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SALICYLIC ACID MODULATES ACCUMULATION OF Cd IN SEEDLINGS OF CD-TOLERANT AND CD-SUSCEPTIBLE SOYBEAN GENOTYPES

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Abstract — The seedlings of Cd-tolerant (Balkan) and Cd-susceptible (L 608) soybean genotypes were used to investigate the effect of salicylic acid pretreatment (10^{-5} M) on Cd toxic action. Cadmium-induced leaf desiccation, chlorophyll degradation, and increase of membrane impairment were more pronounced and partially ameliorated by SA treatment in the Cd-susceptible genotype. Root Cd accumulation was similar in the two genotypes, but SA stimulated Cd accumulation in the tolerant genotype, while in the susceptible genotype this accumulation was inhibited by SA. The Cd-tolerant Balkan genotype was apparently characterized by more efficient control of the oxidative stress induced by Cd, so it did not trigger mechanisms preventing further uptake of this heavy metal. It appears that differences between the genotypes with respect to Cd-tolerance occurred due to different degrees of efficiency of nonspecific defense mechanisms involving salicylic acid as a signal molecule.

Key words: Soybean genotypes., growth parameters, cadmium, salicylic acid, stress

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INTRODUCTION

Contamination of soils with heavy metals is a serious worldwide problem for both human health and agriculture. As agriculture increases, environmental stresses are gaining prevalence. One of the most aggressive and persistent of heavy metals, cadmium can occur in natural environments as a by-product of human activities, among which the application of phosphoric fertilizers is the main way Cd is introduced into agricultural soils (Mc Loughlin et al., 2000). It is easily taken up by roots and transported to other parts of the plant, being toxic to living cells at very low concentrations (Clemens et al., 1999). Plants affected by Cd showed impaired photosynthesis (Siedlecka and Baszynsky, 1993; Baryla et al., 2001), altered mineral nutrition (Moral et al., 1994), and water imbalance (Barceló and Poschenrieder, 1990). The toxic effects of Cd on plants has been extensively reviewed.

Activation of the cellular antioxidant metabolism is part of the general stress response induced by heavy metals (Dietz et al., 1999). It was recently demonstrated that oxidative stress of *Arabidopsis thaliana* seedlings exposed to cadmium is caused by H_2O_2 accumulation (Cho and Seo, 2005), which in turn may be responsible for lipid peroxidation and oxidative damage to proteins and DNA. Antioxidative systems in young Serbian spruce plants are depressed after short-term stress caused by Cd (5 μM and 50 μM), and this effect is dose-independent (Ducic et al., 2008).

Salicylic acid (SA) is an endogenous growth regulator of phenolic nature which participates in the regulation of many physiological processes in plants (Raskin, 1992; Gutierrez-Coronado et al., 1998), playing an important role in protection against different biotic and abiotic stresses (Booker and Miller, 1998; Janda et al., 1999; Mishra and Choudhuri, 1999; Senaratna et al., 2000; Bezrukova et al., 2001; Sharikova et al., 2003). Salicylic acid has been identified as a key signaling molecule involved in plant defense responses against pathogen attack. There is a strong evidence that SA mediates the oxidative burst which precedes the hypersensitive response and the development of systemic acquired resistance (Shiurasu et al., 1997). In addition, it has been shown to play a major role in plant responses to several abiotic stresses, such as UV irradiation and ozone exposure (Yalpani et al., 1994; Rao and Davis, 1999), as well as in chilling tolerance (Kang and Salviet, 2002) and heat acclimation (Dat et al., 1998). However, the role of SA under heavy metal stress is not fully understood. It was shown that exogenous SA ameliorated the lipoxygenase-mediated damaging effects of Pb and Hg on membranes in two cultivars of rice (Mishra and Choudhuri, 1999).

