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Salicylic acid decreases Cd toxicity in sunflower plants

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ABSTRACT

The ameliorative effect of salicylic acid (SA) on cadmium (Cd) toxicity in sunflower plants was studied by investigating leaves protein content and fatty acid composition. Sunflower plants in two leaves stage were exposed to $CdCl_2$ treatment (0, 50,100,150 and 200 µM) and then were treated with salicylic acid (0, 250 and 500 µM) as foliage spraying. One week after the last salicylic acid treatment, plants were harvested and growth parameters and protein content were measured. Oil of leaf was extracted in a Soxhlet system and fatty acid composition were measured by gas chromatography (GC). Statistical analyses showed excess Cd reduced fresh weight and number of leaves and SA increased them compared with the control. Maximum reduction in these parameters was at 200 µmol Cd and 0µmol of SA. Protein content in leaves of sunflower was decreased with increasing concentrations of Cd. Exogenous application of SA increased the amount of protein in sunflower plants exposed to Cd stress. Cd×SA interaction on protein content in leaves was significant. Cd caused a shift in fatty acids composition, resulting in a lower degree of their unsaturation and an increase in saturated fatty acids in sunflower leaves,whereas SA improved them. SA, particularly increased the percentage of linolenic acid and lowered that of palmitic acid by the same proportion. These results suggest that SA alleviated the inhibitory effects of Cd on protein content and could be used as a stabilizer of membrane integrity due to lipids protection of cadmium-induced oxidative stress to improve plant resistance to Cd stress.

Keywords: Cadmium toxicity, Fatty acid, Helianthus annuus L., Protein content, Salicylic acid

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INTRODUCTION

Cadmium (Cd) is one of the most toxic metals in the environment that is toxic to many plant species at low concentrations [23]. Cadmium accumulation in soils may originate from different sources, including air pollutants and soil application of commercial fertilizers, sewage sludge, manure and lime [12].

The high mobility of this metal in soil-plant system allows its easy entry into the food network, which may inciting any toxic effects on plants, animals and humans [18]

Cadmium can cause many toxic symptoms in plants, such as the inhibition respiratory, photosynthesis and nitrogen metabolism , activation or inhibition of enzymes, disturbances in plant–water relationships and the ion metabolism, resulting in low biomass accumulation and growth inhibition [21,27] At cellular level, Cd toxicity lead to the overproduction of reactive oxygen species (ROS) in plants which are highly reactive and toxic and cause damage to membrane integrity due to lipid peroxidation, which may result in generation of highly cytotoxic compounds and reduction of plant development [8].

Varied defense processes in plant cells are activated during exposure to Cd such as complexing of the metal by phytochelatins and metallothioneins, compartmentalization in vacuoles, immobilization at the level of cell wall, exclusion through action of plasma membrane and synthesis of stress proteins [19,26].

One of mechanisms that plants have developed to cope with damages caused by cadmium is related with some stress signaling molecules, such as salicylic acid, jasmonic acid and ethylene [14].All these compounds were induced by Cd treatment, which suggest that they are involved in cell response to Cd toxicity. [22]. Salicylic acid (SA) is a simple phenolic compound involved in the regulation of many processes and physiological functions in plant growth and development, including stomatal movement, seed germination, ion absorption, sex polarization and in eliciting biotic and abiotic stress signaling [10]. Protective action of SA includes the development of anti stress programs and acceleration of growth processes recovery after the removal of stress factors. [16].The protective function of SA mainly includes the regulation of ROS and antioxidants, induction of gene expression [29]. Apparently, SA has broad but divergent effects on stress acclimation and damage development of plants. Thus, SA may act directly as an antioxidant to scavenge the reactive oxygen species and indirectly modulate redox balance through activation of antioxidant responses [22].

It has been shown that SA provides protection in pea plants [21], barley seedlings [15].soybean seedlings[5],hemp plants [24] against Cd stress and it induces adaptive response to copper stress in sunflower [6] or modulates plant responses to salt and osmotic stresses in Maize plants [9] drought and herbicides [22].

The sunflower (Helianthus annuus L.) is one of the four most important oil crops globally and is grown on over 21 million hectares worldwide [30]. The high levels of unsaturated fatty acids with low saturated fat levels in vegetable oils such as sunflower oil have become recognized as good nutritional characteristics for health [11].

