Exogenous Salicylate Application Affects the Lead and Copper Accumulation Characteristics of *Lemna gibba* L.

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Previous studies have shown that salicylates can change the ion permeability of root cells. Therefore the possible effects of exogenous salicylate application on lead (Pb) and copper (Cu) accumulation and its protective role against DNA damage due to metal exposure in *Lemna gibba* were studied. *L. gibba* was exposed to 5, 10, and 25 μM Pb and Cu for six days in the presence and absence of sodium salicylate (SA) (0.1, 0.5, and 1 mM). At all concentrations tested, SA application decreased Pb accumulation. On the other hand, application of 0.5 mM SA increased Cu accumulation. SA did not reduce DNA damage resulting from Pb and Cu toxicity. In summary, SA may be useful for reducing Pb accumulation, and application of SA at 0.5 mM may be useful for the phytoextraction of Cu.

Key words: Lemna gibba, Metal, Salicylate

Introduction

Heavy metal pollution of water has become one of the most important environmental problems throughout the world. Many industries such as steel production, mining, electronics, and motor vehicles release toxic metals into water bodies with inadequate wastewater management. These heavy metals pose a serious threat to the environment, animals, and humans because of their toxicity. The use of metal-accumulating plants for toxic metal phytoremediation is a promising, eco-friendly technology. Aquatic macrophytes may accumulate considerable amounts of heavy metals in their tissues, and accumulation of these heavy metals in plants causes physiological and biochemical changes (Megateli *et al.*, 2009). Copper is an essential metal that interacts with a wide range of physiological and biochemical processes in cells. However, at elevated concentrations, copper inhibits the normal growth and development of plants (Srivastava *et al.*, 2006). Lead is a toxic metal that reacts with biomolecules such as proteins and lipids. It exerts adverse effects on the morphology, growth, and physiology of plants, and it causes enzyme inhibition, water imbalance, and alterations in membrane permeability (Mishra *et al.*, 2006). DNA fingerprinting is a useful biomarker assay in ecotoxicology. Recently, random amplified polymorphic DNA (RAPD) analysis has also been used to detect genome rearrangements induced by genotoxic factors such as heavy metals (Conte *et al.*, 1998; Atienzar *et al.*, 2001). After proper optimization, RAPD analysis is a reliable, sensitive, and reproducible assay with the potential to detect a wide range of DNA damages (*e.g.* DNA adducts, DNA breakage) as well as mutations (point mutations and large rearrangements), and it therefore can be applied in genotoxicity and carcinogenesis studies.

Salicylic acid is a signal molecule that plays an important role in regulating a number of physiological processes (Hayat *et al.*, 2010). Various physiological and biochemical effects of salicylates (SA) on plant systems have been determined. 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 (Senaratna *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

Cassia tora. However, Wang *et al.* (2009) did not find any significant effect of exogenous salicylic acid application on Ni accumulation in *Zea mays*. Many studies have been conducted to evaluate the toxic effects of metals on aquatic plants. Duckweed (Lemnaceae) received much attention because it possesses some physiological properties that make it an ideal test organism, such as its small size, vegetative propagation, and rapid multiplication. Megateli *et al.* (2009) reported that *Lemna gibba* can accumulate large amounts of metals and has great potential for phytoremediation. However, a literature review did not indicate any study addressing the effects of SA on metal uptake and accumulation by duckweeds. The objectives of the present research were (1) to determine the influence of SA on the extent of Cu and Pb accumulation in *L. gibba*, and (2) to investigate the impact of SA on the DNA stability as it is altered by Pb and Cu exposure.

Material and Methods

Sample collection and cultivation

Lemna gibba L. was obtained in June 2009 from Dipsiz spring in Kayseri, Turkey. Prior to the experiment, containers were disinfected by immersion in 1% (v/v) NaClO for 3–5 min. Collected samples were washed in distilled water and acclimatized for 5 d in a climate chamber (23 °C and 14-h photoperiod, 350 μmol m^{-2} s^{-1}).

Experimental design

The experiments were preformed in two series. In Series 1, *L. gibba* fronds (approx. 4 g) were exposed to 10% Hoagland's solution with initial $Pb(NO_3)_2$ concentrations of 5, 10, and 25 μM without added SA. In Series 2, fronds were exposed to a nutrient solution containing sodium salicylate (0.1, 0.5, and 1 mM) and Pb (5, 10, and 25 μM), making 12 treatments in all. Additionally, all experiments were repeated with $CuSO_4$. Each treatment was conducted with three replicates. The experiments were carried out in a climate chamber under the aforementioned conditions for periods of 6 d. Solutions were replaced after 3 d. The change in volume within the flasks due to evaporation was compensated by the addition of double distilled water. At the end of the exposure experiments, fronds were collected and sieved with a plastic griddle. Plants were rinsed with double distilled water, drained and then blotted on paper towels for 2 min.

