

Salicylic Acid Alleviates the Copper Toxicity in *Tagetes Erecta*

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ABSTRACT: The aim of this study is to examine the effects of salicylic acid on the growth, eco-physiological and biochemical characteristics in *Tagetes erecta* 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 under greenhouse conditions. The experimental treatment consisted of four levels of Cu (0, 100, 200, and 400 mg kg⁻¹ in potted soil) and three levels of salicylic acid (0, 1, 2mM) as foliar spray or chelate into soil. Results showed that with increasing levels of copper, reductions in shoot and root growth, leaf area and leaves number were statistically significant at 1 percent level. Analysis of some biochemical indices (chlorophyll content) and eco-physiological indices such as primary fluorescence chlorophyll (Fo), ratio of variable chlorophyll to maximum (Fv/Fm) in treated plants were statistically significant at one percent level. Biomass, leaf area and leaf number and root and shoot elongation decreased with increasing of Cu. Plants took more Cu up with increasing Cu in the copper containment soil. The results showed that copper accumulation was higher in the roots than shoots. The Fluorescence measurements showed that primary fluorescence chlorophyll (Fo) was increased but Fm was decreased progressively with increasing Cu. Salicylic acid significantly increased root and shoot growth, chlorophyll in copper stressed plants. In addition SA reduced the primary fluorescence chlorophyll. Salicylic acid treatments as chelate in concentration of 2mM also increased level of tolerance toward high Cu concentrations as indicated by chlorophyll fluorescence parameters. The results support the conclusion that SA alleviates Cu toxicity.

Keywords: Copper, Salicylic acid, Toxicity, *Tagetes erecta*

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 as organic pollutants can, 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, 1997). It is also an essential component for plants that has known biological function (Harris, 2000). Normal concentration of copper in plant tissues is approximately 5-25 mg/kg. Plant copper concentrations are controlled within a remarkably narrow range and plant copper concentrations above 100 mg/kg are rare even in the presence of high soil copper (Arduini, 1995). It is easily taken up by roots and translocate to different plant parts. The toxic symptoms of metals in plants can be recognized by changes in biochemical and physiological processes or by organ and intact plant responses such as growth inhibition, reduction in yield, chlorosis, alterations of anatomical, morphological and several metabolic activities in different cell compartments, especially chloroplasts (Maksymiec, 2007). These damaging effects include inhibition of photosynthesis, such as biosynthesis of chlorophyll and functioning of photochemical reactions. Photosystem II

(PSII) is extremely sensitive to Cu and its function was inhibited to a much greater extent than that of Photosystem I (PSI). Recent studies have also indicated that copper toxicity due to many aspects of its behavior inhibits iron metabolism and the activities of many enzymes (Arnon, 1949; Mattioni , 1997; Harris, 2000; Hou , 2007). It causes such problems as reduction in absorption of essential elements and reduction in 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 , 1997). This phenomenon has even threatened the health of ecosystems and human beings themselves (Lombardi and Sebastiani, 2005; Grytsyuk , 2006). An economical alternative to conventional methods is phytoremediation, the use of plants to physically remove contaminants from the soil (Chen , 2000). A plants ability to take up contaminants is directly related to the bioavailability of the contaminant such as a heavy metal (Shu , 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 , 1998). Recent studies have indicated that SA has been used as chelate for rapid mobility and uptake of metals from contaminated soils by plants. Use of chelate significantly increased heavy metal uptake and translocation from roots to shoots facilitating phytoextraction of the heavy metals from soil (Salt , 1995; Branquinho , 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; Liu , 2006). Thus, using ornamentals for remediation of contaminated environment has a significant and realistic purpose (Hernández-Apaolaza, 2005; Zhou, 2006). *Tagetes erecta* L. Which is an herbaceous plant belonging to Compositae. Wang (2005) showed that maximum concentrations of Cd accumulated by root in *Tagetes erecta*. Salicylic acid belongs to a group of phenolic compounds that widely exists in plants and now a day is considered as a hormone-like substance. This salicylic acid also plays an important role in plant growth and development (Mazaheri and Manochehri-Kalantari, 2007). SA induces resistance to water deficit and ameliorates the damaging effects of heavy metals, like lead and mercury (Berukova , 2001; Rugh , 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. Results of this study should provide insight for using plant to remediate copper contaminated soil.