In our previous investigations, soybean seedlings were used as a convenient model system in investigations of Cd toxicity and uptake by plants. It was shown that Cd accumulation was closely related with the contents of other ions in roots and shoots, but that the phytotoxic effect could not be directly ascribed to any Cd-induced deficiency of a macro- or micronutrient (Drazic et al., 2005). It was also shown that SA applied simultaneously with Cd suppressed the toxic effect of Cd in a concentration-dependent manner; SA did not decrease Cd uptake, but did change its distribution in plant organs in dependence on the applied concentrations of Cd (Drazic and Mihailovic, 2005). Also, SA-pretreatment caused increased Cd accumulation, accompanied by a decrease of the toxic effect of Cd in alfalfa seedlings (Drazic et al., 2006). The uptake and toxic effects of Cd in plants varies not only among plant species, but also among cultivars (Morishita et al., 1987; Li et al., 1997; Quadir et al., 2004). Thus, significant differences were found in grain Cd levels between two soybean varieties grown on sewage sludge-amended soil (Reddy and Dunn, 1986) and in uptake, distribution, and translocation of Cd among 17 soybean genotypes grown on soil or on nutrient solution (Arao et al., 2003). Differences of Cd toxicity and accumulation were detected in 10 soybean cultivars. Only in the susceptible genotype did Cd at 3 mg kg^{-1} induce significant leaf desiccation and chlorophyll destruction, as well as an increase of phenol concentration in the roots, this effect being significantly suppressed by SA pretreatment. The effect of SA in leaves depended on their developmental stage. The obtained results suggest that differences between genotypes with respect to Cd-tolerance occurred due to different degrees of efficiency of nonspecific defense mechanisms involving salicylic acid as a signal molecule, and not as a part of phenol metabolism (Drazic and Mihailovic, 2008).

The aim of the present study was to determine differences of Cd accumulation in Cd-tolerant and Cd-susceptible genotypes and their response to SA pretreatment in terms of Cd-induced membrane damage.

MATERIAL AND METHODS

Plant material and *growth conditions*

One half of the seeds of Cd-tolerant (Balkan) and Cd-susceptible (L608) soybean cultivars were treated with SA (10^{-5} M) at the beginning of imbibition for 3 h, and the other half was used as the control. All seeds were germinated on wet filter paper in the dark (at 20°C and 100% RH). From day 4, seedlings of uniform size (rootlet length between 2 and 2.5 cm) were cultivated in hydroponics (Römheld and Marschner, 1981) under controlled conditions – day length of 14 h, average light intensity at the leaf surface of 90 μmoles m^{-2} · g^{-1}, and temperature of 25±2°C (day)/14±2°C (night) – in 3-L pots (20 plants per pot). After the plants reached the stage of the first fully developed leaf, they were transferred onto a fresh nutrition solution containing 3, 5, or 7 mg kg^{-1} Cd. After 72 h of Cd treatment, the plants were harvested and the fresh masses of roots and shoots were determined immediately. Plant material was then partly dried at room temperature and then in a drying oven at 40°C until constant mass was reached.

Measurement of pigment contents

Chlorophyll content was determined spectrophotometrically (Moran, 1982) with an *AMINCO DW 2* (Maryland USA) spectrophotometer in leaf disks (10 mm in diameter) from the first trifoliate extracted in dimethylformamyde.

Determination of membrane injuries

For assessment of membrane permeability, ion leakage from leaf disks was measured. Ten disks of 10 mm in diameter (from the trifoliate) were washed with bidistilled water and kept in closed vials containing 20 mL of bidistilled water and incubated at 25°C for 6 h on a shaker. Electrical conductivity (EC) was determined as EC_1. The samples were then kept at 100°C for 2 h and EC was measured after cooling to 25°C as EC_2. Electrolyte leakage (EL) was calculated using the equation: EL (%) = (C_1/C_2) x 100.

The level of lipid peroxidation was estimated by the thiobarbituric method (Ohkawa et al., 1979). Approximately 0.5 g of frozen plant tissue samples was cut into small pieces, homogenized with the addition of 2.5 ml of 5% trichloroacetic acid, and centrifuged at 10000 *g* for 15 min at room temperature. Equal volumes of supernatant and 0.5% thiobarbituric acid in 20% trichloroacetic acid were added to a new tube and incubated at 96°C for 25 min. The tubes were transferred to an ice bath and then centrifuged at 8000*g* for 5 min. The absorbance of the resulting supernatant was recorded at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The concentration of lipid peroxides was quantified and expressed as total thiobarbituric acid-reacting substances (TBARS) in terms of μmoles g^{-1} (f. m.) using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Determination of Cd contents

For determination of Cd uptake and distribution, dry plant material was powdered and mineralized in the presence of strong acids. The element concentration was determined by the AAS method (*Pye Unicam SP 192*, Cambridge, UK) using a *CERTIPUR* (*Merck*, Darmstadt, Germany) Cd standard solution.