Although sunflower is usually regarded as a highly tolerant crop, which can cope with elevated heavy metal concentrations in soil, impairment of growth at initial stages of plant development may result in a poor crop establishment [7]. Previous works have demonstrated that abiotic stresses like metals, UV-B and salt caused variations in the antioxidant defense system and generated oxidative damage in sunflower plants [21]. However, the role of exogenously applied SA under Cd stress on fatty acids profile in sunflower leaves is not still clear and needs further investigations. Based on the above studies, our research has shown the influence of SA on Cd-induced changes of growth and fatty acid composition in sunflower leaves.

MATERIALS AND METHODS

Homogenous seeds of sunflower (Helianthus annuus L. var. Euroflor) were obtained from the Agricultural Research Center, Khoy, Iran. Seeds were sterilized with sodium hypochlorite solution (1%) for 15 minutes, washed thoroughly with distilled water before use. Six seeds were sown and were cultivated in each pot and after emergence; four homogenous seedlings were left in each. To maintain humidity, 100 ml of distilled water was used to each pot every day and 100 ml of Hoagland solution was applied to each pot every week.

Plants were placed in greenhouse conditions under 24.5ºC and 33.5 ºC, respectively, minimum and maximum temperatures, light intensity 13000 luxs provided by fluorescent lamps on top of canopy and 16:8 (light: dark) photoperiod. Two leaves stage plant were exposed to $CdCl_2$ treatment. $CdCl_2$ was added to each pot with various concentrations (0, 50, 100, 150, 200 µM) every week. One week after Cd treatment ended, SA (mixed with tween-20 (a surfactant and spreading agent) with three concentrations (0, 250 and 500 µM) was sprayed on plant leaves with a sprayer (10 ml per plant) every week. Four replicates were performed for each treatment.

Plant growth (fresh weight and number of leaves) analysis

One week after the last salicylic acid treatment, the plants were harvested and Leaves were separated. Number of leaves was counted per plant. Fresh weight of leaves in treated and control plants was estimated (g per plant).

Estimation of Protein content: Protein content in leaves (500 mg) was extracted in with buffers used, grind well the samples with a pestle and mortar in 5–10 ml of buffer and was centrifuged at 8000 rpm for 94 minutes, the supernatant was decanted and proteins were determined according to Lowry et al. (1951). Amount of protein was measured at 750 nm by using bovine serum albumin as the standard protein. Protein content was expressed as mg $(100\ ml)^{-1}$.

Oil extraction

The leaves were dried at 40 ◌̊C for 4 h, using a ventilated oven, to reduce moisture content to 5%. Then dried leaves were crushed with a mortar. One gram of leaf tissue was used to oil extract with petroleum ether for 6 h in a Soxhlet

system (B.chi Universal Extraction System B-811, Germany) according to the AOCS method (AOCS, 1993). The oil extract was evaporated by distillation at reduced pressure in a rotary evaporator at 40 ◌̊C until the solvent was totally removed.

Analysis of fatty acids

The oil extracted with hexane/methanol (3:2, v/v) from the test sample was converted to its fatty acid methyl esters as described by Marquard (1987). The methyl esters of the fatty acids (0.1 µl) were analyzed in a Hewlett-Packard 5890B series gas chromatograph (Perkin Elmer Auto System XL, USA) equipped with a flame ionizing detector (FID), and a fused silica capillary column (MNFFAP (50 m x 0.32 mm i.d.; film thickness = 0.25 µm)).

It was operated under the following conditions: oven temperature program, 120 ◌̊C for 1 min, raised to 250 ◌̊C at a rate of 6 ◌̊C/min and then kept at 250 ◌̊C for 15min; injector and detector temperatures were 250 ◌̊C and 260 ◌̊C, respectively, carrier gas, helium, at flow rate of 40 ml/min; split ratio, 1/20 ml/min. Peak identification was performed by comparing the relative retention times with those of a commercial standard mixture of fatty acid methyl esters. The contents of palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2) acids and linolenic (C18:3) acids were determined by computing integrator on a percentage basis.

Statistical analysis

Statistical analysis was carried out by two-way ANOVA using SPSS, version 18 software. When the effect was significant, means of the studied parameters were compared by Duncan's test at $P \leq 0.001$, $P \leq 0.01$ and $P \leq 0.05$ levels.

RESULTS

Fresh weight and number of leaves

Fresh weight of leaves in sunflower was decreased significantly under the influence of Cd (66.5 % at 200 µmol and 0µmol of SA, respectively, compared with the control plants) (Table 1). Contrary, treatment with 500 µmol SA in plants exposed to Cd, increased leaf fresh weight (Figure1).