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 (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 a. About 1 kg of the soil placed into plastic pots (25 cm in diameter and 20 cm in length) and then various concentration of CuSO_4 (100, 200 and 400 mg/Kg) was added in the experimental polythene bags and mixed it in soil by using hand gloves. Thus metals are properly mixed with the soil. A month later, copper contaminant soil was filled separately into each pot. Experimental pots were prepared.

Table a. 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 (ppm) |
|--------------|------------------|------------------------------|------------------|-----------|-----------------------------|-----------------------------|-------------|
| Loam | 44:38:18 | 1.5 | 21.75 | 0.4 | 7 | 220 | 0.38 |

Plant, harvest and analysis

Seeds of *Tagetes erecta* were germinated in trays of washed and sterilized Perlite (autoclave-sterilized: 121°C, 15 min, at 103 kPa) used for the hydroponic culture. Hoagland's solution used for plant nutrition. After the seedling appeared, small and unhealthy plants were removed. The experimental pots were set up by adding 5 kg of copper contaminant soil sample and one seeding of *Tagetes erecta* was sown separately into each pot. For each concentration of particular metal four pots are prepared. During the experiment, watering was not allowed to exceed the water holding capacity of the soil in order to prevent the leakage of copper from the pot. Morphology changes of plants in each pot were observed for copper toxicity symptoms and shoot and root growth parameters (height, leaves number and leaf area, length of root). Then biochemical indices such as chlorophyll content (Starnier and Hardley, 1967) and eco-physiological indices (primary florescence chlorophyll (Fo), ratio of variable chlorophyll to maximum (Fv/Fm)(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 AM. The collected plant samples were placed in plastic bags, labeled carefully and brought to the laboratory. Now the overall length of plant is measured with the help of cm scale. Each plant was rinsed, cut, and group selected into shoots and roots. Each part was dried in an oven at 65o C 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. 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 AND DISCUSSION

RESULTS

Growth parameters

In this study, the effect of copper toxicity, salicylic acid and interaction between them on height, root length, leaves number, leaf area and fresh and dry weight of shoot and root were statistically significant at 1% level (Table 1). The comparison of means by Duncan's method showed that the shoot and root growth, height, leaf area and leaves number were decreased by increasing copper concentration (Table 2). The highest effect of salicylic acid treatments on growth parameters was 2mM as chelate into soil (Table 2). Toxicity of copper caused inhibition of root growth and copper concentration in root decreased the root length and had a harmful effect on plants. The functions of copper are regarded as being closely associated with those of iron and as being concerned with chlorophyll formation. It can be interpreted that the copper toxicity may cause such problems as reduction in absorption of essential elements such as iron and reduction in the rate of chlorophyll and photosynthesis (3). Similar observations were also made by Arduini , (1995) in *Pinus pinea* L. and *Pinus pinaster* Ait, Rossini , (2010) in *Erica andevalensis*, Shengoil , (2009) in *Commelina communis* and Wei , (2008) in *Chrysanthemum coronarium*.

Table 1. Factorial analysis of the copper toxicity effect and SA on growth

| Source of variance | df | 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) |
|------------------------|----|----------------------|----------------|------------------------------|---------------------------|-------------------------|--------------------------|------------------------|---------------------|
| CuSo ₄ | 3 | 1688.02** | 820.08** | 92509** | 1832.6** | 430.3** | 239.65** | 54.30** | 363.2** |
| SA | 4 | 71.08** | 24.7** | 3217** | 31.6** | 10.7** | 21.1** | 10.66** | 17.7** |
| CuSo ₄ * SA | 12 | 23.5** | 6.3** | 4476** | 2.02** | 1.3** | 1.02** | 0.08* | 3.26** |
| Error | 60 | 0.46 | 0.74 | 1.1 | 0.08 | 0.04 | 0.007 | 0.037 | 0.79 |
| c.v. % | - | 5.2 | 6.7 | 1.1 | 2.02 | 2.92 | 1.23 | 4.3 | 7.8 |