Statistics

All the experimental values reported in this article are the means of at least three individual biological experiments ± standard deviation. The significance of differences was determined using Student's t-test.

Abbreviations: AAS - atomic apsorption spectrometry, Chl - chlorophyll a + b, EC - electrical conductivity, EL - electrolyte leakage, RH - relative humidity, ROS - reactive oxygen species, TBARS - thiobarbituric acid reactive substances, SA - salicylic acid.

RESULTS AND DISCUSSION

Cadmium toxicity was monitored through leaf desiccation (water content change) (Fig. 1), chlorophyll degradation (Fig. 2), electrolyte leakage (Fig. 3), and lipid peroxidation (Fig. 4) in seedlings pretreated with SA and control seedlings. There was a concentration-dependent decrease of water content in leaves of Cd-treated seedlings of both genotypes, this decrease being more marked in leaves of the Cd-susceptible one. Pretreatment with SA at the beginning of seedling development partially protected the leaves of both genotypes from Cd toxicity, but more markedly in the Cd-susceptible L608 genotype (Fig. 1). Significant differences between the genotypes

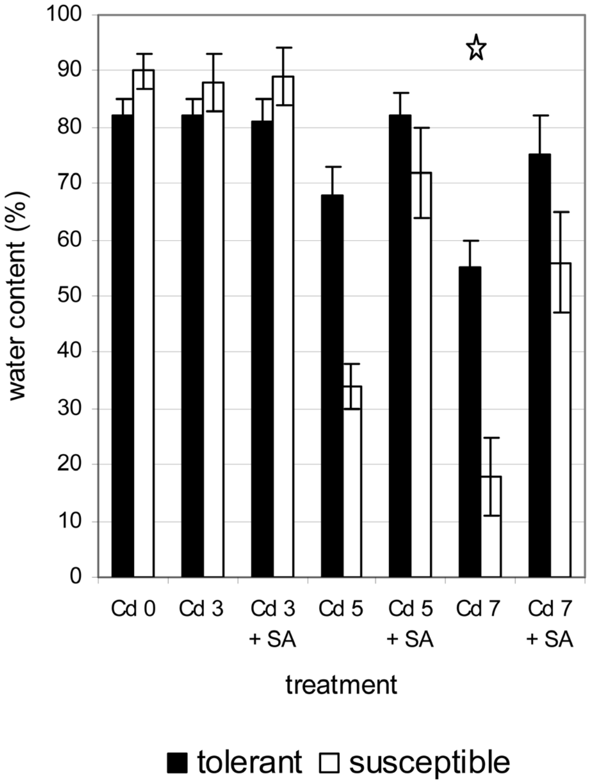

Fig. 1. Changes of water content [%] in leaves of soybean seedlings. Treatment: 3, 5, and 7 mg kg-1 Cd for 72 h. +SA: seeds treated with 10^{-5} M SA at the beginning of imbibition. Means ± SD are presented. *Difference between genotypes significant at the level of $p < 0.01$.

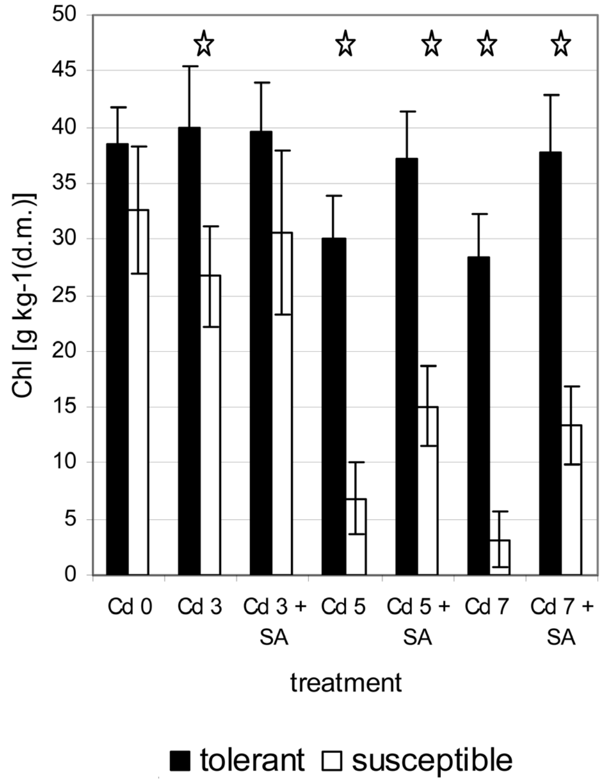

Fig. 2. Content of Chl a + b [g kg-1(d. m.)] in leaves of soybean seedlings. Treatment: 3, 5, and 7 mg kg-1 Cd for 72 h. +SA: seeds treated with 10^{-5} M SA at the beginning of imbibition. The results are given as mean values of three independent experiments ± SD. *Difference between genotypes significant at the level of $p < 0.01$.

