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Salicylic Acid Alleviates the Copper Toxicity in *Zinnia elegans*

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Abstract

Salicylic acid (SA) plays a key role in plant disease resistance and hypersensitive cell death but is also implicated in hardening responses to abiotic stress. The aim of this study is to examine the effects of salicylic acid on the growth, eco-physiological and biochemical characteristics in *Zinnia elegans* plant exposed to copper stress. Effects of copper (Cu) on biomass, root length and shoot height and Cu uptake are also discussed. This experiment was arranged as a factorial experiment based on completely randomized design with four replications in greenhouse conditions. The experimental treatment consisted of four levels of Cu (0, 100, 200, and 400 mg/kg in potted soil) and three levels of salicylic acid (0, 1, 2 mM) as foliar spray and chelate to soil. Results showed that with increasing levels of copper, reductions in shoot and root growth, leaf area and leaf number were statistically significant ($p<0.01$). Analysis of some biochemical indices (chlorophyll content) and eco-physiological indices such as primary florescence chlorophyll (Fo), ratio of variable chlorophyll to maximum (Fv/Fm) in treated plants were statistically significant ($p<0.01$). The results showed that copper accumulation was higher in the roots than shoots. The Fluorescence measurements showed that primary florescence chlorophyll (Fo) was increased but maximum florescence chlorophyll (Fm) was decreased progressively with increasing Cu. Salicylic acid significantly increased root and shoot growth and chlorophyll content in copper stressed plants. SA applying as chelate 2 mM also showed an increased level of tolerance toward high Cu concentrations. The results support the conclusion that SA alleviates Cu toxicity.

Keywords: Copper, Salicylic acid, Toxicity, *Zinnia elegans*.

INTRODUCTION

Heavy metal contamination is a serious environmental problem that limits plant productivity and threatens human health. Metals present a unique problem because they cannot be degraded such as organic pollutants, but must be either physically removed or immobilized (Meagher, 2000; Chaignon and Hinsinger, 2003). Copper contamination is one of the major environmental hazards in contaminated areas (Chaney *et al.*, 1997). It is also an essential nutrient for plants that has known biological function (Harris, 2000). Standard concentration of copper in plant tissues is approximately 5-25 mg/kg. Plant copper concentrations are controlled within a remarkably narrow range (Arduini *et al.*, 1995). It is easily taken up by roots and transport to aerial parts (Maksymiec *et al.,* 2007). Toxicity of copper caused inhibition of root growth and copper concentration in root decreased the root length and had a harmful effect on growth and metabolism in plants (Mattioni *et al.,* 1997; Hou *et al.,* 2007). It causes to reduce absorption of other essential elements and reduce the rate of photosynthesis (Ariyakanon and Winaipanich, 2006). Copper is taken up by plants mainly through the root system and partly in minor amounts through the shoots and leaves (Mattioni *et al*., 1997). This phenomenon has even threatened the health of ecosystems and human beings themselves (Lombardi and Sebastiani, 2005; Grytsyuk, 2006). An economical method is phytoremediation, the use of plants to physically remove contaminants from the soil. Phytoextraction is a technology that works on removing contaminants from the surface layers of soil. The advantages of utilizing plants for remediation of contaminated soils are becoming recognized (Chen *et al.,* 2000). A plants ability to take up contaminants is directly related to the bioavailability of the contaminant such as a heavy metal (Shu *et al*., 2002). Some plants naturally uptake high concentrations of specific contaminants, while other plants can be induced to increase their uptake through the use of chelating agents such as salicylic acid (SA) (Brooks *et al.*, 1998). However, a reference review did not indicate any study addressing the effects of SA on metal uptake by species. Recent studies have also indicated that SA has been used as chelate for rapid mobility and uptake of metals from contaminated soils by plants. Use of chelates significantly increased heavy metal uptake and translocation from roots to shoots. Application of low cost the synthetic and natural composition's on large scale are applied to the soil through irrigation at specific stages of plant growth which might be beneficial to accelerate metal accumulation (Salt *et al.*,1995; Branquinho *et al.,* 2006). Ornamental plants are an important type of higher plants apart from those in the food chain, and are quite crucial if they have hyper-accumulation properties and can be applied to remediation of contaminated soils (Ma, 2003; Wang, 2005; Li *et al*., 2006). Thus, using ornamentals for remediation of contaminated environment has a significant and realistic purpose (Hern´andez-Apaolaza *et al*., 2005; Zhou, 2006). *Zinnia elegans* L. is an herbaceous plant belonging to Asteraceae. Recent studies have indicated that maximum concentrations of heavy metal accumulated by plants in family Asteraceae. Salicylic acid belongs to a group of phenolic compounds that widely exists in plants and nowadays is considered as a hormone-like substance. Salicylic acid also plays an important role in plant growth and development. SA induces resistance to water deficit and ameliorates the damaging effects of heavy metals, like lead and mercury (Berukova *et al.*, 2001; Rugh *et al.*, 2000). The overall objectives of this research were: (1) to determine the concentrations of Cu in plant biomass growing on a copper contaminated soil; (2) to study evolutionary, biochemistry and physiological aspects involved in the metal uptake process and SA treatment.

