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Salicylic Acid Alleviates the Copper Toxicity in Sunflower Seedlings

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Abstract: The effect of salicylic acid (SA: 0.5 mM) on the plant growth, copper accumulation, Cu-bound proteins and protein pattern in roots and shoots of *Helianthus annuus* plants under Cu stress (5 mg L^{-1}) was studied. Cu stress reduced the fresh and dry weights of sunflower plants. Cu markedly accumulated in Cu-stressed plants. However, this accumulation of Cu was higher in roots than in shoots. Chromatography of cell free extract of Cu-binding proteins of cu-stressed plants revealed three main protein peaks in roots and four peaks in leaves. The main peak for copper is coincident with protein of molecular weight of 75 kDa and contains about 89.40 and 80.84% of the total copper in the soluble fraction in roots and leaves, respectively. Proteins pattern shows that, Cu-stress induced the synthesis of new polypeptides of 178 and 97 kDa in plant roots and 105, 81 kDa in case of plant leaves. SA increased the fresh and dry weights of Cu-stressed plants. SA lowered the Cu content both in the roots and shoots of Cu-stressed plants. This was associated an increase in the endogenous SA content in the two organs. Cu plus SA treatment revealed 4 protein peaks with molecular weights about 175, 130, 75 and 35 kDa in roots and 175, 130, 100 and 45 kDa in case of leaves. The main peak for copper is coincident with protein of molecular weight 75 kDa in roots and 100 kDa in leaves, retaining about 82.90 and 74.72% of the total copper in the soluble fraction. Under Cu stress, SA induced the synthesis of 5 new polypeptides with molecular weight ranged from 204 to 26 kDa in roots and 4 new polypeptides ranged from 189 to 6 kDa in case of leaves. The results indicate that salicylic acid can alleviate the adverse effects of copper on the growth of sunflower plants by interfering of SA in translocation of Cu and/or increasing Cu-binding proteins in sunflower.

Key words: Copper toxicity, Cu-binding proteins, *Helianthus annuus*, salicylic acid

INTRODUCTION

Copper loading of agricultural soils may originate from the application of sewage sludge or fungicidal sprays. Although Cu is an essential element for plant growth (Arnon and Stout, 1939), its accumulation in soils may be toxic to plants (Baryla *et al*., 2000). Cu is also known to damage cell membranes (De Vos *et al*., 1989, 1992) and can catalyze the formation of active oxygen species in the cell in Haber-Weiss reaction (Kurepa *et al*., 1997). Since oxidative stress produced in plants exposed to high metal concentration, the formation of phytochelatins is a part of the cellular response to metal toxicity (Mazhoudi *et al*., 1997).

Heavy metals taken up by the roots accumulate in different parts of plants (Salt *et al*., 1995). Jarvis and Jones (1978) concluded that cadmium contents of the roots were much larger than that of the shoots and although older plants contained more cadmium than young plants of the perennial ryegrass (*Lolium perenne* var. S23) grown in 0.01 mg L^{-1} for 15 days.

The metal-binding peptides are synthesized, chelated and inactivated every toxic metal ion entering the cytosol before they can inactivate the enzymes of essential metabolic routes. A side effect from detoxification, phytochelatins play a role in homeostasis of heavy metals in plants and this is the mechanism that regulates the metal-ion availability in plant cells (Thomine *et al*., 2000). Metallothioneins are high molecular weight proteins, which bind heavy metals and are found in animals and plant kingdoms. These proteins also play an important role in detoxification by sequestering metals in plant cells (Murphy and Taiz, 1997; Liu *et al*., 2000).

Plants respond to stress by synthesis of signal molecules. These molecules activate a rate of signal transduction pathways, some of which help the plant to overcome the stress. Elucidation of such signal transduction pathways has been studied through the response to exogenous application of salicylic acid, at the physiological and biochemical levels. Salicylic Acid (SA), one of these signals, is a ubiquitous plant phenolic that controls plant growth and development

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(Raskin, 1992). Several reports support the major role of SA in ameliorating the plant sensitivity to abiotic stresses.