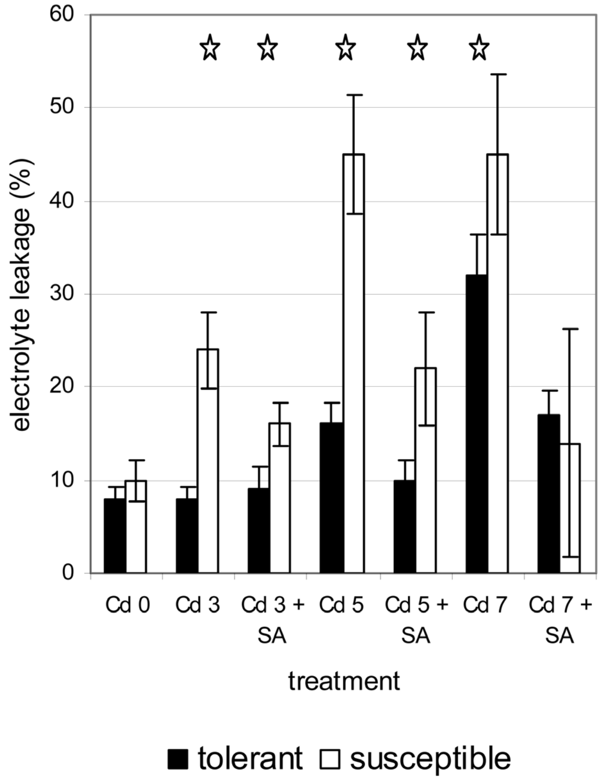

Fig. 3. Electrolyte leakage [%] in leaves of soybean seedlings. Treatment: 3, 5, and 7 mg kg^{-1} Cd for 72 h. +SA: seeds treated with 10^{-5} M SA at the beginning of imbibition. The results are given as mean values of three independent experiments ± SD. *Difference between genotypes significant at the level of $p < 0.01$.

were determined only in the presence of the highest Cd concentration. The results obtained at a concentration of 5 mg kg^{-1} Cd are representative because at 3 mg kg^{-1} Cd there were no visible changes in leaves of the tolerant Balkan genotype, while at 7 mg kg^{-1} Cd leaf necrosis occurred in the susceptible L608 genotype. Pretreatment with SA without Cd did not induce any significant changes in any of the investigated parameters (data not shown).

In the case of chlorophyll content, the pattern of changes induced by Cd is similar but more pronounced than in the case of water content (Fig. 2). Significant differences between cultivars, at the level of $p < 0.01$, were found in all of the treatments, indicating that photosynthetic pigment content is a better indicator of Cd toxic action. Similar results were obtained in two mustard cultivars differing in Cd tolerance (Mobin and Khan, 2007) and in Indian mustard (*Brassica juncea* L.) and mung bean (*Vigna radiata* L.) (Šimonova et al., 2007).

Membrane injuries were measured by monitoring electrolyte leakage (Fig. 3). More serious damage was found in leaves of the susceptible genotype, as expected.

Cadmium-induced changes in all investigated parameters of toxicity were significantly less marked in the Cd-tolerant genotype than in the susceptible one. The employed SA pretreatment significantly decreased the Cd effect in both genotypes, doing so more markedly in the Cd-susceptible one, except in the case of electrolyte leakage from the leaves of susceptible genotype seedlings treated with 7 mg kg^{-1} Cd, where electrolyte leakage was less pronounced than under conditions of SA pretreatment and treatment with 5 mg kg^{-1} Cd. This can be explained in

