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Effects of exogenous salicylic acid pre-treatment on cadmium toxicity and leaf lipid content in *Linum usitatissimum* L.

A. Belkhadi [a], H. Hediji [a], Z. Abbes [a], I. Nouairi [b], Z. Barhoumi [c], M. Zarrouk [b], W. Chaïbi [a], W. Djebali [a,*]

[a] Unité de Recherche de Biologie et Physiologie Cellulaires Végétales, Département de Biologie, Faculté des Sciences de Tunis, Campus Universitaire, 1060 Tunis, Tunisia
[b] Laboratoire de Caractérisation et Qualité de l'Huile d'Olive, Centre de Biotechnologie de Borj-Cedria, BP 901, 2050 Hammam-Lif, Tunisia
[c] Laboratoire d'Adaptation des Plantes aux Stress Abiotiques, Centre de Biotechnologie de Borj-Cedria, BP 901, 2050 Hammam-Lif, Tunisia

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ABSTRACT

The effects of salicylic acid (SA) on cadmium (Cd) toxicity in flax plants were studied by investigating plant growth, lipid peroxidation and fatty acid composition. Cadmium inhibited biomass production as well as the absorption of K, Ca, Mg and Fe. Furthermore, it dramatically increased Cd accumulation in both roots and shoots. The pre-soaking of dry flax grains in SA-containing solutions partially protected seedlings from Cd toxicity during the following growth period. SA treatment decreased the uptake and the transport of Cd, alleviated the Cd-induced inhibition of Ca, Mg and Fe absorption and promoted plant growth. At leaf level, Cd significantly decreased both total lipid (TL) and chlorophyll (Chl) content and enhanced electrolyte leakage and lipid peroxidation as indicated by malondialdehyde (MDA) accumulation. Concomitantly, Cd caused a shift in fatty acid composition, resulting in a lower degree of their unsaturation. SA pre-soaking ameliorated the increased electrolyte leakage as well as Chl, MDA and TL content. SA particularly increased the percentage of linolenic acid and lowered that of palmitic acid by the same proportion. These results suggest that SA could be used as a potential growth regulator and a stabilizer of membrane integrity to improve plant resistance to Cd stress.

1. Introduction

Cadmium (Cd) is a widespread non-redox heavy metal that enters the soil environment from natural (weathering of rocks) as well as anthropogenic sources (mining and industrial activities, urban and agricultural run offs…). Cd pollution is a problem of increasing significance for ecological, nutritional and environmental reasons. It is recognized as an extremely significant pollutant due its high toxicity and large solubility in water (Pinto et al., 2004). Excess Cd disturbs the metabolism of plants and negatively affects the vegetative growth (Das et al., 1997). Numerous studies have indicated that Cd causes nutrient deficiency (Boulila et al., 2006; López-Millán et al., 2009) and induces inhibition of chlorophyll biosynthesis and a decline in the photosynthetic rate (Tukaj et al., 2007; López-Millán et al., 2009). Other membrane-located activities, which are also adversely affected by Cd exposure are cation leakage (Singh et al., 2008), ATP production (Dorta et al., 2003) and ion uptake (Das et al., 1997). At cellular level, Cd toxicity enhanced oxidative stress by increased levels of reactive oxygen species (ROS) (Sharma and Dietz, 2009). However, the levels of oxidative stress vary with the plant species and period of Cd exposure. One of the consequences of ROS activity is the oxidative damage of membrane integrity due to lipid peroxidation processes (Apel and Hirt, 2004; Dixit et al., 2001), which may result in generation of highly cytotoxic compounds and reduction of plant development. Changes in membrane lipid composition may affect the fluidity and also the intrinsic-membrane protein activities as a result of alteration in the lipidic environment in which they are embedded (Quartacci et al., 2000). Following Cd stress, several aspects of lipid biochemistry have often been noted to change. These include qualitative and quantitative alterations in lipids, inhibition of biosynthetic pathways and a reduction in unsaturated fatty acids due to metal-enhanced peroxidation (Djebali et al., 2005; Ben Youssef et al., 2005).

