} fw observed in control. Cad content was decreased in seedlings given treat-

Table 1 Endogenous polyamine content (µg/g fw) in 7days old seedlings of *Raphanus* subjected to Cu-metal and 24-epiBL treatments.

Treatments	Putrescine	Cadaverine	Spermidine	Spermine	Total (µg/g fw)
Control	$38.25^{b} \pm 1.40$	$57.24^{b} \pm 2.1$	$184.92^{b} \pm 8.1$	n.d.	280.41
0.2 mM Cu	$52.84^{a} \pm 2.98$	$5.96^{a} \pm 0.021$	$404.99^{a} \pm 20.9$	1288.75 ± 25.9	1752.54
10^{-7} M epiBL	$105.0^{a,b} \pm 3.40$	$0.358^{a,b} \pm 0.02$	$7.21^{a,b} \pm 1.1$	n.d.	112.568
10^{-9} M epiBL	$73.70^{a} \pm 2.87$	$13.82^{a} \pm 0.98$	$63.25^{a,b} \pm 5.3$	n.d.	150.77
10^{-11} M epiBL	47.56 ± 2.09	$7.07^{a} \pm 0.87$	$193.56^{b} \pm 9.7$	n.d.	248.19
Cu + 10^{-7} M epiBL	$60.37^{a} \pm 3.90$	$8.65^{a} \pm 0.98$	$242.24^{a,b} \pm 13.6$	$2.98^{b} \pm 0.08$	314.24
Cu + 10^{-9} M epiBL	44.72 ± 2.67	$0.36^{a,b} \pm 0.03$	$7.30^{a,b} \pm 0.98$	$899.22^{b} \pm 10.5$	951.601
Cu + 10^{-11} M epiBL	$14.01^{a,b} \pm 1.45$	$2.24^{a} \pm 0.21$	$16.24^{a,b} \pm 1.78$	$1127.25^{b} \pm 20.9$	1159.74

Data presented as mean±SE

($^{a,\,b}$ indicate statistically significant differences from control and metal treatment at $p \leq 0.05$)

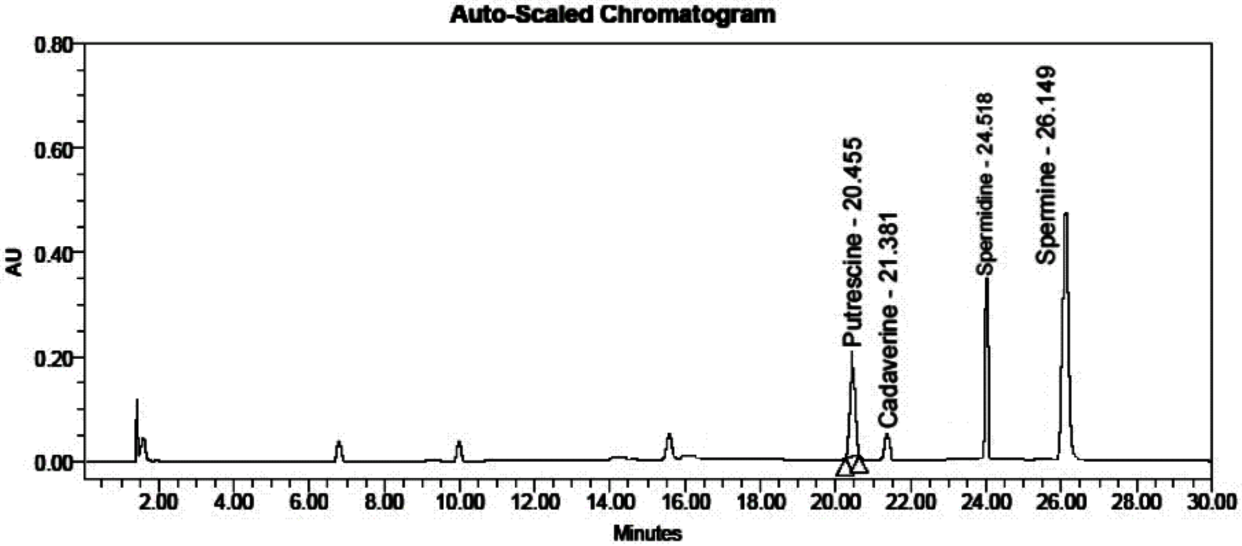

Fig. 1 HPLC chromatogram of a mixture of polyamine standards (putrescine, cadaverine, spermidine and spermine) characterized at 254 nm.

