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Research article

Exogenous malic and acetic acids reduce cadmium phytotoxicity and enhance cadmium accumulation in roots of sunflower plants

Barbara Hawrylak-Nowak [a, *], Sławomir Dresler [b], Renata Matraszek [a]

[a] Department of Plant Physiology, University of Life Sciences in Lublin, Akademicka 15, 20-950 Lublin, Poland
[b] Department of Plant Physiology, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland

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ABSTRACT

There is increasing evidence showing that low molecular weight organic acids (LMWOA) are involved in heavy metal resistance mechanisms in plants. The aim of this study was to investigate the effects of exogenous malic (MA) or acetic (AA) acids on the toxicity and accumulation of cadmium (Cd) in sunflower (*Helianthus annuus* L.). For this purpose, plants were grown in hydroponics under controlled conditions. Single Cd stress (5 μM Cd for 14 days) induced strong phytotoxic effects, as indicated by a decrease in all growth parameters, concentration of photosynthetic pigments, and root activity, as well as a high level of hydrogen peroxide (H_2O_2) accumulation. Exogenous MA or AA (250 or 500 μM) applied to the Cd-containing medium enhanced the accumulation of Cd by the roots and limited Cd translocation to the shoots. Moreover, the MA or AA applied more or less reduced Cd phytotoxicity by increasing the growth parameters, photosynthetic pigment concentrations, decreasing accumulation of H_2O_2, and improving the root activity. Of the studied organic acids, MA was much more efficient in mitigation of Cd toxicity than AA, probably by its antioxidant effects, which were stronger than those of AA. Plant response to Cd involved decreased production of endogenous LMWOA, probably as a consequence of severe Cd toxicity. The addition of MA or AA to the medium increased endogenous accumulation of LMWOA, especially in the roots, which could be beneficial for plant metabolism. These results imply that especially MA may be involved in the processes of Cd uptake, translocation, and tolerance in plants.

1. Introduction

High levels of heavy metals in the environment may have a geological or anthropogenic origin. Some of them are essential micronutrients for plants (Co, Cu, Fe, Mn, Mo, Ni, Zn) but become phytotoxic at elevated concentrations. Other heavy metals (Cd, Pb, Cr, Hg, Ag, Au, U) are not essential for plant functioning and are extremely toxic, even at mild excess (Lambers et al., 2008). Increasing environmental pollution caused by metals, mainly due to industrial and agricultural activities, is becoming a serious problem in the modern world (Sun et al., 2005). Among the various heavy metals, cadmium (Cd) is considered as the most dangerous environmental pollutant due to its high toxicity, mobility, and availability for all living organisms (Das et al., 1997; Sanitá di Toppi and Gabbrielli, 1999). It has been ranked No. 7 among the top 20 toxins (Gill and Tuteja, 2011). The biochemical and physiological basis of Cd phytotoxicity is not always clear. Cadmium, like other heavy metals, can accept a pair of electrons from a coordinate covalent bond, react with $-S^-$ groups, $-OH^-$ groups, amino groups, and carboxylic acid termini, and thus affect sulfhydryl groups and N atoms in proteins causing its inactivation (Lambers et al., 2008). Moreover, oxidative stress has often been discussed as the primary effect of Cd exposure (Podazza et al., 2012). Although Cd is a non-redox active element, it can generate overproduction of reactive oxygen species (ROS), including superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\bullet$), which cause cell damage (Gill and Tuteja, 2011).

Plant organisms can effectively diminish Cd-induced damage by regulating the physiological and biochemical metabolism. In order to survive, plants have to develop specific heavy metal detoxification mechanisms. One of the main Cd tolerance mechanisms by which plants are able to reduce Cd bioavailability in the rhizosphere, and thus reduce the amount of Cd taken up by roots, is exudation of some organic substances, like low molecular weight organic acids (LMWOA), to the rhizosphere (Dong et al., 2007). Metabolism of organic acids plays a crucial role at the cellular level

* Corresponding author.
E-mail address: bhawrylak@yahoo.com (B. Hawrylak-Nowak).

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in several biochemical processes, including energy production, photosynthesis, formation of precursors for amino-acid biosynthesis, cation transport, and at the whole plant level in modulating adaptation to stressful conditions (López-Bucio et al., 2000; Schulze et al., 2002). The hypothesis about the involvement of LMWOA in the detoxification of heavy metals was proposed by Rauser (1999), and there is increasing evidence showing that LMWOA may contribute in the heavy metal tolerance mechanisms (Saber et al., 1999; Ma, 2000; Boominathan and Doran, 2003; Sun et al., 2005; Chiang et al., 2006; Duarte et al., 2007). The LMWOA produced by the root system can modify the physical and chemical properties of both soil and metals, participating in the uptake of essential nutrients and in the detoxification of heavy metals (Duarte et al., 2007). As plant exudates, LMWOA increase extracellular precipitation of heavy metals by the chelation oxidation–reduction reaction in the root zone. Moreover, likewise phytochelatins or amino acids, LMWOA can chelate and sequestrate metals in the vacuole (Dresler et al., 2014). However, the detailed mechanisms of their biological action are still not fully understood. Therefore, knowledge regarding the physiological processes involved in Cd toxicity, accumulation, and distribution within plants under the influence of exogenous LMWOA, is crucial for a better understanding of their role in the detoxification of heavy metals. Moreover, due to their degradable nature, the use of LMWOA can be an efficient alternative to synthetic chelating agents used in the phytoremediation (chelate-assisted phytoextraction or chelate-assisted phytostabilisation) of heavy metal-contaminated areas (Kim et al., 2010; Lu et al., 2013), since many studies have shown that uptake of heavy metals by plants can be considerably enhanced by adding relatively low amounts of LMWOA to the contaminated medium (Han et al., 2006; Freitas et al., 2013; Ehsan et al., 2014).