The present study was conducted to study the effect of exogenous SA on plant growth, protein pattern and Cu accumulation in roots and shoots of Cu-stressed sunflower plants. The cu-binding proteins and the endogenous SA content are of our concern.

MATERIALS AND METHODS

Plant material: Seeds of *Helianthus annuus* L. obtained from the Agronomy Department, Faculty of Agriculture, Assiut University, Assiut, Egypt were germinated on moist filter paper in the dark at room temperature. Four-day-old seedlings were placed in plastic pots containing 1 L nutrient solution. The germinated seeds were selected and transferred to half-strength Hoagland's solution (Arnon and Hoagland, 1940) and the solution pH was maintained at 5.5±0.2. Four day old seedlings were planted in plastic pots (five seedlings per pot), containing 1 L of the freshly prepared nutrient solution.

Cu and salicylic acid treatments: The two-weeks old plants were treated with two concentrations of Cu (0 and 5 mg L^{-1}) supplied as $CuCl_2$ in the presence and absence of 0.5 mM salicylic acid. This was carried out by exposing the plant roots for two weeks to nutrient solution containing 5 mg L^{-1} Cu with Cu free half strength nutrient solution was used as a control. All the solutions were renewed at an interval of every three days and the pH was adjusted to 5.5±0.2. There were three replications per treatment with five plants per replication. After four weeks, two seedlings of each replicate were used for fresh and dry weight determination and then ground to powder for further analysis. The remaining seedlings were divided into roots, stems and leaves and then frozen in liquid nitrogen and stored at -20°C for biochemical analysis.

Cu and salicylic acid determination: The total copper content assayed photometrically by flame atomic absorption spectrophotometry Model (Buck Scientific, Model 210 VGP) in the Unit of Analytical Chemistry in Department of Chemistry, Faculty of Science Assiut University. The data were expressed as mg Cu g^{-1} dry weight. Free salicylic acid was determined according to Siegrist *et al.* (2000) with some modifications. For SA extraction 0.2 g of frozen roots and shoots were homogenized in 0.5 mL of MeoH (85% V/V). SA was analyzed by HPLC using fluorescence detector at 210 and 410 nm emission in the lab of Professor Dr. Karl-Josef Dietz University of Bielefeld, Germany.

Gel filtration and gel electrophoresis: Copper makes complex with proteins called Metallothioneins or metal-binding proteins, which isolated according to (Grill *et al.*, 1985). The purified and concentrated protein of different samples (5 mL each) applied to sephadex G-50 column and the absorbance at 254 nm of every fraction recorded and the Cu in each sample measured using AAS.

Protein pattern was analyzed using SDS-PAGE (12%) according to the method of Laemmli (1970) in the first dimension. Electrophoresis was carried out at 2 mA (100 v) for each well for 60 min and then at 3 mA (200 v) until the tracking dye reached the bottom of the gel. Gels were scanned with Biorad video densitometry model GDS-8000 System and analyzed using gel analyzer Phoretix 1D Advanced V 2.5.

Statistical analysis: The data were statistically analyzed by one-way analysis of variance and the Least Significant Difference (LSD) test was used to separate the means at $p = 0.05$.

RESULTS

Excess of copper in the rooting medium markedly reduced the fresh and dry weights of sunflower plants (Fig. 1). SA application increased both the fresh and dry weight of sunflower plants under control and Cu-stress conditions. The data also revealed that, SA induced higher fresh and dry weight of Cu-stressed plants even than those of control ones (Fig. 1). This indicates that SA could counteracts the inhibitory effects of copper on the plant growth.

To evaluate the effect of salicylic acid on copper accumulation in the organs the stressed sunflower plants, the copper content of both the roots and shoots were determined. The results show that, SA has no effect on the copper content in both the roots and shoots of control plants. Under Cu stress, SA application reduces the accumulation of Cu in shoots, while it has no significant effect in case of plant roots. Plants subjected to copper stress in presence and absence of salicylic acid exhibited higher Cu accumulation in roots than that of shoots. The results also show that endogenous SA content increased in both roots and leaves of control and Cu-stressed plants with exogenous SA application (Fig. 1).