Quantification of Pb and Cu

An aliquot of each sample was dried at 70 °C. Each sample was then digested with 10 mL of pure HNO_3 using a CEM Mars 5 (CEM Corporation, Matthews, NC, USA) microwave digestion system. The digestion conditions were as follows: maximum power, 1200 W (100% power); ramp, 20 min; pressure, 180 psi; temperature, 200 °C; hold time, 10 min. After digestion, the volume of each sample was adjusted to 25 mL using double deionized water. Pb and Cu concentrations were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES, Varian-Liberty II, Mulgrave, Victoria, Australia). The stability of the device was evaluated every ten samples by examining an internal standard. Reagent blanks were also prepared to detect potential contamination during the digestion and analytical procedure. Peach leaves (NIST, SRM-1547) were used as a reference material, and all analytical procedures were also performed on this reference material. The samples were analysed in triplicate. All chemicals used in this study were analytical reagent grade (Merck, Darmstadt, Germany).

Genomic DNA isolation and PCR amplification

DNA was isolated by the CTAB method (Rogers and Bendich, 1985), and the DNA concentration was estimated with a spectrophotometer at 260 nm. Moreover, The RAPD-PCR experiments were conducted according to the method of Williams *et al.* (1990), and randomly chosen seven oligonucleotide primers (Alpha DNA, Montreali, QC, Canada) were used. However, we considered only those three primers which gave the best results. Standard 25 μL reaction mixtures contained 100 ng of genomic DNA, 1.5 U of *Taq* polymerase, 0.4 μM of primer (Primer 1, CCACAGCAGT; Primer 2, TGCCCAGCCT; Primer 3, GAGGGTGGCGGTTCT), 0.2 mM of each dNTP, 2.0 mM of $MgCl_2$, and 2.5 μL of 10X reaction buffer (Fermentas, Hanover, MD, USA). DNA was amplified in a thermal cycler according to the following regime: 2 min of denaturation at 94 °C, 45 cycles of 1 min at 94 °C, 1 min at 36 °C, 2 min at 72 °C, and 1 cycle of 5 min at 72 °C. The products were separated on 2% agarose gel by electrophoresis in Tris/acetic acid/EDTA (TAE)

Table I. Effects of exogenous SA application on Pb and Cu accumulation (μg g^{-1} DW) in *L. gibba*. Values represent means ± SE (n = 3).

SA [mM]	5 μM Pb	10 μM Pb	25 μM Pb	5 μM Cu	10 μM Cu	25 μm Cu
0	756 ± 14.8	989 ± 23.4	1577 ± 49.2	262 ± 6.6	664 ± 21.5	1054 ± 38.9
0.1	504 ± 19.2	967 ± 15.1	1215 ± 24.5	285 ± 5.3	691± 17.5	1119 ± 31
0.5	497 ± 11.6	935 ± 22.7	1074 ± 22.9	414 ± 5.7	759 ± 20.6	1165 ± 38.3
1	387 ± 6.3	873 ± 27.7	1048 ± 23.6	297 ± 4.1	677 ± 20	1095 ± 40.5

buffer and stained with ethidium bromide. The resultant gels were then photographed on a UV-transilluminator. GeneRuler 100 bp DNA Ladder Plus (Fermentas) was used as the DNA molecular weight standard. For accuracy and comparison of the results, the controls and the Pb/SA- and Cu/SA-treated PCR products, respectively, were run in the same gel. The process of amplification of each DNA sample was repeated at least three times in order to ensure reproducible results.

Genomic template stability test

Each change observed in RAPD profiles, such as disappearance or appearance of bands and variation in band intensities in comparison to controls, was given the arbitrary score of +1, and the average was calculated for each metal based on the number of primers used. Primers that did not produce changes in RAPD profiles or that were too difficult to score were not used in the calculation. Genomic template stability (GTS, %) was calculated using the equation GTS (%) = $(1 - a/n) \cdot 100$, where a is the number of RAPD changes detected in each treated sample, and n is the total number of bands in the control. Changes in this value were calculated as a percentage of the control (set to 100%).

Statistics

The data were expressed as mean values with standard errors (SE). The Kolmogorov-Simirnov test and Levene's test were used to ensure the normality assumption and the homogeneity of variances, respectively. Two-way analysis of variance (two-way ANOVA) was used to assess the significance of the effects of metal concentration and SA concentration, as well as of their interaction, on accumulation. All pairwise mean comparisons were made using post-hoc analyses (Tukey's test). Additionally, we addressed the effect size of each factor in the ANOVA model by calculating the partial η^2 value. We used 0.05 as the statistical significance threshold. All statistical analyses were performed with the SPSS 15.0 software package.