MATERIALS AND METHODS

Soil and plant preparation

To initiate the experiment under controlled condition the soil was air-dried, crushed, mixed thoroughly and passed through a 2 mm sieve. Physical and chemical properties of soil (i.e., soil texture, moisture content, soil pH, cation exchange capacity, organic matter content and the nutritional status) were analyzed. The physical and chemical properties of soil used in this study are shown in Table 1. About 1 kg of the soil placed into plastic pots (25 cm in diameter and 20 cm in

Table 1. The physical and chemical properties of soil studied.

Soil texture	Sand: Silt: Clay	EC (ds. m^{-1})	CEC (cmol/kg)	OC (%)	p (mg.kg^{-1})	K (mg.kg^{-1})	Cu (mg.kg^{-1})
Loam	44:38:18	1.5	21.75	0.4	7	220	0.38

length) and then various concentration of $CuSO_4$ (100, 200 and 400 mg/kg) was added in the experimental polythene bags and mixed in soil. A month later, copper contaminant soil was filled separately into each pot.

Plant, harvest and analysis

Seeds of *Zinnia elegans* were germinated in trays of washed and sterilized perlite (autoclave-sterilized: 121°C, 15 min, at 103 kPa) used for the hydroponic culture (Erowid, 2007). Hoagland's solution (Hoagland, 1948) used for plant nutrition. After the seedlings appeared, small and unhealthy plants were removed. The experimental pots were set up by adding 5 kg of copper contaminant soil sample and one seedling of *Zinnia elegans* was sown separately into each pot. Four pots are prepared for each metal treatment. During the experiment, exceed watering was not allowed in order to prevent the leakage of copper from the pot. Shoot and root growth parameters (height, leaf number and leaf area, length of root) were measured. Then biochemical indices such as chlorophyll content (Starner and Hardley, 1967) and eco-physiological indices (primary florescence chlorophyll (Fo), ratio of variable chlorophyll to maximum (Fv/Fm) (OSI with Opti Sciences Inc ADC, UK) were recorded. Plant samples were gently removed from the pots 75 days for the measurement of various growth parameters and eco-physiological and biochemical analysis. Plants were harvested early in the morning between 8.0 to 9.0 A.M. The collected plant samples were placed in plastic bags, labeled carefully and brought to the laboratory. The overall length of plant is measured. Each plant was rinsed, cut, and group selected into shoots and roots. Each part was dried in an oven at 65oC for 72 hours. Both wet and dry weight was recorded. All dried parts were ground using mortar, mixed thoroughly, and digested with 0.1 N HCl. Sample solutions were analyzed for copper by flame atomic absorption. The soil was air-dried, sieved through a 2.0 mm screen and digested with 0.1 N HCl. All values reported in this study are the mean of four replicates (Tan, 1996). Analysis of variance (ANOVA) was carried out using the statistical software, SAS, to determinate if there were significant differences in metal accumulation as a result of metal treatments. Significant differences between the means assessed by Duncan test at $P<0.05$.

RESULTS

Growth parameters

In this study, the effect of copper toxicity, salicylic acid and interaction between them on height, root length, leaf number, leaf area and fresh and dry weight of shoot and root were statistically significant ($p<0.01$) (Table 2). The comparison of means by Duncan's method showed that the shoot and root growth, height, leaf area and leaf number decreased by increasing in concentration of copper (Table 3). The highest effect of salicylic acid treatments on growth parameters was 2Mm as chelate into soil (Table 3). Toxicity of copper caused inhibition of root growth and copper con-

Table 2. Analysis of variance of the growth parameter.