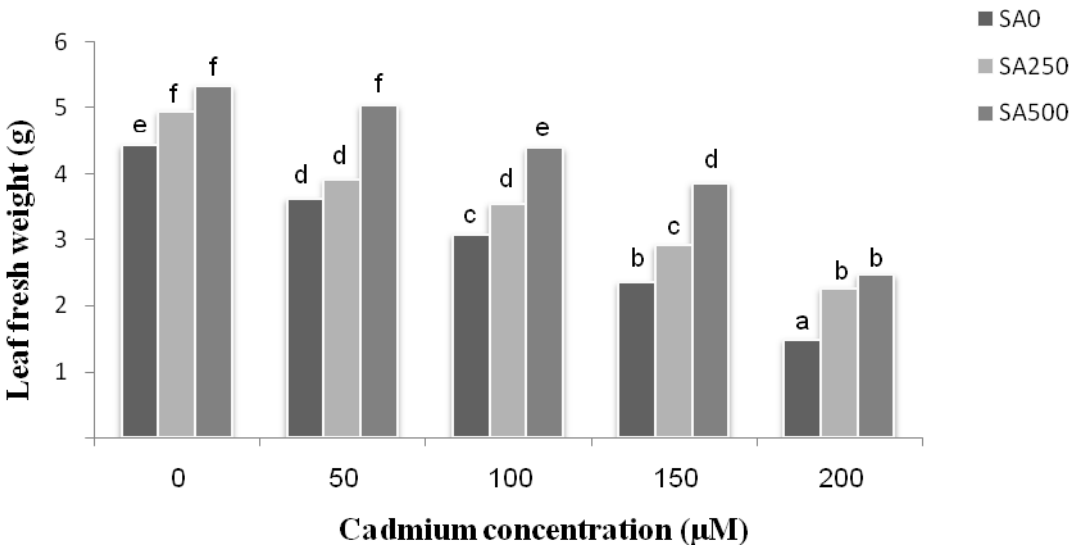

Fig 1. Effects of Cd and SA on leaf fresh weight in sunflower, data are means of four replicates. Means with common letters are significantly different according to Duncan's multiple range test.

Leaf number decreased proportionally with increasing Cd concentration, and the reduction in the values of this parameter under 200 µmol of Cd and 0µmol of SA was 22.53% compared with the control plants (Figure 2). SA treatment decreased Cd toxicity on leaf number (Table1).

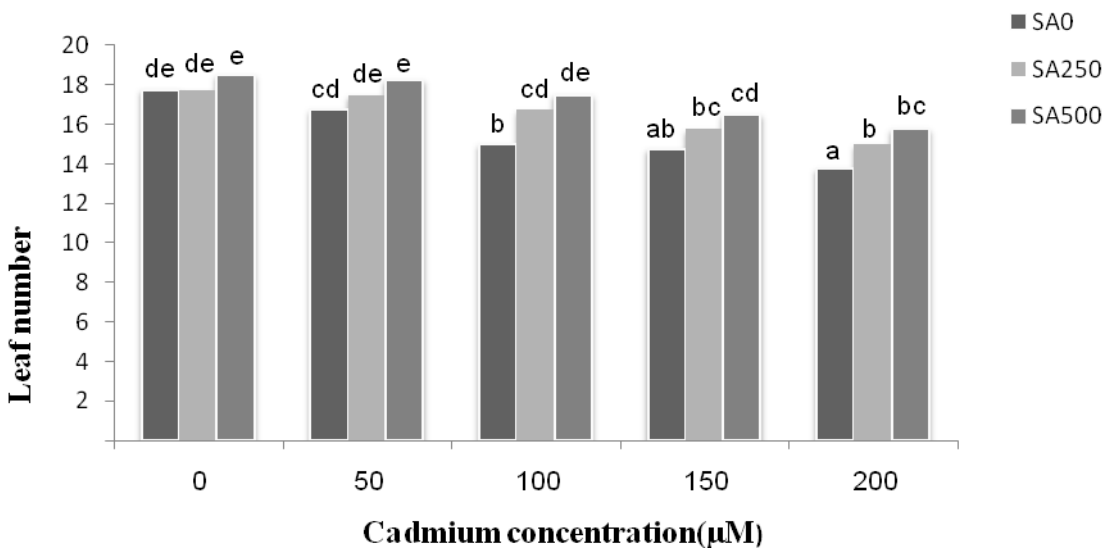

Fig 2. Effects of Cd and SA on leaf number in sunflower, data are means of four replicates. Means with common letters are not significantly different: ns (not significant) according to Duncan's multiple range test.