ment of 24-epiBL alone (10^{-7}, 10^{-9} and 10^{-11} M), more significantly at 10^{-7} M 24-epiBL (0.358 µg g^{-1} fw). A similar trend was recorded in seedlings, given combinations of Cu metal and 24-epiBL, with considerable decrease found in combination of Cu metal with 10^{-9} M 24-epiBL (0.361 µg g^{-1} fw).The Spd content got enhanced significantly in the seedlings given 0.2 mM Cu-metal treatment (404.99 µg g^{-1} fw) when compared with control (184 µg g^{-1} fw) (**Table 1, Fig. 2**). The treatments of 24-epiBL alone revealed reduction in Spd synthesis from 190.34 µg g^{-1} fw in case of 10^{-11} M 24-epiBL to 63.25 µg g^{-1} fw (10^{-9} M) and 7.21 µg g^{-1} fw in 10^{-7} M 24-epiBL concentration. The combinations of 0.2 mM Cu-metal with different concentrations of 24-epiBL indicated still lower content of Spd, significant reduction in Cu-metal with 10^{-9} (7.30 µg g^{-1} fw) and 10^{-11} (16.24 µg g^{-1} fw) M 24-epiBL was observed. However the combination of Cu metal with 10^{-7} M 24-epiBL showed an enhancement in Spd level to 242.24 µg g^{-1} fw (**Table 1, Fig. 3**).

The Spm which was not detected in control seedlings was reported in seedlings undergone Cu-metal stress, level of Spm found was 1288.75 µg g^{-1} fw. But no Spm was found in the seedlings given treatments of 24-epiBL alone (10^{-7}, 10^{-9} and 10^{-11} M). A decrease in Spm content was recorded in seedlings where Cu metal treatment was supplemented with 24-epiBL (10^{-7}, 10^{-9} and 10^{-11} M), this reduction was maximum (2.98 µg g^{-1} fw) in 10^{-7} M 24-epiBL over metal treatment alone.