Source of variance	df	Height of plant	No. leaf	Leaf area	Fresh weight of shoot	Dry weight of shoot	Fresh weight of root	Dry weight of root	Length root
CuSO4	3	1350.01**	241.1**	170014**	435.22**	102.1**	429.3**	141.3**	294.9*
SA	4	59.66**	17.08**	17712.3**	19.8**	5.3**	12.7**	4.07**	8.1**
CuSO4* SA	12	34.64**	3.42**	5320.6**	7.5**	1.87**	2.9**	0.77**	1.16*
Error	60	1.42	0.84	0.097	0.05	0.019	0.01	0.02	0.74
c.v. (%)	-	8.2	11.2	0.4	3.8	4.7	1.74	4.9	10.11

*P < 0.05; **P < 0.01; ns: not significant

Table 3. Comparison of treatments on growth stage of *Zinnia elegans*.

Treatments	Height of plant (cm)	Number of leaf	Leaf area (mm^2)	Fresh weight of shoot (g)	Dry weight of shoot (g)	Fresh weight of root (g)	Dry weight of root(g)	Length of root (cm)
0 (mg/kg)	27.5 a	11 a	8896.3 a	11.86 a	5.78 a	11.7 a	6.7 a	10.7 a
100	15.2 b	10.75 a	4849 b	7.76 b	3.72 b	7.5 b	4.2 b	6.5 b
200	10.4 c	7.14 b	3276.3 c	3.31 c	1.61 c	3.17 c	1.77 c	3.4 c
400	4.45 d	3.65 c	2270.01 d	1.46 d	0.72 d	1.33 d	0.75 d	2.1 d
0 SA	13.62 c	6.87 c	3455.3 c	4.77 e	2.24 d	4.47 e	2.5 d	4.5 c
1 mM foliage	11.5 d	8.86 a	4321.5 b	5.47 d	2.71 c	5.77 d	3.31 c	5.7 b
2 mM foliage	13.43 c	9.37 a	4578.6 b	5.74 c	2.77 c	6.22 c	3.57 b	5.7 b
1 mM soil	16.18 b	7.36 c	5797.5 a	7.16 b	3.46 b	6.38 b	3.62 b	6.06 a
2 mM soil	17.25 a	8.18 b	5961.7 a	7.35 a	3.63 a	6.79 a	3.78 a	6.42 a

Mean separation by Duncan's Multiple Range Test at P = 0.05. The same letters within a column are not significantly different.

centration in root decreased the root length and had a harmful effect on plants. The functions of copper are regarded as being closely associated with iron's function. It can be interpreted that the toxicity of copper to reduce the absorption of iron thus chlorophyll and photosynthesis is reduced (Ariyakanon and Winaipanich, 2006). Similar observations were also made by Arduini *et al.*, (1995) in *Pinus pinea* L. and *Pinus pinaster* Ait, Rossini *et al*., (2010) in *Erica andevalensis*, Shengoil *et al.*, (2006) in *Commelina communis* and Wei *et al.*, (2008) in *Chrysanthemum coronarium.*

Eco-physiological and biochemical parameters

The results showed that salicylic acid, copper toxicity levels and their interaction were statistically significant at 1% level (Table 4). Based on results, the toxicity of copper decreased all fractions of chlorophyll in treated plants considerably (Table 5). Chlorophyll (Fo) and ratio of variable chlorophyll to maximum (Fv/Fm) were significant ($p < 0.05$) (Table 4). Exposing plant to different levels of Cu resulted in changes of the chlorophyll fluorescence parameters, Fo and Fm, (Table 5). Increasing Cu concentrations resulted in increase of Fo values. In contrast, Fo values decreased significantly for the whole range of Cu concentrations (Table 5). Examination of Fv/Fm values clearly showed that this parameter had a high correlation with Fv (Table 5). In control leaves, Fv/Fm values were approximately 0.8 (Table 5). Maximum all fractions of chlorophyll, Fv and Fv/Fm were observed in salicylic acid 2Mm treatment (Table 5). The results suggested that application of salicylic acid 2Mm as chelate in soil can greatly reduce the negative effects of copper toxicity on studied parameters. Research in this field (Arduini *et al.*, 1995; Ariyakanon and Winaipanich, 2006; El-Tayeb *et al.*, 2006; Hou *et al.*, 2007) in *Pinus, Brassica juncea* and *Bidens alba,* sunflower and Duckweed (*Lemna minor*) showed that with the increasing concentration of CuSO4, chlorophyll and photosynthesis decreases. In addition SA reduced the Fo (primary florescence chlorophyll) (Patsikka *et al.,* 2002). In recent years, the ratio of Fv/Fm is often used as a stress indicator and describes the potential yield of the photochemical reaction. Chlorophyll fluorescence allows us to study the different functional levels of photosynthesis indirectly (Goedheer, 1964; Bilger *et al.,* 1984; Govindjee, 1995). A typical measurement on intact leaf by the saturation