Cell-free extracts of roots and leaves of sunflower plants grown under various treatments were subjected to gel filtration chromatography. Root cell-free extracts from control plants shown in Fig. 2, revealed 5-protein peaks (1, 2, 3, 4 and 5) with molecular weights of 175, 130, 100, 75

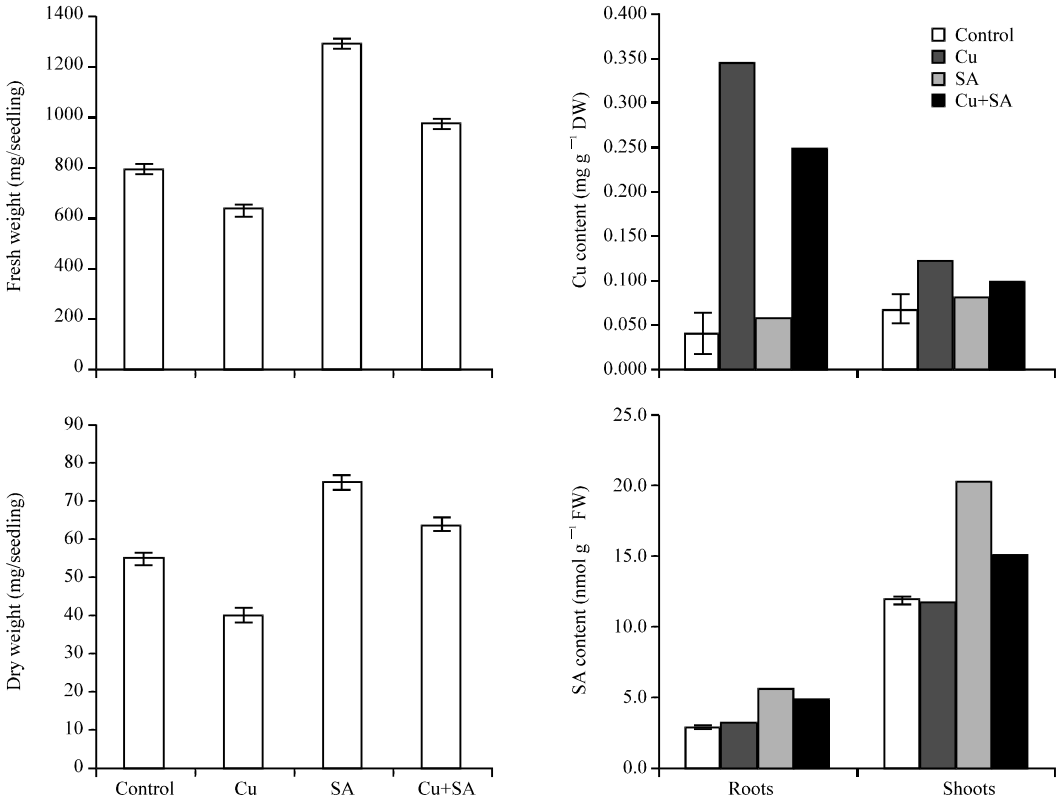

Fig. 1: Effect of exogenous salicylic acid (0.5 mM) on fresh and dry weights (mg seedling^{-1}), copper content (mg g^{-1} DW) and salicylic acid content (nmol SA g^{-1} FW) in roots and shoots of copper-stressed sunflower plants