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

Table 2. Comparison of treatments on growth

| Source of variance | 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) |
|--------------------|----------------------|----------------|------------------------------|---------------------------|-------------------------|--------------------------|------------------------|---------------------|
| 0ppm | 25.92a | 21.75a | 18971a | 28.33a | 13.83a | 11.7a | 6.7a | 17.1a |
| 100ppm | 13.14b | 12.45b | 10236.6b | 12.59b | 6.034b | 7.5b | 4.3b | 11.57b |
| 200ppm | 8.3c | 9.1c | 6202.1c | 9.11c | 4.51c | 5.42c | 4.3b | 9.65c |
| 400ppm | 5d | 7.4d | 3352.6d | 7.36d | 3.72d | 3.66d | 2.72c | 7.05d |
| 0 SA | 9.9e | 10.6c | 7439e | 11.85c | 5.58c | 5.28e | 3.127e | 9.5b |
| 1mM foliage | 12.31d | 13.56a | 10459.8b | 14.68b | 7.27b | 6.88d | 4.38d | 11.53a |
| 2mM foliage | 13.8c | 13.75a | 11199.56a | 15.04a | 7.26b | 7.04c | 4.61c | 11.81a |
| 1mM soil | 14.59b | 12.56b | 9448.25d | 15.01a | 7.37b | 7.9b | 4.94b | 11.75a |
| 2mM soil | 15.15a | 12.87b | 9906.31c | 15.16a | 7.63a | 8.23a | 5.25a | 12.125a |

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

Eco-physiological and biochemical parameters

The results showed that salicylic acid, copper toxicity levels and interaction between them were statistically significant at 1% level (Table 3). Results showed that, the toxicity of copper decreased all fractions of chlorophyll in treated plants considerably (Table 4). Chlorophyll (Fo) and ratio of variable chlorophyll to maximum (Fv/Fm) were significant at 1% level (Table 3). Exposing plant to different levels of Cu resulted in changes of the chlorophyll fluorescence parameters, Fo and Fm, (Table 3). Increasing Cu concentrations resulted in increase of Fo values. In contrast, Fo values decreased significantly for the whole range of Cu concentrations (Table 3). Examination of FV/FM values clearly showed that this parameter had a high correlation with FV and had similar responses (Table 4). In control leaves, FV/FM values were approximately 0.8. Maximum all fractions of chlorophyll, Fv and Fv/Fm were observed in salicylic acid 2mM treatment (Table 4). The results suggested that treatment of salicylic acid 2mM as chelate into soil can greatly reduce the negative effects of copper toxicity on studied parameters. Research in this field (Arduini , 1995; Ariyakanon and Winaipanich, 2006; El-Tayeb , 2006; Hou , 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 fluorescence chlorophyll) (Patsikka , 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 (processes at the pigment level, primary light reactions, thylakoid electron transport reactions, dark-enzymatic stromal reactions and slow regulatory processes)(Goedheer, 1964; Govindjee, 1995). A typical measurement on intact leaf by the saturation pulse method is that the plant was dark adapted for 20 min prior to the measurement. Upon the application of a saturating flash (8000 mmol m⁻²s⁻¹for 1s), fluorescence raises from the ground state value (Fo) to its maximum value, Fm. In healthy leaves, this value is always close to 0.8, independently of the plant species studied (Maxwell and Johnson, 2000). Changes in the dawn Fv/Fm may, however, give important information concerning the effect on the plant of environmental stress. Nesterenko , (2001) and Neves , (2005) reported that in healthy leaves with high Chlorophyll had a maximum (Fv/Fm). The similar results have been reported by Faust (1989).

Table 3. Factorial analysis of the Cu toxicity and SA on ecophysiological & biochemical parameters

| Source of variance | df | Total chlorophyll | Chlorophyll a | Chlorophyll b | F0 | Fv | Fv/Fm |
|------------------------|----|-------------------|---------------|---------------|---------|----------|----------|
| CuSO ₄ | 3 | 0.00013** | 0.00009** | 0.00006** | 416.25* | 636289** | 0.29** |
| SA | 4 | 0.00023** | 0.00004** | 0.00004** | 66.83** | 6200.4** | 0.008** |
| CuSO ₄ * SA | 12 | 0.00005** | 0.00001** | 0.00001** | 30.93** | 1920.9** | 0.0003** |
| Error | 60 | 0.000001 | 0.000003 | 0.000001 | 0.219 | 0.083 | 0.00004 |
| c.v. % | - | 5.3 | 9.8 | 9.3 | 0.29 | 0.07 | 1 |