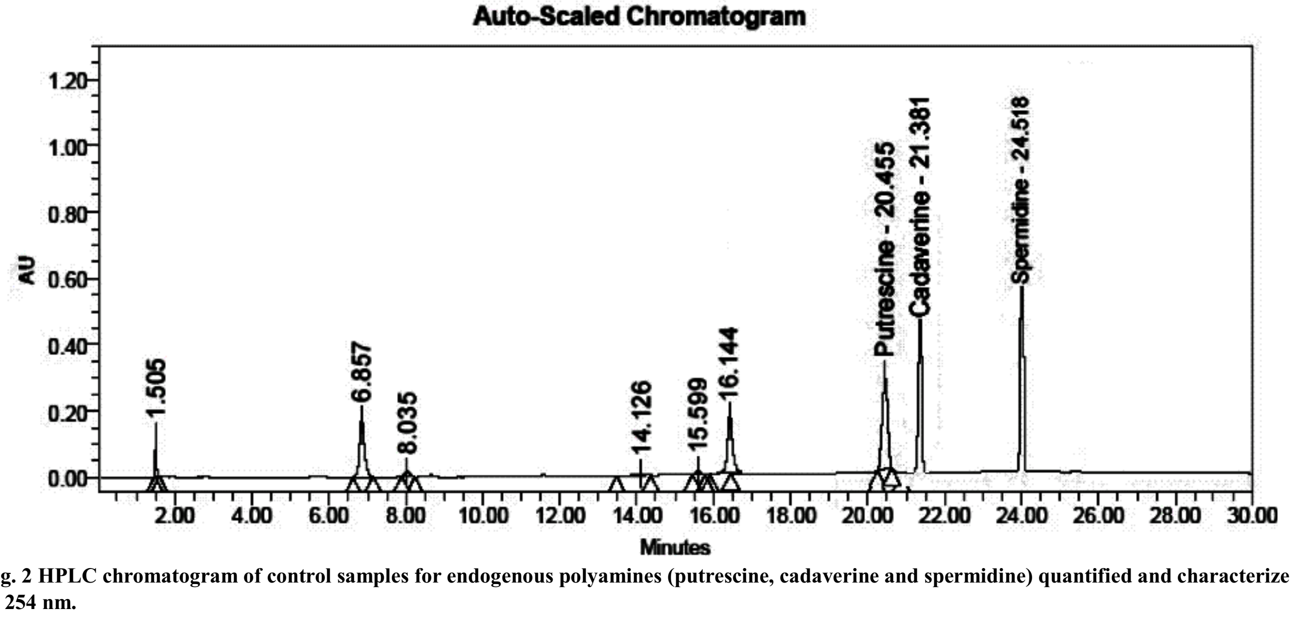

Fig. 2 HPLC chromatogram of control samples for endogenous polyamines (putrescine, cadaverine and spermidine) quantified and characterized at 254 nm.

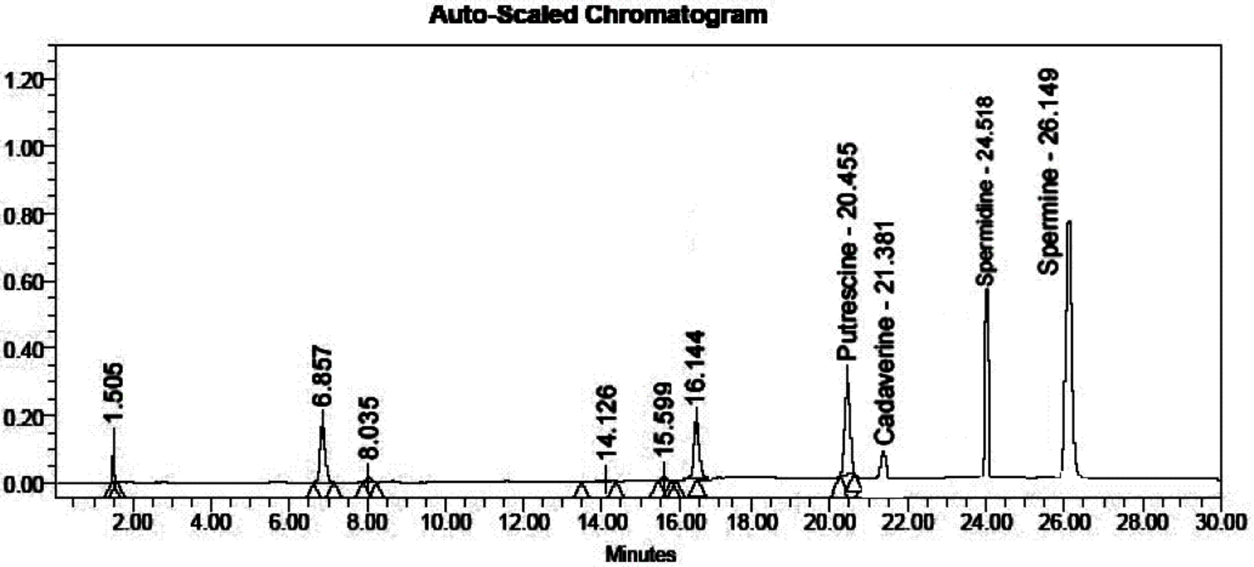

Fig. 3 HPLC chromatogram of Cu-metal treated samples for endogenous PAs (putrescine, cadaverine, spermidine and spermine) quantified and characterized at 254 nm.

Polyamine ratios

Cu metal treatment lowered the value of Put/Spd ratio from 0.206 in control to 0.130 in seedlings under metal stress. A significant increase in Put/Spd ratio (14.563) was recorded for seedlings given treatment of 10^{-7} M 24-epiBL alone. This ratio further got enhanced when seedlings were given treatments of combination of Cu metal with 10^{-9} M 24-epiBL (6.126) over control seedlings (**Table 2**). The Spd/Spm ratio got lowered under metal stress alone and in combination with different concentrations of 24-epiBL (10^{-7}, 10^{-9} and 10^{-11} M). Maximum reduction was found in seedlings given treatment of Cu metal and 10^{-9} M 24-epiBL (0.0081). However evident decrease in Put/Cad ratio (0.668 to 8.865) was found in seedlings under Cu-metal effect when compared to control. The Put/Cad ratio recorded a considerable increase under the influence of 24-epiBL (**Table 2**). This rise (293.29) was evident in concentration of 10^{-7} M 24-epiBL alone. The same trend was observed in seedlings given treatments of Cu metal in combination with 24-epiBL, with maximum enhancement (123.878) at Cu-metal in combination with 10^{-9} M 24-epiBL.