Table 4. Analysis of variance of the eco-physiological and biochemical parameters.

Source of variance	df	Total chlorophyll	Chlorophyll a	Chlorophyll B	F0	Fv	Fv/Fm
$CuSO_4$	3	0.0005 **	0.0006**	0.0004**	370.62**	75676**	0.241**
SA	4	0.00006**	0.00006 **	0.000046**	92.15**	40453**	0.011*
$CuSO_4$* SA	12	0.00002**	0.000007**	0.000005**	19.62**	12516**	0.003**
Error	60	0.0000008	0.000002	0.0000008	0.2	1.31	0.00006
c.v. (%)	-	4.18	8.4	5.6	0.28	0.27	1.17

*P < 0.05; **P < 0.01; ns: not significant

Table 5. Comparison of treatments on eco-physiological and biochemical parameters.

Treatments	Total chlorophyll	Chlorophyll a	Chlorophyll b	F0	Fv	Fv/Fm
0 (mg/kg)	0.0273 a	0.0255 a	0.0214 a	153.2 c	686.4 a	0.8 a
100	0.0238 b	0.0223 b	0.0187 b	161 b	413.4 b	0.7 b
200	0.0183 c	0.0162 c	0.0145 c	162.1 a	332.9 c	0.6 c
400	0.0162 d	0.0132 d	0.0113 d	162.1 a	233 d	0.54 d
0 SA	0.0183 d	0.0161 c	0.0137 c	163.39 a	351.66 d	0.625 e
1 mM foliage	0.022 b	0.0194 b	0.0167 b	158.91 c	387.49 c	0.622 d
2 mM foliage	0.0233 a	0.0208 a	0.0179 a	157.24 e	410.01 b	0.676 c
1 mM soil	0.0205 c	0.0193 b	0.0164 b	160.57 b	466.59 a	0.682 b
2 mM soil	0.0288 a	0.0209 a	0.0178 a	158.28 d	466.66 a	0.695 a

Mean separation by Duncan's Multiple Range Test at P = 0.05. The same letters within a column are not significantly different.

pulse method is used. The plant was dark adapted for 20 min prior to for the measurement. Upon the application of a saturating flash (8000 mmol m^{-2} s^{-1}), fluorescence raises from the ground state value (Fo) to its maximum value (Fm). In this condition QA, the first electron acceptor of PSII, is fully reduced. This allows the determination of the maximum quantum efficiency of photosystem II (PSII) primary photochemistry, given by Fv/Fm=(Fm-fo)/Fm. In healthy leaves, this value is always close to 0.8, independently of the plant species (Maxwell and Johnson, 2000). A lower value indicates that a proportion of PSII reaction centers are damage. Changes in the Fv/Fm give important information concerning the effect of environmental stress on plant. Nesterenko *et al.*, (2001) and Neves *et al.*, (2005) reported that in healthy leaves with high Chlorophyll had a maximum (Fv/Fm). The similar results have been reported by Faust.

Copper accumulation

Analysis of variance and mean comparison showed that the salicylic acid, copper toxicity levels and their interactions were statistically significant ($p < 0.05$) (Table 6). Maximum concentration of copper in shoot and root was recorded in 196.22, 249.08 mg/kg, respectively (Table 7). At all copper concentrations, SA application as chelate in soil increased Cu accumulation. So application of SA at 2mM chelate in soil can be useful for the phytoextraction of Cu. How-

Table 6. Analysis of variance of the copper concentration.

Source of variance	df	Cu shoot	Cu root
$CuSO_4$	3	226937.62**	311485**
SA	4	39495.8**	87763**
$CuSO_4$* SA	12	9804.53**	36286**
Error	60	962	377
c.v.(%)	-	15	10

*P < 0.05; **P < 0.01; ns: not significant

Table 7. Comparison of treatments on concentration of copper.