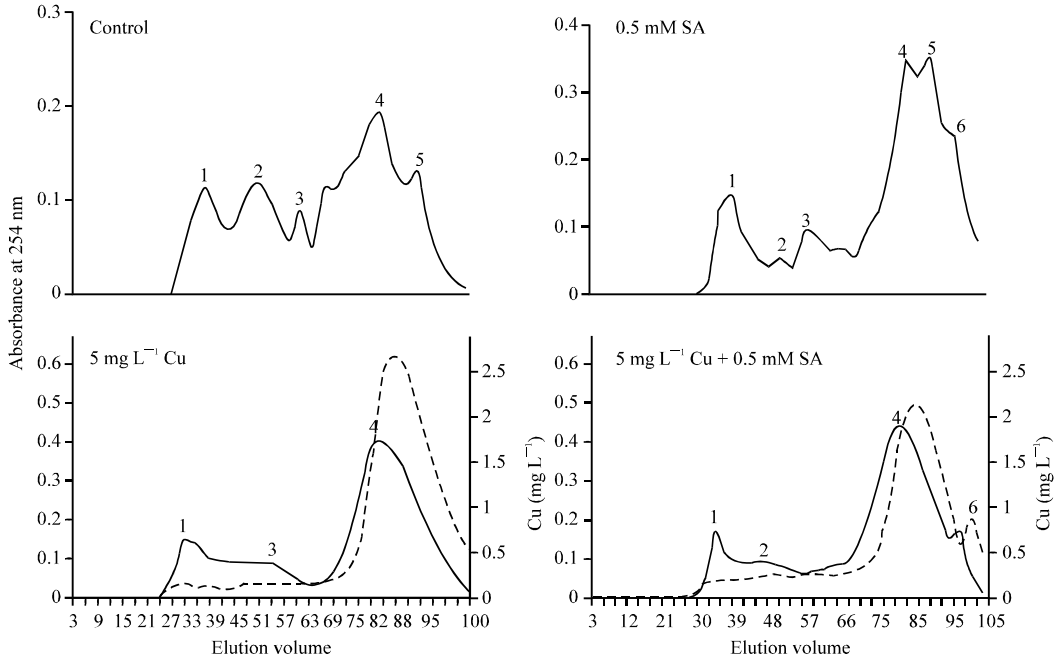

Fig. 2: Fractionation of root cell free extract of *Helianthus annuus* plants grown at 0 (control) and 5 mg L^{-1} copper for two weeks with or without SA (0.5 mM), applied on sephadex G.75 column (2.4×60 cm). Flow rate 0.5 mL min^{-1}, Elution volume 3 mL. Copper was measured using AAS

Table 1: The molecular weights of protein peaks and the Cu-bound protein (μg/peak) in roots and leaves of *Helianthus annus* plants as influenced by Cu (5 mg L^{-1}) and salicylic acid (0.5 mM) treatments

			Roots						Leaves			
Peak No.	Elution volume	Mw (kDa)	0	Cu	SA	Cu+SA	Elution volume	Mw (kDa)	0	Cu	SA	Cu+SA
1	33	175-170	+	+	+	+	33	175-170		+	+	+
Bound Cu			-	0.602		0.780			-	0.233	-	0.217
2	51	130-125	+	-	+	+	51	130-125	+	-	+	+
Bound Cu			-	-	-	1.174			-	-	-	0.351
3	63	100-95	+	+	+	-	63	100-95	+	+	+	+
Bound Cu			-	0.754	-	-			-	0.141	-	2.88
4	75	75-65	+	+	+	+	75	75-65	+	+	-	-
Bound Cu			-	16.003	-	11.234			-	2.959	-	-
5	95	45-40	+	-	+	+	95	45-40	+	-	-	+
Bound Cu			-	-	-	-			-	-	-	0.209
6	102	35-25	-	-	+	+	102	35-25	-	+	-	-
Bound Cu			-	-	-	1.971			-	0.130	-	-