Protein content

Protein content was found to be significantly decreased after Cd treatments at the higher concentrations tested. When SA was applied there was a increasing in protein content in leaves. The minimum content of protein in sunflower leaves (66.29 %) was at 200 µmol of Cd and 0 µmol of SA compared with the control. The maximum content of protein in leaves (61.79 %) was at 0 µmol Cd and 500 µmol of SA concentration compared with the control (Figure 3).

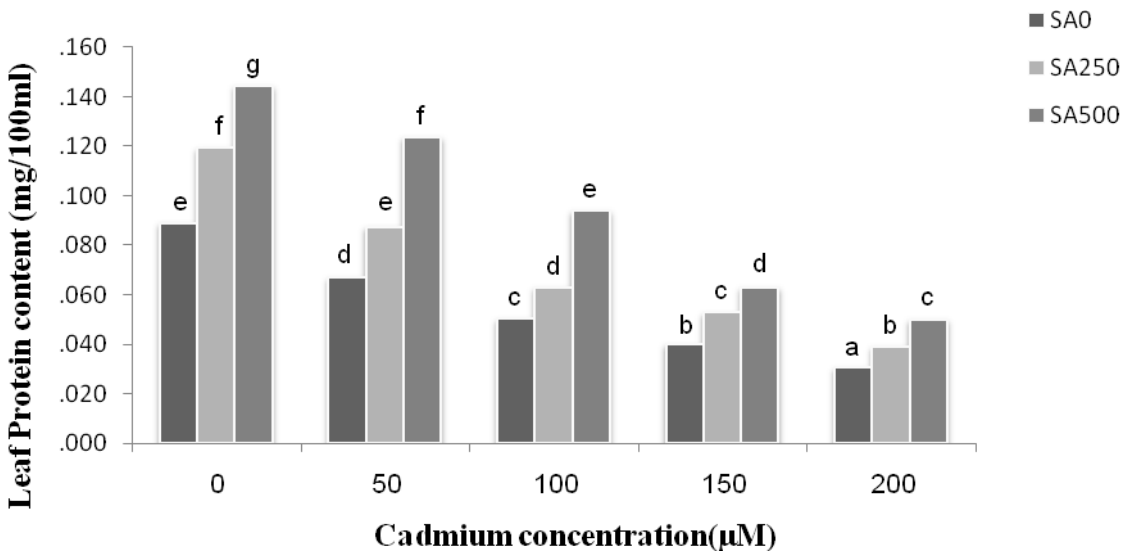

Fig 3. Effects of Cd and SA on Leaf protein content in sunflower, data are means of four replicates. Different small letters of the same type of column indicated significant difference between lines according to Duncan's multiple range test.

In Cd treatment, protein content in leaves of sunflower is shown in decreasing trend with increasing concentrations of Cd. However, exogenous application of SA further increased the amount of protein in sunflower plants exposed to Cd stress. Cd×SA interactions on protein content in leaves were significant (Table 1).

Table1- Effect of Cd and SA on leaf number and fresh weight, protein content of leaves of sunflower. Values (means ± Std) followed by different letters in the same columns are significantly different according to the Duncan's test. ns: not significant,* P≤0.05, ** P≤0.01, *** P≤0.001.

Treatments		fresh weight (g)	Leave number	Protein content (mg $(g)^{-1}$FW)
Cd	SA			
0 µM	0 µM	4.5±0.306	17.50±0.957	0.089±0.003
	250 µl	4.899±0.307	17.75±0.500	0.119±0.001
	500 µl	5.29±0.224	18.75±0.577	0.144±0.005
50 µM	0 µM	3.614±0.316	16.75±0.957	0.067±0.005
	250 µl	3.937±0.432	17.50±0.577	0.087±0.005
	500 µl	4.996±0.203	18.5±0.500	0.123±0.005
100 µM	0 µM	3.101±0.206	15.00±0.816	0.050±0.007
	250 µl	3.489±0.280	16.75±0.957	0.063±0.004
	500 µl	4.402±0.381	17.75±0.577	0.094±0.006
150 µM	0 µM	2.405±0.082	14.75±0.957	0.040±0.007
	250 µl	3±0.100	15.75±0.957	0.052±0.005
	500 µl	3.864±0.293	16.50±0.577	0.063±0.007
200 µM	0 µM	1.5±0.022	13.75±0.957	0.030±0.005
	250 µl	2.308±0.235	15.00±0.816	0.039±0.006
	500 µl	2.51±0.287	15.75±0.500	0.050±0.007
ANOVA				
Cd		14.118***	20.183***	0.012***
SA		7.504***	14.517***	0.008***
Cd×SA		0.170*	0.558 ns	0.000***