Table 2 Polyamine ratios of 7-days old *Raphanus* seedlings subjected to Cu-metal and 24-epiBL treatments.

Treatments	Put /Cad ratio	Put / Spd ratio	Spd / Spm ratio
Control	0.668^{b}	0.206	184.920^{b}
0.2mM Cu	8.860^{a}	0.130	0.314^{a}
10^{-7} M epiBL	$293.290^{a,b}$	$14.563^{a,b}$	$7.210^{a,b}$
10^{-9} M epiBL	$5.332^{a,b}$	1.165	$63.250^{a,b}$
10^{-11} M epiBL	6.720^{a}	$0.245^{a,b}$	$193.560^{a,b}$
Cu + 10^{-7} M epiBL	6.970^{a}	0.2492	$81.288^{a,b}$
Cu + 10^{-9} M epiBL	$123.878^{a,b}$	$6.126^{a,b}$	$0.00811^{a,b}$
Cu + 10^{-11} M epiBL	6.254^{a}	$0.8626^{a,b}$	$0.01440^{a,b}$

($^{a, b}$ indicate statistically significant differences from control and metal treatment at $p \leq 0.05$)

Antioxidative enzyme activities

Radish metabolism was significantly affected by exogenous application of 24-epiBL. This effect was observed with changes in the activities of antioxidative enzymes. The guaiacol peroxidase (GPOX) activity was reduced significantly from (0.28 U/mg protein fw) in control seedlings to 0.068 U/mg protein fw in seedlings under Cu stress (**Table 3**). The GPOX activity was not significantly increased under the influence of 24-epiBL. On the other hand, activity of CAT was lowered from 0.16 U/mg protein fw (control) to 0.072 U/mg protein fw (Cu-metal treatment) (**Table 3**). The CAT activity got enhanced under the effect of 24-epiBL alone, with small rise (0.18 U/mg protein fw) in 10^{-11} M 24-epiBL. The combination of Cu metal and 24-epiBL showed restoration of CAT activity (0.21 U/mg protein fw) significantly in seedlings given treatments of Cu metal and 24-epiBL (10^{-7} M) combination. The superoxide dismutase (SOD) activity got enhanced from 3.4 U/mg protein fw (control) to 8.08 U/mg protein fw (Cu-metal) (**Table 3**). But a considerable decrease in SOD activity was recorded under the influence of 24-epiBL, more significantly (2.3 U/mg protein fw) at 10^{-7} M 24-epiBL. However SOD activity got enhanced when metal stress was supplemented by 24-epiBL (10^{-7}, 10^{-9} and 10^{-11} M). Maximum increase in SOD activity (9.9 U/mg protein fw) was observed for seedlings treated with Cu metal in combination with 10^{-7} M 24-epiBL. A significant reduction in protein content from 20.25 mg/fw (control) to 10.23 mg/fw (Cu-stress) had been recorded. 24-epiBL treatment increased protein content considerably with maximum increase (38.43 mg/fw) at 10^{-11} M 24-epiBL. Similarly, 24-epiBL (10^{-7}, 10^{-9} and 10^{-11} M) treatment in combination with Cu metal increased protein content (**Table 3**) over metal stress alone.

Table 3 Effect of Cu-metal and 24-epibrassinolide treatments on protein contents (mg/g fw) and specific activities (U/mg fw) of antioxidative enzymes.

Treatments	Protein content (mg/g fw)	Guaiacol Peroxidase (U/mg fw)	Catalase (U/mg fw)	Superoxide dismuatse (U/mg fw)
Control	$20.25^{b} \pm 1.1$	0.28 ± 0.002	0.16 ± 0.02	3.40 ± 0.2
0.2 mM Cu	$9.38^{a} \pm 1.32$	0.068 ± 0.009	0.072 ± 0.012	$8.08^{a} \pm 0.89$
10^{-7} M epiBL	$21.38^{b} \pm 2.08$	0.23 ± 0.01	0.10 ± 0.01	2.30 ± 0.3
10^{-9} M epiBL	$27.25^{b} \pm 2.85$	0.30 ± 0.02	0.17 ± 0.02	3.10 ± 0.25
10^{-11} M epiBL	$29.12^{a,b} \pm 1.98$	0.245 ± 0.009	0.18 ± 0.03	3.56 ± 0.1
Cu + 10^{-7} M epiBL	$11.23^{a} \pm 0.98$	0.089 ± 0.005	0. 098 ± 0.002	5.60 ± 0.25
Cu + 10^{-9} M epiBL	13.67 ± 1.21	0.091 ± 0.002	0.101 ± 0.02	7.30 ± 1.01
Cu + 10^{-11} M epiBL	$18.91^{b} \pm 1.45$	0.20 ± 0.01	0.21 ± 0.01	$9.90^{a} \pm 0.99$