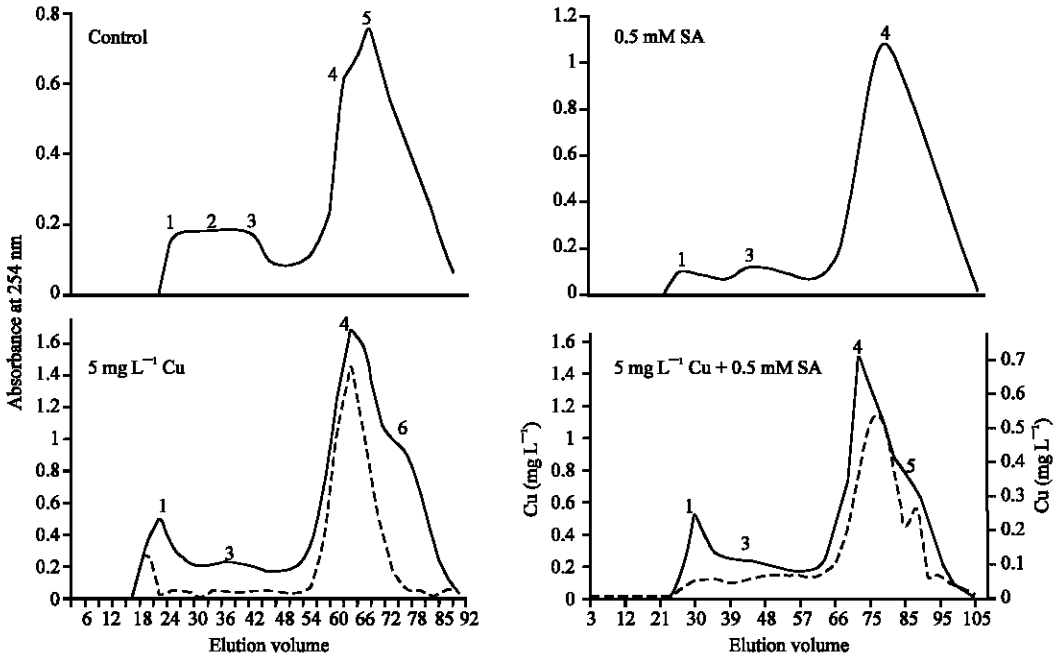

Fig. 3: Fractionation of leaf cell free extract of *Helianthus annuus* plants grown at 0 copper (control) and 5 mg L^{-1} copper for two weeks with or without SA (0.5 mM), applied on sephadex G.75 column (2.4×60 cm). Flow rate 0.5 mL min^{-1}, Elution volume 3 mL. Copper was measured using AAS

and 45 kDa (Table 1). In root cell-free extracts from plants treated with 5 mg L^{-1} of Cu, the elution diagram revealed three main peaks (1, 3 and 4). It also shows that one main peak for copper coincident with protein peak 4, which represent about 89.40% of the total copper in the soluble fraction. The elution diagram of root cell-free extracts from plants treated with SA, revealed 6-protein peaks with molecular weights of 175, 130, 100, 75, 45 and 35 kDa. Plants treated with copper and salicylic acid exhibited four protein peaks (1, 2, 4 and 6). It also shows that one main peak for copper coincident with protein peak 4, which retained about 82.90% of the total copper in the soluble fraction.

The chromatographs of leaf cell-free extracts of variously treated plants are given in Fig. 3. These elution diagrams revealed five protein peaks (1, 2, 3, 4 and 5) with molecular weights of 175, 130, 100, 75 and 45 kDa for control plants (Table 1). In case of plants treated with 5 mg L^{-1} of Cu, there is one peak for copper coincident with protein peak 4 and peak 4 of fractionated protein was maximal and represents about 80.84% of total copper in soluble fraction. SA application to control plants resulted in three protein peaks (1, 3 and 4) with molecular weights of 175, 130 and 100 kDa. In case of plant treated with 5 mg L^{-1} of Cu plus SA (Fig. 3), there are four protein peaks and the main peak

Table 2: Summarize the molecular weights (KDA) of the electrophoretic protein bands of sunflower plants roots and leaves shown in Fig. 4. Lane 1(control), lane 2 (Cu), lane 3 (SA) and lane 4 (Cu + SA)

		Roots						Leaves			
Band No.	Mw (KDa)	0	Cu	SA	Cu+SA	Band No.	Mw (kDa)	0	Cu	SA	Cu+SA
1	204	-	-	-	+	1	200	+	+	+	+
2	199	+	-	+	-	2	189	-	-	-	+
3	189	+	+	-	-	3	180	-	-	+	-
4	178	-	+	-	-	4	179	+	+	-	-
5	174	+	-	-	-	5	135	+	+	-	-
6	169	-	-	+	+	6	123	-	-	-	+
7	116	+		-	-	7	121	+	-	-	-
8	107	-	-	-	+	8	105	-	+	-	-
9	106	-	-	+	-	9	96	-	-	+	-
10	97	-	+	-	-	10	81	-	+	-	-
11	52	-	-	-	+	11	46	-	-	-	+
12	49	+	-	-	-	12	44	+	+	+	+
13	45	+	-	-	-	13	35	+	-	-	-
14	29	-	-	-	+	14	6	-	-	-	+
15	26	-	-	-	+						
16	24	-	-	+	-						