Results and Discussion

Effect of SA on Pb accumulation

The effects of SA on Pb and Cu accumulation were assessed. Two-way ANOVA revealed a significant SA-Pb interaction (Table I). In addition, a significant interaction was found regarding the strength of association (effect size). Pb accumulation was strongly affected by the Pb concentration (η^2 = 0.986, $P < 0.01$) and by the presence of SA (η^2 = 0.933, $P < 0.01$) (Table II). *Post-hoc* comparisons demonstrated that SA at all applied concentrations significantly reduced Pb accumulation ($P < 0.05$) relative to control values. The largest decrease in Pb accumulation was 95% in plants exposed to 5 μM Pb + 1 mM SA.

As expected, the accumulation of Pb increased with increasing concentrations of Pb in the culture solution. Similar findings have already been reported by Axtell *et al.* (2003) with *Lemna minor* plants. Cadmium (Cd), like Pb, is a highly toxic trace element that is not essential. Regarding the tolerance to metals, plants have a variety of mechanisms to maintain and regulate cellular metal homeostasis (Hall, 2002). Most of these mechanisms involve reduced uptake caused by altered absorption. Similar to our results, Popova *et al.* (2009) found that salicylic acid pretreatment reduced root accumulation of Cd in pea seedlings. Chen *et al.* (2007) stated that exogenous salicylic acid induced Pb tolerance in rice by enhancing antioxidant defense activities. Our study has shown that increasing concentrations of exogenous SA reduce the accumulation of Pb from the growth medium by *L. gibba*. According to earlier studies, we can say that exogenous SA supply protects the cell membranes by stabilizing their permeability, which leads to a reduction in Pb absorption. As a result, there is less damage to the cell membrane,

Table II. Summary of two-way analysis of variance.

Parameter	*df*	*F*	*P*	Effect size*	Parameter	*df*	*F*	*P*	Effect size*
Pb	2	839	<0.01	0.986	Cu	2	1036.4	<0.01	0.989
SA	3	111.4	<0.01	0.933	SA	3	12.8	<0.01	0.616
Pb x SA	6	19.1	<0.01	0.827	Cu x SA	6	0.6	0.724	0.131
Error	24				Error	24			

* Effect size is given in partial η^2; *df*, degrees of freedom; *F*, *F* ratio; *P*, probability interaction between metal and SA.

and consequently less Pb enters the cytoplasm. These results suggest that the use of exogenous SA effectively reduces Pb accumulation.

Effect of SA on Cu accumulation

Similarly, it was determined that Cu accumulation was strongly affected by the Cu concentration ($\eta^2 = 0.989$, $P < 0.01$) and by the presence of SA in the solution ($\eta^2 = 0.616$, $P < 0.01$) (Table II). However, a significant SA-Cu interaction was not observed ($\eta^2 = 0.131$, $P > 0.724$). Application of 0.5 mM SA increased the Cu accumulation. However, a significant difference was not observed between 0, 0.1, and 1 mM SA (Table I). A maximum increase of 58% was observed at 5 μM Cu + 0.5 mM SA.

Cu accumulation was concentration-dependent, and similar results have been reported by other researchers (Srivastava *et al.*, 2006; Monferran *et al.*, 2009). In the present study, we observed that Cu accumulation in plants treated with 5 mM SA increased relative to control plants. Thus, the application of exogenous SA might be useful for the phytoextraction of metals. These results are in agreement with the findings of Gunes *et al.* (2007), who found that concentrations of Cu increased significantly with increasing levels of exogenous SA in maize plants. Aly and Soliman (1998) studied the effect of SA on the iron uptake in soybean genotypes. They found that SA increased the Fe content in shoots. Cu, unlike Pb, is an essential element that is involved in photosynthetic electron transport, mitochondrial respiration, cell wall metabolism, oxidative stress responses, and hormone signaling (Yruela, 2005). Therefore, exogenous SA application may have increased the Cu accumulation. As a result, we can say that the effect of SA on metal accumulation appears to be related to the type of metal.

Effect of SA on GTS

For the RAPD analyses, seven oligonucleotide primers of 60–70% GC content were used to screen the *L. gibba* genome for alterations. However, only three primers produced specific and stable results with a total number of 14 bands of 200–1100 bp for Cu-SA and 20 bands of 100–1500 bp for Pb-SA. The changes occurring in the RAPD profiles of *L. gibba* following Cu and Pb treatment included the disappearance and/or appearance of DNA bands compared with the control group. *L. gibba* showed 11 and 15 new bands after treatment with Cu-SA and Pb-SA, respectively, with three primers.