Data presented as mean±SE
($^{a, b}$ indicate statistically significant differences from control and metal treatment at $p \leq 0.05$)

Table 4 Morphological parameters of 7 days-old *Raphanus* seedlings given treatments of Cu-metal and 24-epibrassinolide.

Treatments	Shoot length (cm)	Root length (cm)	Percent germination%
Control	$3.42^b \pm 0.32$	$6.60^b \pm 0.98$	98
0.2 mM Cu	2.65 ± 0.2	$3.41^a \pm 0.56$	81
10^{-7} M epiBL	$3.67^b \pm 0.9$	$4.93^{a,b} \pm 0.87$	98
10^{-9} M epiBL	$3.72^b \pm 0.86$	$6.93^b \pm 0.99$	98
10^{-11} M epiBL	$4.21^{a,b} \pm 0.78$	$7.32^{a,b} \pm 0.89$	98
Cu + 10^{-7} M epiBL	2.61 ± 0.34	$4.12^{a,b} \pm 0.54$	92
Cu + 10^{-9} M epiBL	3.02 ± 0.12	$4.98^{a,b} \pm 0.49$	94
Cu + 10^{-11} M epiBL	3.31 ± 0.3	$6.32^b \pm 0.71$	96

Data presented as mean±SE

($^{a,\,b}$ indicate statistically significant differences from control and metal treatment at $p \leq 0.05$)

Seedling growth

The shoot and root length of *Raphanus* seedlings was markedly reduced in comparison to untreated control seedlings (**Table 4**). Cu metal stress reduced the shoot length by 22.48% and root length by 48.33% when compared with controls. However 24-epiBL treatment increased the shoot and root length significantly at 10^{-7}, 10^{-9} and 10^{-11} M 24-epiBL conc and in combination with Cu metal. Maximum increase was found in 10^{-9} M 24-epiBL alone with 8% and 5% enhancement in shoot and root length respectively. Whereas combination of Cu metal with 10^{-11} M 24-epiBL showed minimum decrease in shoot (3.21%) and root (4.22%) length in comparison to metal treated seedlings. Percent germination got decreased by 12% under Cu-metal treatment, where as 24-epiBL treatment exogenously enhanced percent germination by 98%. In addition to these effects Cu metal stress also resulted in poor root formation, interveinal chlorosis and development of necrotic spots on the leaf lamina.

DISCUSSION

Brassinosteriods (BRs) have been reported to regulate growth and developmental processes (Clouse 2008). In the present investigation seedling growth showed considerable reduction under Cu metal stress. This reduction in terms of shoot length was 22.48 and 48.33% for root length when compared to control values. However, Cu metal when supplemented with different concentrations of 24-epiBL revealed improvement in shoot and root lengths. Maximum improvement in shoot (3.21%) and root length (4.22%) was recorded in seedlings treated with 10^{-7} M 24-epiBL when compared to metal treated seedlings. PAs which are reported to play an important role in stress management showed their significant involvement under Cu metal stress. It was observed that Cu metal treatment resulted in the enhancement of Put conc by 38% ad Spd level by 119% and it also stimulated the synthesis of Spm which was not expressed in untreated control plants. However only Put content was lowered under metal stress by 89.58% over control values. But when metal treatment was supplemented with different concentrations of 24-epiBL, remarkable reduction in the contents of PAs was recorded. A significant decrease in Put level was found in 10^{-11} M 24-epiBL conc Maximum reduction in the contents of Cad and Spd was recorded in 10^{-9} M 24-epiBL treatment. On the other hand, significant decrease in Spm content was recorded in 10^{-7} M 24-epiBL treatment.