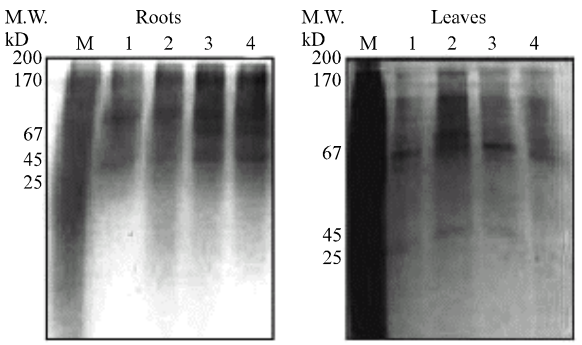

Fig. 4: SDS-PAGE protein profile of *Helianthus annuus* treated with 0 and 5 mg L^{-1} copper in presence and absence 0.5 mM salicylic acid. (M = Marker protein), Lane 1 = Control; Lane 2 = 5 mg L^{-1} Cu; Lane 3 = 0.5 mM SA; Lane 4 = 5 mg L^{-1} Cu+ 0.5 mM SA

for copper coincident with protein peak 4 which represented about 74.72% of total Cu in the soluble fraction.

Protein pattern of roots and leaves cell-free extracts of sunflower plants grown at 0 and 5 mg L^{-1} of Cu in presence and absence of 0.05 mM SA are shown in Fig. 4. In roots, analysis of protein profile revealed the molecular weights of different protein bands according to their densities as shown in Table 2. Lane 1 shows the electrophoretic bands of control plants. This lane has 6 polypeptides with Mw of 199, 189, 116, 107, 149 and 45 kDa. Polypeptides in the roots of Cu-stressed plants are present in lane 2, which contain 3 polypeptides of Mw 189, 178 and 97 kDa. Lane 3, shows electrophoretic bands of plant treated with SA only, this lane contain 4 bands with high weights, 199, 169, 106 and 24. An individual protein band of high molecular weight 204 kDa was appeared in roots of Cu-stressed plants treated with SA in lane 4, which has also 5 bands of Mw 169, 107, 52, 29 and 26 kDa.

Figure 4 also shows the electrophoretic protein bands of leaves of plants grown at 0 and 5 mg L^{-1} of Cu with or without SA. Densitometry computer program showing the MWs of protein bands as given in Table 2. The data reveals that a great variation in molecular weight of polypeptides presents in control and variously treated sunflower plants. Lane 1 shows the protein bands of control plants that have 6 polypeptides of molecular weights ranged from 200, 179, 135, 121, 44 and 35 kDa. Leaves of Cu-stressed plants contain 6 polypeptides of Mw 200, 179, 135, 105, 81 and 44 kDa. Four protein bands with molecular weight of 200, 180, 96 and 44 kDa appeared in sunflower leaves treated with SA only. Six protein bands present in leaves of plants treated with Cu and SA in combination, these proteins have molecular weights 200, 189, 123, 46, 44 and 6 kDa.

DISCUSSION

Excess of Cu in the rooting medium markedly decreased the growth of sunflower seedlings. A decrease in growth of cucumber plants in response to Cu stress has been reported by Alaoui-Sosse *et al.* (2004). This decrease could be attributed to its interference with metabolic processes associated with normal development (Van Asshe and Clijster, 1990; Lidon and Henriques, 1992). In the present study, SA application enhanced plant growth under both control and Cu-stress conditions (Fig. 1). In consistence with this, Metwally *et al.* (2003) and Drazic and Mihailovic (2005) who found that exogenous SA alleviated the inhibitory effect of Cd on plant growth of barley and soybean, respectively.