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

Table 4. Comparison of treatments on ecophysiological and biochemical parameters

| Source of variance | Total chlorophyll | Chlorophyll a | Chlorophyll b | F0 | Fv | Fv/Fm |
|--------------------|-------------------|---------------|---------------|---------|----------|---------|
| 0ppm | 0.0277a | 0.018a | 0.0162a | 152.97d | 638.51a | 0.81a |
| 100ppm | 0.0252b | 0.0163b | 0.0145b | 161.62c | 329.48b | 0.66b |
| 200ppm | 0.0244c | 0.0149c | 0.0140b | 162.1b | 313.138c | 0.6c |
| 400ppm | 0.0214d | 0.0134d | 0.0119c | 162.46a | 233.03d | 0.52d |
| 0 SA | 0.018c | 0.0128b | 0.0112b | 162.37a | 344.22e | 0.0604d |
| 1mM foliage | 0.0252b | 0.0168a | 0.0152a | 159.36c | 384.9c | 0.66b |
| 2mM foliage | 0.0255b | 0.0167a | 0.0153a | 157.49e | 392.51a | 0.67a |
| 1mM soil | 0.0273a | 0.0163a | 0.0147a | 161.38b | 381.06d | 0.65c |
| 2mM soil | 0.0272a | 0.0162a | 0.0146a | 158.37d | 389.91b | 0.653bc |

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

Copper accumulation

Analysis of variance and mean comparison showed that the salicylic acid, copper toxicity levels and their interactions were statistically significant at 1% level (Table 5). The maximum concentration of copper in shoot and root was recorded in 196.22, 249.08 ppm, respectively (Table 6). At all copper concentrations, SA application as

chelate into soil increased Cu accumulation. So application of SA at 2mM chelate into soil can be useful for the phytoextraction of Cu. However, Wang , (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 , 2001), drought (Senaranta , 2002), herbicides (Ananieva , 2004) and metals (Krantev , 2008). It is known that exogenous salicylic acid alleviates the toxic effects generated by Cd in barley (Metwally , 2003) and in maize plants (Pal , 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 , (2003) stated that exogenous salicylic acid causes a reduction in aluminum accumulation in rice.

Table 5. Factorial analysis of the copper toxicity and SA on concentration of copper in *Tagetes erecta*

| Source of variance | df | Cu shoot | Cu root |
|--------------------|----|-----------|------------|
| CuSo4 | 3 | 88434.8** | 249694 ** |
| SA | 4 | 3887.49** | 14212.59** |
| CuSo4* SA | 12 | 410.26** | 2750.8** |
| Error | 60 | 4.92 | 19.17 |
| c.v. % | - | 1.8 | 2.2 |

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

Table 6. Comparison of treatments on concentration of copper in *Tagetes erecta*

| Source of variance | Cu shoot | Cu root |
|--------------------|----------|---------|
| 0ppm | 25.94d | 37.44d |
| 100ppm | 137.81c | 225.65c |
| 200ppm | 155.8b | 234.38b |
| 400ppm | 177.5a | 295.92a |
| 0 SA | 96.65e | 151.7e |
| 1mM foliage | 122.26d | 194.19d |
| 2mM foliage | 130.56c | 197.35c |
| 1mM soil | 132.17b | 221.33b |
| 2mM soil | 134.66a | 227.17a |

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

DISCUSSION

Copper, present 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 , 2004; Drazic and Mitailovic, 2005; Krantev , 2008; Liu , 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 wheat 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 *Tagetes erecta* was 196.2 and 249.08 mg/kg in the experimental pots with 200 mg Cu/kg. *Tagetes erecta* grew and showed low sign of toxicity at the studied Cu contaminated soil (100 mg/kg). Thus, it could be classified as a copper tolerant species. In recent years, researchers discovered that *Tagetes erecta* can accumulate moderate levels of environmentally important metals including Cu and Pb. Wang (2005) reported that *Tagetes erecta* can accumulate Cu in shoot parts of the plant. In addition, there was a significant difference between copper accumulation and SA as chelate into 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, 1997; El-Tayeb , 2006; Hussein , 2007). Additionally, SA application can improve other important traits such as leaf area, dry matter, pigment content and photosynthesis rate (Khan , 2003; Khodary, 2004; Hussein , 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 (Table 3). It seems that SA in foliar spray and chelate into soil concentrations reinforces metabolic responses similar to a growth regulator, influencing photosynthetic parameters and copper uptake relations (Senaranta , 2002; Hayat , 2005 and

2010). For instance, SA application can improve the plant tolerance against heavy metal containment soils (Reeves and Baker, 2000; Srivastava, 2006). In addition to the physiological effect of SA on plant traits and its overall stature (Fariduddin, 2003; Khan, 2003; Khodary, 2004; Hussein, 2007), its role in alleviating the Cu toxicity have been demonstrated (Metwally, 2003; Drazic and Mitailovic, 2005; Krantev, 2008). The results were indicative that SA protects plants against stress caused by Cu stress. For *Tagetes erecta*, 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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