BRs treatment alone remarkably lowered the levels of PAs, with significant decrease observed in 10^{-11} M for Put and Cad and 10^{-7} M 24-epiBL for Spd content, Spm was not detected when seedlings were given treatments of 24-epiBL. The enhanced titers of Spd and Spm under metal treatment suggests their active involvement in the amelioration of stress generated by Cu metal than Cad and Put. These results also suggest that 24-epiBL may overcome the metal stress by somehow down regulating the production of PAs. The decrease in the titers of low molecular weight PAs by 24-epiBL was associated with the small number of amine groups than high molecular weight Spd and Spm, clearly indicating faster scavenging of oxidants or the free radicals generated due to Cu-toxicity. Shevyakova *et al.* (2006) recorded enhanced production of Put and Spd temporarily and gradual and constant increase in Spm content when leaves and roots of *Mesembryanthemum crystallinum* were subjected to chloride salinity stress (**Table 5**). Apple callus subjected to salinity stress (NaCl 200mM) detected enhanced contents of Put, Spd (**Table 5**) and Spm (Liu *et al.* 2006). Pirinstsos *et al.* (2004) found increased levels of Put, Spd and Spm in *Pseudevernia furfuracea* Zopf. and *Evernia prunastri* L. grown in heavy metals polluted area (**Table 5**).The increased Putrescine/Cadaverine ratio under Cu stress signified the importance of Put in Cu-stress management than Cad (**Table 2**). Similar observations were recorded by Lin and Kao (1999) observed enhanced production of Putrescine and significant increase in putrescine/cadaverine ratio of detached rice leaves subjected to copper stress. The reduction in cadaverine content has also been reported to induce oxidative burst which led to enhanced activity of superoxide dismutase (Kuznetsov *et al.* 2009). This may be assumed by the increased superoxide dismutase enzyme activity over other antioxidative enzymes in the present investigation. The reduced value of spermidine/spermine ratio was another important observation, as Cu-stress stimulated the synthesis of spermine which was not expressed under control conditions. These results clearly suggest that Cu-metal stress has been selective in the induction of PA synthesis. This has also signified the extraordinary ability of spermine in metal stress management than other PAs.

The decrease in the activities of guaiacol peroxidase, catalase and glutathione reductase, under Cu-metal stress was observed (**Table 3**). However significant enhancement in the activity of superoxide dismutase was (**Table 3**) accompanied by increase in the endogenous titers of PAs, which may be playing an important role in protecting the plants against oxidative stress induced by Cu-metal.

Table 5 Comparative account of endogenous content of PAs (nm/g fw) in apple callus, *Mesembrynthemum crystallinum* L., *Pseudevernia furfuracea* Zopf. and *Evernia prunastri* L. under salinity and heavy metal stresses.

Treatments	Putrescine	Cadaverine	Spermidine	Spermine	Study material	References
Control	$3623.9^b \pm 197.8$	n.d.	$58.3^b \pm 0.3$	$1.6^b \pm 0.1$		
NaCl (200 mM)	$5097^a \pm 773.5$	n.d.	$69.4^b \pm 2.0$	$12.1^a \pm 0.1$	Apple callus (P)	Liu *et al.* 2006
Control	$13.2^b \pm 0.6$	$24.0^b \pm 1.3$	$58.8^b \pm 2.4$	$90.1^b \pm 4.2$		
NaCl (400 mM)						
6 h	$5.5^a \pm 0.2$	$16.2^a \pm 0.7$	44.7 ± 1.9	95.2 ± 4.3		
24 h	$5.2^a \pm 0.1$	18.6 ± 0.8	43.2 ± 2.0	$138.0^a \pm 6.2$	*Mesembrynthemum*	
48 h	$4.9^a \pm 0.1$	$11.4^a \pm 0.6$	$138.0^a \pm 6.2$	110.1 ± 4.8	*crystallinum* L. (H)	Shevyakova *et al.* 2006
Control	6.5*	n.d.	16.6*	17.5*	*Pseudevernia furfuracea* Zopf. (L)	
Heavy metal pollution site	95.6*	n.d.	92.2*	45.6*	*Evernia prunastri* L. (L)	Pirintsos *et al.* 2004

Data presented as mean±SE

($^{a,\,b}$ indicate statistically significant differences from control and metal treatment at $p \leq 0.05$)

Abbreviations used: (P) plant, (H) halophyte, (L) lichen, (*) Pearson's Coefficient of PAs with respect to controls.