Different plant species and varieties show a wide range of plasticity in Cd tolerance, reaching from the high degree of sensitivity to the hyper accumulating phenotype of some tolerant plants. To avoid Cd toxicity plants adopt various defense strategies including phytochelation and sequestration as well as induction of antioxidant machinery and stress proteins (Cobbett and Goldsbrough, 2002; Vazquez et al., 2006). Other detoxification mechanisms that plants have developed to cope with damages caused by Cd are related with some stress signalling molecules, such as salicylic acid and nitric oxide. Salicylic acid (SA) is considered as a hormone-like substance, which plays an important role in regulating a number

* Corresponding author. Fax: +216 71 885 480.
E-mail address: wahbi.djebali@fst.rnu.tn (W. Djebali).

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of physiological processes and plant resistance to biotic and abiotic stresses (Mishra and Choudhuri, 1999; Hayat et al., 2009). SA is implicated in the regulation of plant growth, seed germination, fruit yield, systemic acquired resistance against some pathogenic infections and hypersensitive cell death and hardening responses to abiotic stressors (Gaffney et al., 1993; Metraux et al., 1990; Klessig and Malamy, 1994). SA appears to regulate the delicate balance between pro- and antideath functions during hypersensitive response (Klessig et al., 2000). Moreover, ion uptake and transport, photosynthetic rate, stomatal conductance and transpiration could also be affected by SA application (Hayat et al., 2009). In fact, SA reversed the closure of stomata caused by abscissic acid, affected membrane depolarization, stimulated photosynthetic machinery, and increased chlorophyll content (Rai et al., 1986; Hayat et al., 2009). Thus, convincing data have been obtained concerning the SA induced increase in plant resistance to chilling injury (Janda et al., 1999), low and high temperature (Senaratna et al., 2000) as well as salt stress (Shakirova, 2007). Further, it has been shown that SA modifies plant responses to osmotic stresses (Borsani et al., 2001), ozone or UV light (Sharma et al., 1996), drought (Senaratna et al., 2000) and herbicides (Ananieva et al., 2004). There are also evidences that SA ameliorates the damaging effects of heavy metals by broad but different effects (Drazic and Mihailovic, 2005; Metwally et al., 2003; Mishra and Choudhuri, 1999). SA pre-treatment alleviated Pb-induced membrane damage in rice (Mishra and Choudhuri, 1999), Hg-induced oxidative stress in *Medicago sativa* (Zhou et al., 2009) and Cd toxicity in barley and maize plants (Metwally et al., 2003; Pal et al., 2002). The protective function of SA mainly includes the regulation of ROS and antioxidants, induction of gene expression (Shah, 2003), and absorption and distribution of elements (Metwally et al., 2003; Mishra and Choudhuri, 1999). Apparently, SA has broad but divergent effects on stress acclimation and damage development of plants. However, the role of exogenously applied SA under Cd stress on the mineral nutrition and membrane integrity of plant is not still clear and needs further investigations. Based on the above studies, our investigations aimed at exploring the interaction of SA and Cd stress by using a single SA-induced priming event by pre-soaking of the seeds. The influence of SA on Cd-induced changes of growth, nutrient distribution and leaf lipid content and fatty acid profiles in flax seedlings has been studied.

2. Materials and methods

2.1. Growth conditions

Flax (*Linum usitatissimum* L., var. Vicking) grains were soaked for 8 h either in 250 and 1000 μM SA (sodium salt) or in water as a control. Flax seeds were germinated on moistened filter paper for four days at 25 °C in the dark. The seedlings were selected for uniformity and transferred into plastic beakers (6 l capacity, seven plants per beaker) filled with nutrient solution containing 1 mM $MgSO_4$, 2.5 mM $Ca(NO_3)_2$, 1 mM KH_2PO_4, 2 mM KNO_3, 2 mM NH_4Cl, 50 μM EDTA–Fe–K, 30 μM H_3BO_3, 10 μM $MnSO_4$, 1 μM $ZnSO_4$, 1 μM $CuSO_4$ and 30 μM $(NH_4)_6Mo_7O_{24}$. The nutrient solution was buffered to pH 5.5 with HCl/KOH, aerated, and changed twice per week. After an initial growth period of 2 days, $CdCl_2$ was added at concentrations of 50 and 100 μM. Cadmium doses used in this work are chosen appropriately to expose the plants from moderate to high levels of Cd. Plants were grown in a growth chamber at a day/night cycle of 16 h/8 h, at 23 °C/18 °C, respectively, a relative humidity close to 75% and a light intensity of 200 μmol photons m^{-2} s^{-1}. After 12 days of growth in hydroponics, i.e. 4 d after soaking the grains, the plants were harvested, growth parameters determined, and material was frozen at −80 °C for biochemical analysis.