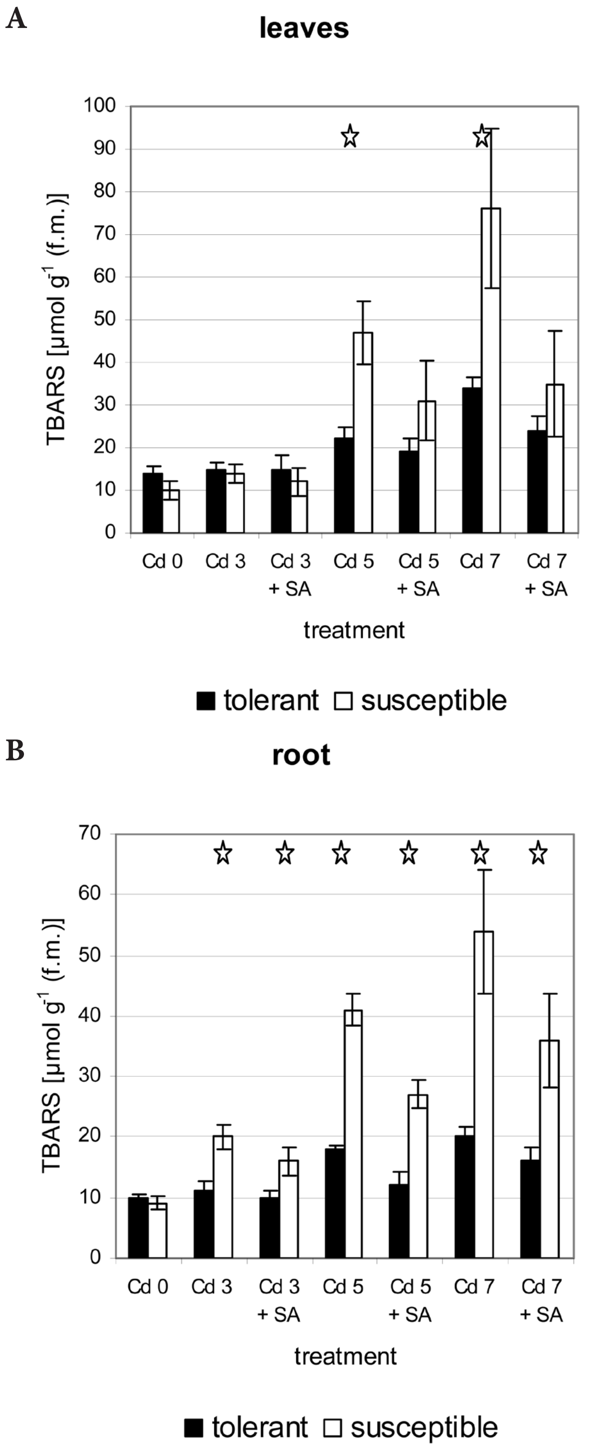

Fig. 4. Content of TBARS [µmoles g-1 (f. m.)] in roots (A) and leaves (B) of soybean seedlings. Treatment: 3, 5, and 7 mg kg^{-1} Cd for 72 h. +SA: seeds treated with 10^{-5} M SA at the beginning of imbibition. The results are given as mean values of three independent experiments ± SD. *Difference between genotypes significant at the level of $p < 0.01$.