CONCLUSIONS

The present study indicates possible role of BRs in regulating the synthesis of selective PAs in *Raphanus* seedlings under Cu metal stress. Further enhanced activity of SOD also helped in ameliorating metal stress in the seedlings. The behavior of PAs and antioxidative enzymes under the influence of metal alone and in combination of 24-epiBL suggests the active involvement of BRs in stress protection via regulating the production of PAs and antioxidative enzyme activities. It opens a future area to study regulation of PAs in plants under metal stress by brassinosteriods.

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REFERENCES

Aebi HE (1983) Catalase In: *Methods of Enzymatic Analysis* (Vol 3), Verlag Chemie, Weinham, pp 273-282

Ali B, Hayat S, Ahmad A (2007) 28-homobrassinolide ameliorates the saline stress in chickpea (*Cicer arietinum* L.). *Environmental and Experimental Botany* **59**, 217-223

Almeida JM, Fidalgo F, Confraria A, Santos A, Pires H, Santos I (2005) Effect of hydrogen peroxide on catalase gene expression, isoform activities and levels in leaves of potato sprayed with homobrassinolide and ultrastructural changes in mesophyll cells. *Functional Plant Biology* **32**, 707-720

Arteca RN, Arteca JM (2008) Effects of brassinosteriods, auxins and cytokinins on ethylene production in *Arabidopsis thaliana* plants. *Journal of Experimental Botany* **59**, 3019-3026

Chary NS, Kamala CT, Raj DS (2008) Assessing risk of heavy metals from consuming food grown on sewage irrigated soils and food chain transfer. *Ecotoxicology and Environmental Safety* **69**, 513-524

Clouse SD (2008) The molecular intersection of brassinosteriods-regulated growth and flowering in *Arabidopsis*. *Proceedings of the National Academy of Sciences USA* **105**, 7345-7346

Fontaniella B, Mateos JL, Vicente C, Legaz ME (2001) Improvement of the analysis of dansylated derivatives of polyamines and their conjugates by high-performance liquid chromatography. *Journal of Chromatography A* **919**, 283-288

Groppa MD, Benavides MP (2008) Polyamines and abiotic stress: recent advances. *Amino Acids* **34**, 35-45

Ha HC, Sirisoma NS, Kuppusamy P, Zweier JL, Woster PM, Casero RA (1998) The natural polyamine spermine functions directly as a free radical scavenger. *Proceedings of the National Academy of Sciences USA* **95**, 11140-11145

Halliday KJ (2004) Plant hormones: The interplay of brassinosteriods and auxin. *Current Biology* **14**, 1008-1010

Halliwel B, Gutteridge JMC (1984) Oxygen radicals, transition metals and diseases. *Biochemical Journal* **219**, 1-14

Haubrick LL, Assmann SM (2006) Brassinosteriods and plant function: some clues, more puzzles. *Plant Cell and Environment* **29**, 446-457

Hayat S, Ali B, Hasan SA, Ahmad A (2007) Brassinosteriod enhance the level of antioxidants under cadmium stress in *Brassica juncea. Environmental and Experimental Botany* **60**, 33-41

Jagger CE, Symons GM, Ross JJ, Smith JJ, Reid JB (2005) The brassinosteriods growth response in pea is not mediated by changes in gibberellin content. *Planta* **221**, 141-148

Jagger CE, Symons GM, Ross JJ, Smith JJ, Reid JB (2008) Do brassinosteroids mediate the water stress response? *Physiologia Plantarum* **133**, 417-25

Kopittke PM, Dart PJ, Menzies NW (2007) Toxic effects of low concentrations of Cu on nodulation of cowpea (*Vigna unguiculata*). *Environmental Pollution* **145**, 309-315

Kono Y (1978) Generation of superoxide radical during autoxidation of hydroxylamine and an assay for superoxide dismutase. *Archives of Biochemistry and Biophysics* **186**, 189-195

Kumar V, Giridhar P, Chandrashekar A, Ravishankar GA (2008) Polyamines influence morphogenesis and caffeine biosynthesis in *in vitro* cultures of *Coffea canephora* P. ex Fr. *Acta Physiologia Plantarum* **30**, 217-223