2.2. Determination of mineral element concentrations

Harvested plants were washed thoroughly with running distilled water, divided into roots, stems and leaves, and dried to constant weight. Dried plant tissues were ground and digested in HNO_3:$HClO_4$ (3:1, v/v) at 100 °C. Cadmium and mineral contents were estimated by atomic absorption spectrophotometry (Perkin-Elmer, AAnalyst 300) using air–acetylene flame.

2.3. Determination of lipid peroxidation and membrane permeability

The level of lipid peroxidation in the plant tissues was determined as 2-thiobarbituric acid (TBA) reactive metabolites chiefly malondialdehyde (MDA) as described previously by (Buege and Aust, 1972). In brief, 0.25 g of tissue was homogenized in 5 mL of 0.1% (w/v) trichloroacetic acid. The homogenate was spun at 10,000*g* for 5 min. To 1 mL aliquot of the supernatant, 4 mL of 20% (w/v) trichloroacetic acid containing 0.5% (w/v) thiobarbituric acid was added. The mixture was heated at 95 °C for 30 min and then quickly cooled in an ice bath. After centrifugation at 10,000*g* for 10 min, the absorbance of the supernatant was measured at 532 nm. The value was corrected for the nonspecific absorption at 600 nm. The level of lipid peroxidation is expressed as nmol of MDA formed using an extinction coefficient of 155 mM cm^{-1}. Membrane permeability of the leaves was determined as follows. Leaf tissue (100 mg) was vibrated for 30 min in deionised water followed by measurement of conductivity of bathing medium (EC1). Then, the samples were boiled for 15 min and the final conductivity (EC2) of the medium was measured. The percent leakage of electrolytes was calculated by using the formula: $(EC_1/EC_2)100$.

2.4. Determination of chlorophyll and carotenoid contents

Chlorophyll content in the leaves (100 mg FW) was extracted in 80% chilled acetone and estimated by the method of Arnon (1949). Carotenoid concentration in the same extract was calculated by the formula given by McKinney (1941).

2.5. Lipid extraction and fatty acid determination

The lipids were extracted according to the method of Allen and Good (1971). Leaf tissues were fixed in boiling water for 5 min to denature phospholipases and then homogenised in chloroform:methanol mixture (2:1, v/v). The homogenate was centrifuged at 3000*g* for 15 min. The lower chloroformic phase containing lipids was aspirated and evaporated at 40 °C under vacuum using a rotary evaporator or with nitrogen gas. The residue was immediately redissolved in 2 mL of toluene:ethanol mixture (4:1, v/v) for conservation. Fatty acids from total lipids (TL) were methylated by the method of Metcalfe et al. (1966). Fatty acid methyl esters of the total lipids were analyzed by gas chromatography using a Hewlett Packard chromatography model 4890D equipped with an Innowax capillary column (30 m × 0.53 mm i.d.) maintained isothermally at 210 °C. For measuring the amounts of fatty acids, heptadecanoic acid (17:0) was added as an internal standard. Calculation of fatty acid quantities was done using an integrator.

2.6. Data analysis

All the presented data are means of at least two independent experiments on at least 6 plants each. For each parameter, data were subjected to a one-way ANOVA analysis. When the effect was significant ($P=0.05$), differences between means were evaluated for significance by using Tukey's (HSD) test.

3. Results

3.1. Plant growth

Fresh weights of roots, stems and leaves are decreased significantly under the influence of Cd (45.6, 58.3 and 36.1% at 100 μM Cd, respectively) (Table 1). Contrarily, fresh masses of these organs increased by SA treatment. Pre-soaking with SA before exposure to Cd increased root, stem and leaf fresh masses in Cd-dependent manner. Moreover, SA enhanced root and shoot tissue elongations in plants submitted or not to Cd (Table 1). However, Cd and SA in concentrations applied here do not lead to significant changes of water balance (Data not shown).

3.2. Cd accumulation and distribution

In the analysed flax seedlings, Cd is accumulated mainly in root (approximately, 55–83% of total plant content) (Fig. 1). In roots, Cd accumulated in a concentration dependant manner, but in stems and leaves its accumulation was in a dose-independent