The data showed that, Cu stress induced an increase in the Cu ion concentration in sunflower seedlings (Fig. 1). However, this accumulation of Cu was higher in roots than in shoots. These results are generally in accordance with those obtained by some authors (El-Enany, 2000; Gonnelli *et al*., 2001; Alaoui-Sosse *et al*., 2004; Demirevska-Kepova *et al*., 2004). Application of salicylic acid to the control plants has no effect on the copper content in roots and shoots of sunflower plant. Under Cu stress, SA application has no effect on Cu accumulation in plant roots while it caused a significant decrease in Cu accumulation in case of plant shoots. Consistently, Pal *et al*. (2002) found that Cd content was significantly lowered in *Zea mays* plants when SA and Cd were applied together.

Cu stress increased endogenous salicylic acid content in roots, while it has no significant effect on SA content in case of shoots (Fig. 1). Application of exogenous SA increased SA content in both the roots and shoots of control and Cu-stressed plants. SA has been shown to accumulated in response to several other biotic and abiotic stresses. SA has been also shown to increase by 4 to 5 fold after exposure of *Arabidopsis* to ozone for 3-6 h (Sharma *et al*., 1996). Furthermore, SA accumulated during exposure of tobacco to ultraviolet (Yalpani *et al*., 1994). Thus, SA might be a common link among various stress-activated pathways.

The adaptation of plants to toxic concentration of metals depends upon various mechanisms operating at the plant, cell and intercellular levels. Protein fractionation of roots and leaves of control and variously treated plants revealed six protein peaks, coincident with Cu maxima (Fig. 2 and 3). These results are in agreement with the findings obtained by Neumann *et al*. (1997). The mechanism of metal tolerance based on metal-binding proteins could operate in conjunction with other sub-cellular mechanisms (Palma *et al*., 1990; Wojtaszek *et al*., 1997). However, Cu was retained by roots in a high quantity compared to its accumulation in the sunflower leaves (Fig. 1). This may be explained as a specific strategy of the plant to storage and to inactivate the excess of toxic metals in cell walls of roots. In this respect Cuypers *et al*. (2005) concluded that Cu treatment increased the level of proteins in the roots of *Phaseolus vulgaris* plants, three proteins apparently reacting in a dose-dependent manner to Cu exposes. The level of an intracellular pathogenesis-related proteins and a newly identified protein homologous to Pvpp and Pvpr was increased with increasing in Cu concentration. Our results indicate that, SA application increased the Cu-bound proteins, which associated with an increase in endogenous SA content in both roots and leaves of the Cu-stressed plants (Table 1 and Fig. 1). In consistence with this, Schaller *et al*. (2000) who found that, the accumulation of PR protein transcripts was parallel by an increase in leaf salicylic acid content.

Proteins pattern of roots and leaves reveal that, new polypeptides with 178 and 97 kDa in plant roots and 105, 81 kDa in case of leaves were exhibited in response to Cu stress. Protein kinases are involved in stress signaling in plants and that many general mechanisms of signal transduction are common among all eukaryotic cells (Stone and Walker, 1995). The most prevalent of these are calcium-dependent protein kinases, which participate in transduction of stress signals in plants (Sheen, 1996). Under Cu stress, SA application resulted in the synthesis of new polypeptides with molecular weight of 204-26 kDa in roots and 189-6 kDa in case of leaves. Mikolajczyk *et al*. (2000) demonstrated that SA induced protein kinase of Mw 48 kDa. The accumulation of specific proteins is a common response of plants to various environmental stresses (Przymusinski *et al*., 2004; Cuypers *et al*., 2005). Przymusinski *et al*. (2001) found that Pb stimulates the accumulation of 16 kDa polypeptide, which was identified as a member of PR-10 pathogenesis related protein. They also indicated that signaling substances like SA, H_2O_2, or ethylene are capable to induce the synthesis of stress-proteins. It worth notice that, SA application specified with the synthesis of new polypeptide with high molecular weight of 204 kDa in roots of Cu-stressed plants.

The results of this study indicate that, the adverse effects of copper stress on growth of sunflower plants could be alleviated by SA application by inducing the production of stress proteins and/or stimulation Cu incorporation into Cu-binding proteins. Such effects of salicylic acid may play a role in the adaptive response to Cu pollution and indicate that SA may increase the ability of roots and shoots of plants to inactivate the excess of toxic elements in the cell walls.

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