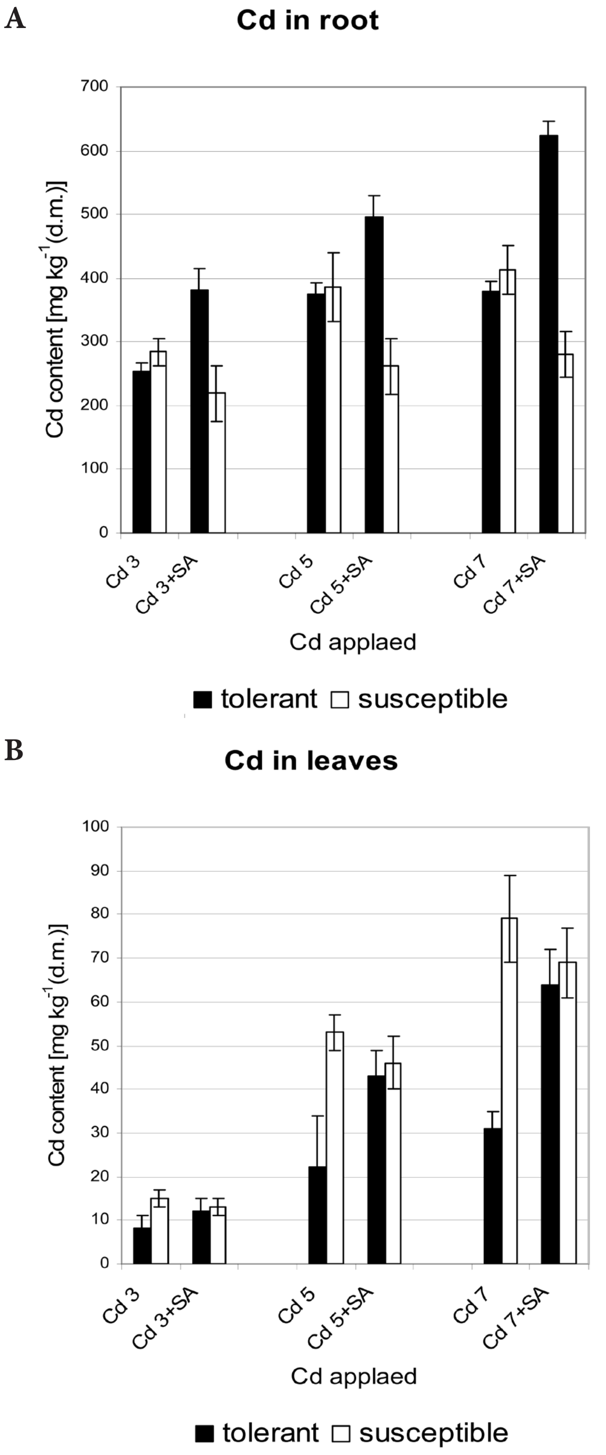

Fig. 5. Cadmium accumulation in roots and leaves of Cd-tolerant (Balkan) and Cd-susceptible (L608) soybean seedlings treated with 3, 5, and 7 mg kg-1 Cd for 72 h. +SA: seedlings pretreated with 10^{-5} M SA. The results are given as mean values of three independent experiments ± SD. *Difference between genotypes significant at the level of $p < 0.01$.

terms of the already mentioned dry tissue necrosis. Great variations encountered between the experiment repetitions also support this explanation.

Lipid peroxidation was measured by changes in TBARS content. These changes were similar in leaves and roots: in the Cd-tolerant genotype, a significant increase of TBARS concentration was induced by Cd only at the highest concentration. In roots of the susceptible one, such an increase was induced at the lowest Cd concentration, and SA caused a decrease of these changes (Fig. 4).

On the basis of the differences in TBARS content, it can be assumed that the antioxidative system in the tolerant genotype is much more effective than in the susceptible one. Our results are in accordance with results obtained for barley (Metwalley et al., 2003). Panda and Patra (2007) recently reported that Cd-induced oxidative damage in rice leaves – measured from enhanced thiobarbituric acid reactive substance (TBARS), hydrogen peroxide, and superoxide anion contents – was ameliorated by 16-h SA presoaking treatment, in addition to which efficiency of the antioxidant enzymes was significantly elevated.

Equal concentrations of Cd were found in roots of the tolerant and susceptible genotypes (Fig. 5) at all three of the applied Cd concentrations. Pretreatment with SA led to an increase of Cd accumulation in roots of the tolerant genotype and to a decrease of this accumulation in roots of the susceptible one. In leaves of the susceptible genotype, more Cd was accumulated than in leaves of the tolerant genotype. Under conditions of SA pretreatment, Cd accumulation was stimulated in leaves of the tolerant genotype and inhibited in leaves of the susceptible one, but not significantly. This result is in accordance with those obtained for *Brassica juncea* and *Vigna radiata* (Šimonova et al., 2007). In this case, high correlation of Cd concentration with toxic effects indicates that the response is dose-dependent. However, increased Cd-tolerance accompanied by increased metal concentration indicates that a different tolerance mechanism prevails.