According to the RAPD profiles obtained with the three primers, the GTS values decreased with increasing concentrations of Cu and Pb. When we compared the RAPD-PCR profiles of control, 5, 10, and 25 μM Pb treated samples, it was obvious that the GTS values decreased with increasing doses of Pb (Fig. 1A). Conversely, the GTS values increased with application of 0.1, 0.5, and 1 mM SA along with the increasing Pb doses, as compared with the profile of the control group. When different concentrations of SA (0.1, 0.5, and 1 mM) were applied with increasing concentrations of Cu (5, 10, and 25 μM), the GTS values decreased in 10 μM Cu with increasing concentrations of SA (Fig. 1B). At 25 μM Cu with 0.1 or 0.5 mM SA, a reduction in GTS was observed, but 1 mM SA resulted in an increase in GTS. In the same way, while 25 μM Cu together with 0.1 mM SA reduced the value of GTS, 0.5 and 1 mM SA increased the value of GTS.

Changes in the DNA caused by genotoxic chemicals may be monitored using different biomarker assays both at the biochemical and the molecular levels. RAPD profiles detect alterations in the genomic DNA with the use of arbitrarily primed PCR reactions, and they clearly show promise in the detection of pollutant-induced changes in the DNA. GTS is related to the level of DNA damage and the efficiency of DNA repair and replication (Atienzar *et al.*, 2001). Our results indicated that GTS was affected by Cu and Pb application. According to the RAPD profiles results obtained

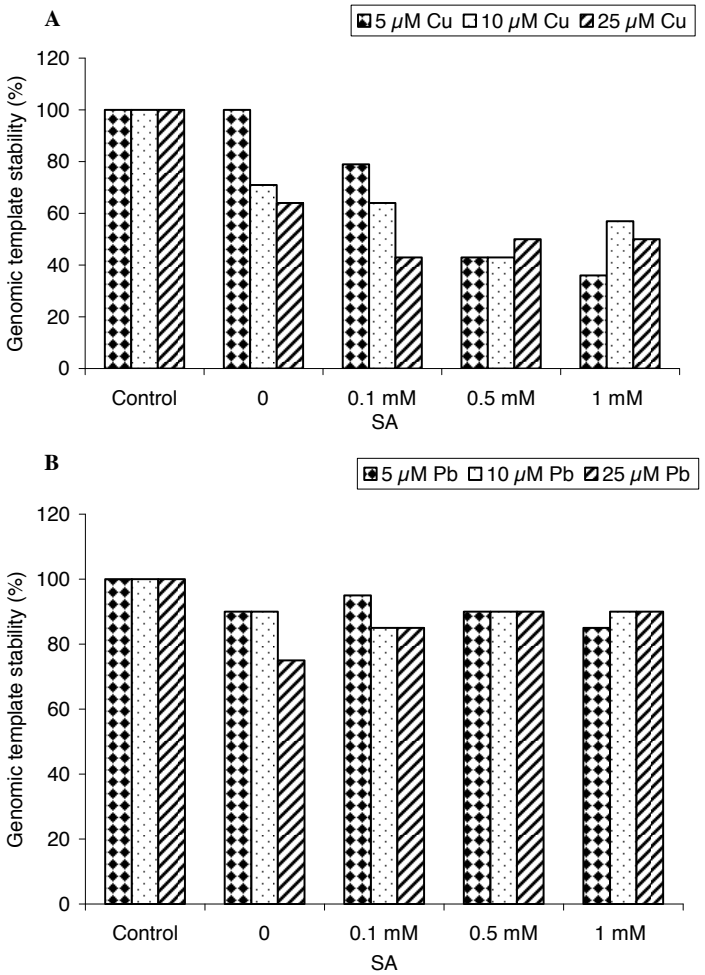

Fig. 1. Effects of exogenous SA application on genomic template stability in *L. gibba* exposed to different (A) Cu and (B) Pb concentrations.

with primers 1, 2, and 3, the GTS values decreased with increasing concentrations of Cu and Pb. Similar effects on GTS were reported due to heavy metal treatment of barley (Liu *et al.*, 2005). When we compared the RAPD profiles of control, 5, 10, and 25 μM Pb treated samples, it was obvious that the GTS values decreased with increasing doses of Pb. Low-level application of SA (0.1 and 0.5 mM) together with Cu resulted in a reduction in the GTS values. However, application of SA at a higher level (1 mM) modestly increased the GTS value. It may be concluded that SA increases Cu accumulation, however, it does not prevent the decrease of the GTS values. Additionally, it was determined that SA was not effective in decreasing DNA damage resulting from Pb exposure.

Conclusions

In conclusion, SA affected Pb and Cu uptake and accumulation by *L. gibba*. While SA application together with Pb decreased the Pb accumulation, SA application together with Cu increased the Cu accumulation at low concentrations and then decreased the Cu accumulation at high concentrations. In addition, the use of SA may be practical in reducing Pb accumulation. Also, SA application might be useful for the phytoextraction of Cu. However, SA does not effectively decrease DNA damage resulting from Pb and Cu toxicity.

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