Kurepin LV, Qaderi MM, Back TG, Reid DM, Pharis RP (2008) A rapid effect of applied brassinolide on abscisic acid concentrations in *Brassica napus* leaf tissue subjected to short-term heat stress. *Plant Growth Regulation* **55**, 165-167

Kuznetsov VV, Shevyakova NI (2007) Polyamines and stress tolerance of plants. *Plant Stress* **1**, 50-71

Kuznetsov VV, Stetsenko LA, Shevyakova NI (2009) Exogenous cadaverine induces oxidative burst and reduces cadaverine conjugate content in the common ice plant. *Journal of Plant Physiology* **166**, 40-51

Lin CC, Kao CH (1999) Excess copper induces an accumulation of putrescine in rice leaves. *Botanical Bulletin Academia Sinica* **40**, 213-218

Liu J-H, Nada K, Honda C, Kitashiba H, Wen X-P, Pang X-M, Moriguchi T (2006) Polyamine biosynthesis of apple callus under salt stress: importance of arginine decarboxylase pathway in stress response. *Journal of Experimental Botany* **57**, 2589-2599

Liu J-H, Kitashiba H, Wang J, Ban Y, Moriguchi T (2007) Polyamines and their ability to provide environmental stress tolerance to plants. *Plant Biotechnology* **24**, 117-126

Liu J-H, Moriguchi T (2007) Changes in free polyamine titers and expression of polyamine biosynthetic genes during growth of peach *in vitro* callus. *Cell Biology and Morphogenesis* **26**, 125-131

Lowry OH, Rosebrough MJ, Farr AL, Randall RJ (1951) Protein measurements with the Folin phenol reagent. *The Journal of Biological Chemistry* **193**, 265-275

Luna CM, Gonzalez CA, Trippi VS (1994) Oxidative damage caused by an excess of copper in oat leaves. *Journal of Plant Physiology* **35**, 11-15

Pang X-M, Zhang Z-Y, Wen X-P, Ban Y, Moriguchi T (2007) Polyamines, all-purpose players in response to environment stresses in plants. *Plant Stress* **1**, 173-188

Pirintsos SA, Kotzabasis K, Loppi S (2004) Polyamine production in Lichens under metal pollution stress. *Journal of Atmospheric Chemistry* **49**, 303-315

Putter J (1974) Peroxidase In: Bergmeyer HU (Ed) *Methods of Enzymatic Analysis*, Verlag Chemie, Weinhan, pp 685-690

Ruben A, Marco F, Cuevas JC, Patron M, Ferrando P, Carrasco P, Tiburcio AF, Altabella T (2006) Involvement of polyamines in plant response to abiotic stress. *Biotechnology Letters* **28**, 1867-1876

Sasse JM (2003) Physiological action of brassinosteriods: An update. *Journal of Plant Growth Regulation* **22**, 276-278

Seiler N, Nodgen B (1979) Determination of the naturally occurring monoacetyl derivatives of di- and polyamines. *Journal of Chromatography B* **164**, 155-168

Shevyakova NI, Shorina MV, Rakitin VY, Kuznetsov VV (2006) Stress-dependent accumulation of spermidine and spermine in the halophyte *Mesembryanthemum crystalinum* under salinity conditions. *Russian Journal of Plant Physiology* **53**, 739-745

Tang W, Newton RJ (2005) Polyamines reduce salt-induced oxidative damage by increasing the activities of antioxidant enzymes and decreasing lipid peroxidation in Virginia pine. *Plant Growth Regulation* **46**, 31-43

Tassoni A, Franceschetti M, Bagni N (2008) Polyamines and salt stress response and tolerance in *Arabidopsis thaliana* flowers. *Plant Physiology and Biochemistry* **46**, 607-613

Wen XP, Pang XM, Matsuda N, Kita M, Inoue H, Hao YJ, Honda C, Moriguchi T (2008) Over-expression of the apple spermidine synthase gene in pear confers multiple abiotic stress tolerance by altering polyamine titers. *Transgenic Research* **17**, 251-263

Zapata PJ, Serrano M, Pretel MT, Botella MA (2008) Changes in free polyamine concentrations induced by stress in seedlings of different species. *Plant Growth Regulation* **56**, 167-177