Phytoremediation potential of copper contaminated soils in *Calendula officinalis* and effect of salicylic acid on the growth and copper toxicity

Maryam Afrousheh¹, Mahmud Shoor^{*2}, Ali Tehranifar², Vahid Reza Safari³

¹Horticultural Science department, Ferdowsi University of Mashhad, International Campus

²Department of Horticultural Science and Landscape, Ferdowsi University of Mashhad

³Department of Horticultural Science, Shahid Bahonar University of Kerman

*E-mail address: shoor@ferdowsi.um.ac.ir.

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ABSTRACT

The present research was carried out to assess the effectiveness *Calendula officinalis* on removing the Cu from the contaminated soil and to examine the effects of salicylic acid (SA) on growth and some eco-physiological, biochemical characteristics in plants exposed to copper stress. 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, 2mM) as foliar spray and 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. Although the copper concentration in shoots of *Calendula officinalis* could not exceed 1,000 mg/kg dry weight, the results showed that copper accumulation was higher in the shoots than in the roots. The results of the concentration of copper showed that translocation (TF) and bio-concentration factor (BCF) was greater than 1. Thus, *Calendula officinalis* could be classified as a copper tolerant species. Application of SA was found to alleviate negative effects generated by heavy metals like copper in plants. SA significantly increased growth and resistance index in copper stressed plants. SA as chelate in concentration of 2mM had the greatest effect on studied parameters. Results showed that salicylic acid can increase the efficiency of phytoremediation successfully.

1. INTRODUCTION

Many soils are contaminated with heavy metals due to industrial dumping of toxic waste directly onto land or into water resources (Yell Yang, 2000; Alloway, 1990). 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 *et al.*, 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 in high soil copper (Arduini *et al.*, 1995 ;Ariyakanon and Winaipanich, 2006). Copper toxicity due to many aspects of its behavior inhibits iron metabolism and the activities of many enzymes (Arnon, 1949; Mattioni *et al.*, 1997; Harris, 2000; Hou *et al.*, 2007). Existence of polluted material with copper in soil causes the major reduction of agricultural products. 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 *et al.*, 2006). With respect to heavy metal contamination, phytoremediation is a technology that works on removing contaminants from the surface layers of soil (Chaney *et al.*, 1997; Brooks *et al.*, 1998; Khan *et al.*, 2000; Chen *et al.*, 2000; Ensley, 2000). The success of

phytoremediation for removal of contaminants is dependent of several physiological characteristics of the plant. The main factors controlling the ability of phytoremediation are plant species, metal availability to plant roots, metal uptake by roots, metal translocation from roots to shoots and plant tolerance to toxic metals (Baker and Brooks, 1989; Shu *et al.*, 2002; Market, 2003; McGrath and Zhao, 2003). Efficient removal of the contaminant is possible through the continuous growth and harvesting of a high biomass-producing, hyper-accumulator species (Raskin *et al.*, 1997). 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 (Brooks *et al.*, 1998). However, a literature review did not indicate any study addressing the effects of SA on metal uptake and accumulation by species. 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. 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). For this study, three assumptions were made regarding the plants ability to remove copper. First, the translocation factors must be invariably higher than one ($TF > 1$). This indicates an efficient ability to transport metals from roots to shoots and, most likely, the existence of tolerance mechanisms to cope with high concentrations of metals (McGrath and Zhao, 2003). Second, bio-concentration factor (BCF). Plants with both bio-concentration factor and translocation factor greater than one (TF and $BCF > 1$) have the potential to be used in phytoextraction. Besides, plants with bio-concentration factor greater than one and translocation factor less than one ($BCF > 1$ and $TF < 1$) have the potential for phytostabilization (Yoon *et al.*, 2006). Third, the enrichment factor (EF) that the plants' ability to remove copper, the basis for this study. Both enrichment factor (EF) and translocation factor (TF) have to be considered while evaluating whether a particular plant is a metal hyper-accumulator (Ma *et al.*, 2001). The enrichment factor is calculated as the ratio plant shoot concentration to soil concentration ($[Metal]_{shoot} / [Metal]_{Soil}$) (Branquinho *et al.*, 2006). The enrichment factor must be higher than one ($EF > 1$), meaning higher metal concentrations in the plant than in the soil, which emphasizes the degree of plant metal uptake (McGrath and Zhao, 2003; Yanqun *et al.*, 2005). There has been a continuing interest in searching for plants that are tolerant towards heavy metals (Shu *et al.*, 2002; McGrath and Zhao, 2003). Therefore, a hyper-accumulator plant should have $EF > 1$ or $TF > 1$, as well as total accumulation $> 1000 \text{ mg kg}^{-1}$ of Cu, Co, Cr, Ni or Pb, or $> 10000 \text{ mg kg}^{-1}$ of Fe, Mn or Zn (Baker and Brooks, 1989; Baker, 1994) and (Market, 2003). In phytoremediation, screening out effective hyper-accumulators has become important, however, limited hyper-accumulators have been reported (Liu *et al.*, 2004; Meers *et al.*, 2005). Thus, it is necessary to search for more hyper-accumulators to remedy contaminated soils effectively (Zhou and Song, 2004). Recently, heavy metals are known as pollutants that are more dangerous than the other pollutants. With an increase in the contamination of urban area, more attention has been paid to the role of ornamental plants in remedying contaminated soils and water (Ma, 2003; Hern'andez-Apaolaza *et al.*, 2005; Wang, 2005; Liu *et al.*, 2006; Zhou, 2006). *Calendula officinalis* L. Which is an herbaceous plant belonging to compositae with potential of heavy metals absorption and is able to tolerate high concentrations of heavy metals (Liu *et al.*, 2008) is selected for this research study. 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 and induces resistance to abiotic stress (Rugh *et al.*, 2000; Berukova *et al.*, 2001; Mazaheri Tirani and Manochehri-Kalantari, 2007). The objective of this work was to investigate whether salicylic acid could be a protectant to ameliorate the influence of copper stress on *Calendula officinalis* and thereby increasing its copper tolerance. In other words, the study aims at investigating if this plant has the potential to absorb heavy metals and tolerate high concentrations of copper.