The detected differences between soybean genotypes with respect to the response of seedlings to the toxic action of Cd may be due to genetically determined differences in Cd uptake, transport, and storage in various organs and tissues (Zhao et al., 2002) or to their different systems of defense against stress (Smeets et al., 2005). Genetic differences are specific for individual heavy metals and plant species, while differences in the defense system are systemic/unspecific. The low correlation between phytotoxicity and Cd accumulation in roots and leaves indicates that the tolerance mechanism is not based on Cd uptake. Similar findings were obtained in 17 soybean genotypes grown in hydroponics and under field conditions (Arao et al., 2003), 10 genotypes of *Brassica juncea* (Quadir et al., 2004), and six species of fast-growing shrubs belonging to the family Salicaceae (Lunačkova et al., 2003). In the present study SA exerted a protective effect which was more significant in the susceptible L608 genotype. This indicates that SA activates a systemic protective mechanism similar to these found for other stresses (Gaffney et al., 1993). It is well known that exposure to Cd leads to oxidative stress (Schutzendubel et al., 2001), and that plants have several enzymatic and non-enzymatic antioxidant defense systems which allow scavenging of ROS (reactive oxygen species) and protect the plant cells from oxidative damage. Salicylic acid is known to play an important role in modulating the trans-membrane redox balance, thereby counteracting the negative effects of reactive oxygen intermediates caused by oxidative stress (Yang et al., 2004) by increasing the activity of antioxidant enzymes (Singh and Usha, 2003). It has been demonstrated that Cd can induce oxidative stress in nodules, roots, and leaves of soybean characterized by accumulation of lipid peroxides, decrease in content of soluble antioxidants, and disturbance of the enzymatic system (Balestrasse et al., 2001; Noriega et al., 2004). An increase of TBARS after exposure to metal was observed in *Phaseolus vulgaris* (Somashekaraiah et al., 1992), *Helianthus annuus* (Gallego et al., 1996), and *Pisum sativum* (Lozano-Rodriguez et al., 1997), while methyl salicylate stimulates the antioxidative capacity and total phenolic, H_2O_2, and malonyl aldehyde contents in maca (*Lepidium meyenii*) calli (Wang et al., 2007).

Root Cd accumulation is similar in the two genotypes, but the response to SA pretreatment is completely different: in the tolerant genotype (Balkan), SA stimulates Cd accumulation, while in the susceptible genotype this accumulation is inhibited by SA. The role of root protection of the fragile aboveground parts from phytotoxic action of Cd is especially evident with genotypes tolerant to Cd. It was found that Cd-tolerant plants such as *Silene vulgaris* exhibit a higher ratio of root to shoot Cd content than in sensitive plants (de Knect et al., 1992), as was also been shown in our experiment (Fig. 5). The presented results indicate that differences between the genotypes with respect to Cd-tolerance are not due to differences of Cd accumulation and that SA pretreatment induced different Cd transport from roots to shoots only in the tolerant genotype.

On the basis of the obtained results, it can be assumed that only in the tolerant genotype (Balkan) did Cd induce moderate oxidative stress that activates defense mechanisms preventing further Cd uptake. Salicylic acid pretreatment activates mechanisms of systemic acquired resistance in a way which prevents the plant from recognizing stress and Cd accumulation proceeds freely, but primarily in roots and in the metabolically inactive form. In the Cd-susceptible genotype (L608), the system preventing oxidative stress injuries was poorly developed, but more sensitive to SA pretreatment.

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УТИЦАЈ САЛИЦИЛНЕ КИСЕЛИНЕ НА АКУМУЛАЦИЈУ КАДМИЈУМА У Cd-ОСЕТЉИВИМ И ТОЛЕРАНТНИМ КЛИЈАНЦИМА СОЈЕ

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Праћен је утицај претретмана салицилном киселином (SA) (10^{-5} M) на токсично деловање и акумулацију Cd у клијанцима соје генотипа релативно отпорног (Балкан) и релативно неотпорног (L608) на Cd. Промене свих испитиваних параметара токсичности: сушење листа преко смањења количине воде, деструкција фотосинтетичког апарата преко смањења концентрације хлорофила, оштећења ћелијских мембрана преко цурења елекролита и оксидативни стрес преко промене пероксидације липида су израженије у клијанцима осетљивог генотипа. Промене свих испитиваних параметара су значајно умањене у клијанцима који су третирани SA на самом почетку клијања. Акумулација Cd у корену и листу контролних клијанаца је слична и у корелацији је са детектованим променама. Генотипови се суштински разликују по утицају SA на акумулацију Cd: у корену клијанаца толерантног SA изазива повећање акумулације Cd, а у клијанцима осетљивог смањење. Може се претпоставити да "отпорнији" генотип ефикасније контролише оксидативни стрес изазван Cd тако да се не укључује одбрамбени механизам који спречава даљи улазак Cd у биљку. Ове генотипске разлике су вероватно последица разлике у ефикасности неспецифичних механизама детоксикације који обухватају и SA као сигнални молекул.