Fatty acid composition

Results shown in Table 2 are expressed as a percentage of total leaf fatty acids.Linolenic (0.55%), linoleic (51.2%), oleic(25.6%), stearic (4.5%) and palmitic (13.2%) acids were the major fatty acids (Figure 4).The main difference in the fatty acid composition of sunflower leaves between the control and contaminated plants was a decrease in the percentage of tri-unsaturated fatty acid ; linolenic acid (62.3%) and its precursors , oleic acid (72.2%) under 150 µmol Cd and linoleic acid (2.5%) under 200 µmol Cd as compared with control plants . An increase in the percentage of saturated fatty acids including stearic acid (58.3%) and palmitic acid (67%) under 200 µmol Cd was observed as compared with control plants . A decrease of 2.5, 1.1 and 3.5-fold was noted, respectively, for linolenic acid (C18:3), linoleic acid (C18:2) and oleic acid (C18:1). An increase of 2.4 and 1.6- fold was noted, respectively, for stearic acid (C18:0) and palmitic acid (C16:0) (Table 2).

Treatment with SA (250 µmol) with or without Cd treatment, significantly increased the amount of linoleic acid (24.7%) and linolenic acid (45.2%) and decreased that of stearic acid (8.7%). SA treatment at 500 µmol enhanced the content of oleic acid (9.3%) and decreased that of palmitic acid (48.6%) in leaves with or without Cd treatment (Table 2). Infact, Cd induced a decrease in total content of unsaturated fatty acids and an increase in saturated fatty acids in leaves of sunflower plants. The presence of an antioxidant such as SA with or without Cd treatment significantly increased the total content of unsaturation fatty acids and decreased the amount of saturated fatty acids.

Table 2. Effects of Cd and SA on leaf fatty acids profile (% of total lipids) of sunflower plants.
Values (means ± Std) followed by different letters in the same columns are significantly different according to the Duncan's test.

Treatments		**Palmitic acid**	**Stearic acid**	**Oleic acid**	**Linoleic acid**	**Linolenic acid**
Cd	**SA**					
0 µM	0 µM	12.85±0.002	4.38±0.009	25.41±0.026	50.78±0.875	0.46±0.001
	250 µM	16.20±0.012	6.77±0.08	20.05±0.013	54.45±0.633	0.73±0.028
	500 µM	13.89±0.077	4.11±0.005	28.10±0.033	51.19±0.422	0.39±0.009
50 µM	0 µM	13.34±0.001	7.88±0.010	23.33±0.01	51.95±0.901	0.19±0.500
	250 µM	14±0.011	4±0.014	14.10±0.012	65.79±0.009	0.24±0.020
	500 µM	6.55±0.008	4.90±0.002	25.08±0.021	60.83±0.010	0.40±0.007
100 µM	0 µM	13.56±0.009	8.81±0.021	11.77±0.002	64.02±0.008	0.16±0.030
	250 µM	15.79±0.041	5.40±0.044	9.99±0.005	65.90±0.025	0.77±0.027
	500 µM	14.28±0.02	6.75±0.03	12.14±0.06	63.98±0.199	0.56±0. 344
150 µM	0 µM	13.47±0.005	8.81±0.007	10.39±0.19	65.53±0.132	0.18±0.812
	250 µM	16.90±0.026	5.94±0.009	6.83±0.008	67.81±0.028	0.87±0.023
	500 µM	15.82±0.029	7.92±0.034	8.35±0.100	64.78±0.425	0.63±0.121
200 µM	0 µM	21.89±0.009	10.49±0.044	9.84±0.821	49.81±0.006	0.36±0.128
	250 µM	18.07±0.120	8.63±0.020	14.17±0.255	53.60±0.001	0.55±0.002
	500 µM	16.12±0.113	5.82±0.06	8.73±0.362	66.12±0.009	0.79±0.010

DISCUSSION AND CONCLUSION

Application of different levels of $CdCl_2$ in sunflower plants adversely decreased their growth pattern (leaf number, stem and root length, fresh weight of stem, root and leaf) as compared with control plants (Table 1).These results are in agreement with those of Tukaj et al. [25] in green microalga and Lopez-Millan et al. [12] in tomato who showed that cadmium caused a significant reduction in growth parameters.