2. MATERIAL AND METHODS

2.1. 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 property (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 CuSO₄ (0, 100, 200 and 400 mg) 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

Sand: Silt: Clay	EC (ds/m)	pH	CEC (cmol/kg)	OC (%)	P (mg/kg)	K (mg/kg)	Cu (ppm)
44:38:18	1.5	7.3	21.75	0.4	7	220	0.38

2.2. Plant, harvest and analysis

Seeds of *Calendula officinalis* 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 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 *Calendula officinalis* were 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). Plant samples were gently removed from the pots 75 days for the measurement of various growth parameters 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 65°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 (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.

3. RESULTS

3.1. Growth parameters

In this study, the effect of copper toxicity, salicylic acid and interaction between them on height, root length, leaves number, leaf area, resistant index (root length in untreated plant to treated plant) 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 decreased by increasing in concentration of copper (Table 3). The highest effect of salicylic acid treatments on growth parameters was 2 mM as chelate into soil (Table 3). 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 (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*.

3.2. Concentration of copper

Analysis of variance and mean comparison showed that the salicylic acid, copper toxicity levels and their interactions were statistically significant at 1% level (Table 2). Maximum Concentration of copper in shoot and root was recorded in 200, 400 ppm, respectively (Table 4). As translocation factor, enrichment factor and bio-concentration factor was significant in 200 ppm than the other treatments. The results of the concentration of copper showed that translocation (TF) and bio-concentration factor (BCF) was greater than 1. It showed positive effect of salicylic acid as chelate into soil on translocation, enrichment and bio-concentration factor of *Calendula officinalis* (Table 4). At all concentrations tested, 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 *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.*,2000), 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.

4. 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 *et al.*, 2004; Drazic and Mihailovic, 2005; Krantev *et al.*,2008; Liu *et al.*,2009). *Calendula officinalis* grew well and showed no sign of toxicity at the studied Cu contaminated soil (100 mg/kg). The results showed that the maximum concentrations of copper in shoot and root of *Calendula officinalis* was 348.5 and 459.12 mg/kg in the experimental pots with 200 mg Cu/kg. Copper accumulation was higher in the shoots than in its roots in 200 mg Cu/kg. For the 400 mg Cu/kg amended soils, the copper accumulation decreased due to the lower biomass of plants. A plant's ability to accumulate metals from soils can be estimated using the BCF and a plant's ability to translocate metals from the roots to the shoots is measured using the TF. Although the copper concentration in shoots of *Calendula officinalis* could not exceed 1,000 mg/kg dry weight, the total amount of copper accumulated in shoots and roots was high (459.12, 348.5 mg/kg). The results of the concentration of copper showed that translocation (TF) and bio-concentration factor (BCF) was greater than 1 and was suitable for phytoextraction of Cu. Thus, it could be classified as a copper tolerant species. In recent years, researchers discovered that *Calendula officinalis* can accumulate moderate levels of environmentally important metals including Cd and Pb. Liu *et al.*, (2006) reported that *Commelina communis* can accumulate Cd in 100 ppm in root 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. Previous studies have shown that salicylates can change the ion permeability of root cells. 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 and dry matter (khan *et al.*, 2003; Khodary, 2004; Hussein *et al.*,2007). 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 *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 traits and its overall stature (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 indicative that SA protects plants against tens caused by Cu stress. For *Calendula officinalis*, SA as chelate to soil should be applied to increase the solubility of copper in the soil solution and copper accumulation in the plants.