Treatments	Cu shoot (mg/kg)	Cu root (mg/kg)
0 (mg/kg)	17.37 d	43.78 d
100	125.3 c	174.45 c
200	196.22 b	249.08 b
400	267.1 a	338.8 a
0 SA	132.18 c	159.36 b
1 mM foliage	107.29 d	138.4 b
2 mM foliage	110.06 d	145.4 b
1 mM soil	191.32 b	280.5 a
2 mM soil	216.6 a	273.95 a

Mean separation by Duncan's Multiple Range Test at P = 0.05.

ever, Wang *et al.* (2009) did not find any significant effect of exogenous salicylic acid application on Ni accumulation in *Zea mays*. It has been shown that SA can modulate plant responses to a wide range of oxidative stresses, such as salt and osmotic stresses (Borsani *et al.*, 2001), drought (Senaranta *et al.,* 2002), herbicides (Ananieva *et al.*, 2004), and metals (Krantev *et al.*, 2008). It is known that exogenous salicylic acid alleviates the toxic effects generated by Cd in barley (Metwally *et al.,* 2003) and in maize plants (Pal *et al.*, 2002). Shi and Zhu (2008) found that exogenous salicylic acid alleviated the toxicity generated in *Cucumis sativus* by manganese exposure, and they observed a reduction in reactive oxygen species (ROS) level and lipid peroxidation. Yang *et al.,* (2003) stated that exogenous salicylic acid causes a reduction in aluminum accumulation in rice.

DISCUSSION

Copper, in polluted soils, can be accumulated in all plant parts affecting growth and development. Its accumulation can be toxic to the plant mainly via oxidative damage causing morphological and physiological changes (Wang *et al*., 2004; Drazic and Mihailovic, 2005; Krantev *et al.,* 2008; Li *et al*., 2009). Leaf chlorosis is one of the most commonly observed consequences of Cu toxicity. The leaves were significantly affected by Cu treatment. Chlorophyll contents (a, b and total) declined with increasing Cu concentrations. These results indicated that *Zinnia elegans* has a great ability to accumulate Cu, primarily in roots, and to prevent the transfer of excess Cu to the leaves. The results showed that the maximum concentrations of copper in shoot and root of *Zinnia elegans* was 196.2 and 249.08 mg/kg in the experimental pots with 200 mg Cu/kg. *Zinnia elegans* can accumulate moderate levels of environmentally important metals. Thus, it could be classified as a copper tolerant species. Wei *et al.*, (2001) reported that *Zinnia elegans* can accumulate Cu in shoot parts of the plant. In addition, there was a significant difference between copper accumulation and SA as chelate in soil at the 99% confidence level. Salicylic acid (SA) or hydroxy benzoic acid is a growth regulator produced mainly by roots with positive effect on yield and its components (Kumar *et al*., 1997 and 1999; El-Tayeb *et al.*, 2006; Hussein *et al*., 2007). Additionally, SA application can improve other important traits such as leaf area, dry matter, pigment content and photosynthesis rate (Khan *et al.*, 2003; Khodary, 2004; Hussein *et al*., 2007). The chlorophyll content of the leaves was significantly influenced in the various concentration of copper sulfate and the treatment plants with SA had higher contents of all fractions of chlorophyll. It seems that SA in foliar spray and chelate in soil concentrations reinforces metabolic responses similar to a growth regulator, influencing photosynthetic parameters and copper uptake relations (Senaranta *et al.,* 2000; Hayat *et al.*, 2005, 2010). For instance, SA application can improve the plant tolerance against heavy metal containment soils (Reeves and Baker, 2000; Srivastava *et al.*, 2006). In addition to the physiological effect of SA on plant (Fariduddin *et al*., 2003; Khan *et al.*, 2003; Khodary, 2004; Hussein *et al.*, 2007), its role in alleviating the Cu toxicity have been demonstrated (Metwally *et al.,* 2003; Drazic and Mihailovic, 2005; Krantev *et al.*, 2008). The results were indicated that SA protects plants against tension caused by Cu stress. For *Zinnia elegans*, SA as chelate should be applied to increase the solubility of copper in the soil solution and copper accumulation in the plants.

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