The beneficial effect of SA was seen on all growth parameters in sunflower. The same positive effect of SA on growth in the presence of Cd was reported by Metwally et al. [15] that being exposed to cadmium, reduced root and shoot length and fresh weight in barley seedlings and SA treatment decreased Cd toxicity. These results in response to Cd stress and SA are also in agreement with those of Popova et al. [22] in pea plant and Shi et al. [24] in hemp plants. The reduction in growth could be a consequence of the Cd-interference with a number of metabolic processes associated with normal development such as photosynthetic pigments production, membrane lipid composition, water uptake and mineral nutrition that would result in deficiency in essential elements and ultimately reduction in biomass production [1]. Cadmium growth inhibition could also be due to the inhibition of cell division and elongation rate of cells that results in a decline in biomass production. This result mainly occurs by an irreversible inhibition of proton pump responsible for the process [4].

Metal toxicity in plants may result from the binding of metals to protein sulphydryls, which in turn would cause a modification of protein structures and inhibition of enzymatic activities involved in growth. These alterations usually lead to growth inhibition and cell death [7]. SA is needed for the adaptation process and the induction of stress tolerance[22]. We assume that the beneficial effects of SA during a growth period can be related to avoidance of cumulative damage upon exposure to cadmium or modification of compartmentalization. Alternatively, SA could be involved in the expression of specific proteins or defense-related enzymes [10]. SA can also form a complex with Cd that may provide Cd tolerance [17].

We showed that Cd stress caused a decrease in protein contents in sunflower. SA induced a considerable increase in the content of protein fractions in various organs of control and Cd stressed plants. This may be due to the interactive effect of Cd and SA [6].
In sunflower cells, cadmium induced oxidative stress. Reactive oxygen species react with proteins and generate oxidation products such as carbonyl groups on protein molecules. Cadmium produced oxidation of proteins in sunflower tissues. Our results indicate that cadmium induced increase in protease specific activity. Protein degradation removes abnormal proteins, facilitates the recycling of amino acids, and regulates protein activity by elimination of molecules that are no longer needed [21]. This work was investigated whether salicylic acid could be a protectant to ameliorate the influence of Cd stress on sunflower and thereby increasing its Cd tolerance.

In sunflower plants, leaf fatty acids composition showed significant changes with Cd stress and this oxidative damage was alleviated by SA treatment. The analysis of fatty acid composition in Cd treated plants supports this observation (Table 2). A decrease in the percentage of unsaturated fatty acids ; C18:3, C18:2 and C18:1and an increase in the amount of saturated fatty acids such as C16:0 and C18:0 was observed under Cd stress as compared with control plants . On the other hand, the accumulation of C16:0 and C18:0 by Cd treatment, could be an indication that there are some alterations in biosynthesis pathway between these two acids.

This confirmed that Cd toxicity in sunflower plants was linked to free radical processes in membrane components leading to alterations in membrane stability and increasing their permeability. The peroxidation of unsaturated lipids in biological membranes is the most prominent symptom of oxidative stress in animals and plants [3]. Furthermore, the protective effect of SA on leaf membrane integrity could be related to changes in lipid content and fatty acid profiles (Table 2). Given the known effects of Cd on photosynthesis then it is not surprising that the supply of carbon for fatty acid synthesis and lipid assembly as lipid biosynthetic pathways is altered [22]. SA application seems to reduce the Cd effect on lipid unsaturation. In SA-treated sunflower plants significant decrease in C16:0 and C18:0 were observed. the amount of linolenic (C18:3), linoleic (C18:2) and oleic (C18:1) acids was increased (Table 2).This could be an indication that the desaturase activity by the transformation of C18:0 to C18:1, C18:2 and C18:3 was enhanced. The increase of the unsaturated fatty acids observed under the influence of SA lead to increase the fluidity of lipid membranes that probably affects their permeability and stability. Membrane unsaturation has been shown to be closely related to the heavy metal tolerance in many higher plants [20]. Also, it has been suggested that the high level of unsaturation of thylakoid lipids may be required to maintain the degree of fluidity needed for the diffusion of lipophilic compounds and/or may confer a suitable geometry to the lipid molecules [28].

These results exhibit the beneficial effect of SA treatment on leaf lipid metabolism probably in relation with

chlorophyll synthesis, photosynthetic activity and carbon supply of sunflower plants exposed to Cd [28].

Therefore SA treatment of Cd stressed sunflower plants could stimulate their Cd tolerance via amelioration of growth parameter and lipid profile.

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