Table 1. Factorial analysis of the copper toxicity effect and SA on growth stage of *Calendula officinalis*.

Source of variance	df	Height of plant (cm)	Number of leaf	Leaf area (cm 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)	resistance index
CuSO ₄	3	1516**	352.1**	165742**	78.19**	19.13**	37.7**	14.14**	** 202.7	** 2.35
SA	4	25.9**	18.14**	13384**	0.93**	0.255**	0.79**	0.24**	7.6**	0.086**
CuSO ₄ * SA	12	6.42**	1.45**	4449**	1.018**	0.24**	0.25**	0.07**	1.56**	0.017**
Error	60	0.2	0.22	0.0996	0.026	0.01	0.0023	0.0022	0.32	0.003
c.v. %	-	3.3	5.1	0.22	3.26	4.2	1.32	2.45	8.3	8.3

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

Table 2. Factorial analysis of the copper toxicity and SA on *Calendula officinalis*.

Source of variance	df	Cu shoot	Cu root	Translocation factor (TF: shoot/root)	Bio-concentration factor (BCF: root/soil)	Enrichment factor (EF: shoot/soil)
CuSO ₄	3	226927.62**	311485 **	0.9404**	1.1577**	7.95**
SA	4	39495.86**	87763**	0.04618**	0.7710**	1.404**
CuSO ₄ * SA	12	9804.53**	36286**	0.2377**	0.1433**	0.707**
Error	60	62	177	0.0144	0.0126	0.0329
c.v. %	-	5	7	6.1	7	8.11

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

Table 3. Comparison of treatments on growth stage of *Calendula officinalis*

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)	resistance index
0ppm	24.95a	13.8a	26349a	7.3375a	3.63a	5.64a	3.31a	10.77a	1.158a
100ppm	15.5b	10.05b	153038b	5.94b	2.88b	3.56b	1.99b	7.03b	0.75b
200ppm	9.85c	9.6c	7812.12c	3.38c	1.64c	2.87c	1.6c	6.4c	0.69c
400ppm	4.6d	3.65d	6504.2d	3.33c	1.65c	2.62d	1.47d	3d	0.32d
0 SA	12.5d	7.37b	9281.5e	4.62d	2.26c	3.394e	1.95e	5.65d	0.6d
1mM foliage	12.62d	9.81a	13958.4d	4.9c	2.39b	3.577d	2.04d	6.81c	0.73c
2mM foliage	13.37c	9.75a	13989.88c	5.08b	2.51a	3.647c	2.08c	6.87bc	0.74cb
1mM soil	14.87b	9.62a	16350.75b	5.14ab	2.53a	3.737b	2.14b	7.25ab	0.78ab
2mM soil	15.25a	9.81a	16381.3a	5.24a	2.58a	4a	2.28a	7.41a	0.8a

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

Table 4. Comparison of treatments on concentration of copper of *Calendula officinalis*.

Source of variance	Cu shoot	Cu root	Translocation factor (TF:shoot/root)	Bio-concentration factor (BCF:root/soil)	Enrichment factor (EF:shoot/soil)
0ppm	7.5d	4.06d	1.78a	0.397d	0.211d
100ppm	199.45c	117.75c	1.697b	1.994b	1.177b
200ppm	459.12a	348.5b	1.33c	2.298a	1.745a
400ppm	455.7b	449.6a	1.017d	1.139c	1.125c
0 SA	243.937e	217.1c	1.175c	1.2e	1.006c
1mM foliage	256.65d	195.9d	1.399b	1.23d	0.8593e
2mM foliage	264.68c	219.8c	1.444b	1.26c	0.9712d
1mM soil	317.62b	246.47b	1.478b	1.75b	1.165b
2mM soil	319.37a	270.62a	1.783a	1.83a	1.322a

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

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