Sunflower (*Helianthus annuus* L.) has been considered as a good candidate for bioaccumulation and phytoremediation of heavy metals (Chiang et al., 2006; Niu et al., 2012). Therefore, the objectives of this study were to investigate the Cd phytotoxicity and accumulation in sunflower plants exposed to Cd individually or simultaneously with exogenous malic acid (MA) or acetic acid (AA). The parameters regarding the plant growth, photosynthetic pigments concentration, hydrogen peroxide (H_2O_2) accumulation, and root activity were studied under Cd stress with and without LMWOA application. Moreover, the levels of endogenous Cd and LMWOA were determined.

2. Materials and methods

2.1. Plant material and growth conditions

The experiments were carried out on sunflower (*H. annuus* L.) grown in a controlled-climate phytotron room. Sunflower seeds germinated in germination rolls, between two layers of moistened filter paper, for 7 days at 25 °C. The healthiest, best-developed seedlings were transferred to 1 dm^3 glass jars (two plants per jar) filled with 1.5-times strength Hoagland's II nutrient solution (Hoagland and Arnon, 1950). The initial pH of the growth media was adjusted to 6.0 using diluted NaOH. The pH of the nutrient solution was measured every three days in order to monitor the changes in its value during the experiment. One concentration of Cd (5 μM, as $CdCl_2$) was combined with two (250 or 500 μM) malic acid (MA) or acetic acid (AA) levels. Detailed treatments were as follows: control (0 Cd/0 MA or AA), 5 μM Cd, 5 μM Cd + 250 μM MA, 5 μM Cd + 500 μM MA, 5 μM Cd + 250 μM AA, 5 μM Cd + 500 μM AA. Plants were cultured under the following conditions: photosynthetic photon flux density at the level of the top of the plants of 250–270 μmol m^{-2} s^{-1}, 14-h day length, temperature 24/20 °C (day/night) and relative humidity of 50–60%. After 14 days of plant growth under the differentiated conditions, the biometric parameters (fresh weight and length of roots and shoots, leaf area) and the physiological parameters (photosynthetic pigments, H_2O_2, and organic acid concentrations) were determined and histochemical analysis (visualization of the activities of root tips, visualization of H_2O_2 accumulation in leaf blades) was performed as described below. Thereafter, plant materials were dried at 70 °C and ground before analysis of the Cd concentrations.

2.2. Determination of biometric parameters

The effects of Cd on the sunflower growth were evaluated by determining root and shoot lengths and fresh weights (FW) as well as leaf areas (LA). Fresh first and second true leaves were scanned using a CI-202 laser area metre (CID Bio-Science, USA).

2.3. Determination of physiological parameters

2.3.1. Concentration of photosynthetic pigments

The photosynthetic pigment concentrations were determined in the first and second true leaves by the extraction of leaf samples in 80% (v/v) acetone. The absorbance of the resulting extracts was measured at 663 nm, 646 nm, and 470 nm. The concentration of chlorophyll *a* and *b* and total carotenoids (xanthophylls + carotenes) was determined from the equations given by Lichtenthaler and Wellburn (1983).

2.3.2. H_2O_2 concentration

The concentration of H_2O_2 in the second true leaves was measured colorimetrically by the modified method of Jana and Choudhuri (1982). H_2O_2 was extracted by homogenising 250 mg of plant tissue with 3 mL of phosphate buffer (50 mM, pH 6.5). The homogenates were centrifuged at 6000 rpm for 25 min. To determine H_2O_2 concentrations, 3 mL of the supernatant obtained was mixed with 1 mL of 0.1% titanium dioxide in 20% H_2SO_4 (w/v). Then, the mixture was centrifuged at 6000 rpm for 15 min. The intensity of the yellow colour of the supernatant was measured at 410 nm. The concentrations of H_2O_2 were calculated using the extinction coefficient of 0.28 μM^{-1} cm^{-1}.

2.3.3. Concentration of endogenous organic acids

The organic acid concentrations in the leaf and root homogenates were determined by capillary electrophoresis (CE). Preparation of plant samples and CE separation conditions was performed according to Dresler et al. (2014). Briefly, the organic acids were extracted from fresh plant tissues by homogenisation in distilled water. After incubation (30 min, 50 °C), the homogenate was centrifuged at 10,000 rpm for 5 min and filtered through 0.20 μm nylon filters. Measurements were performed on an Agilent 7000 capillary electrophoresis system (Agilent Technologies) equipped with a diode array spectrophotometric detector (190–600 nm). A fused-silica capillary with 75 μm id with effective length of 72 cm (Agilent Technologies) was used. The data obtained were analysed using ChemStation data analysis software (3D-CE ChemStation, Agilent Technologies).

2.4. Histochemical analysis

2.4.1. Visualization of root activity by the TTC method

The technique used is based on visual detection of the activity of dehydrogenases in plant root tips with the use of the 2,3,5-triphenyltetrazolium chloride (TTC) dye method with slight modifications (Clemensson-Lindell, 1994; Kurzbaum et al., 2010). Briefly, for determination of dehydrogenase activity, the root tips (about 10 mm long) were gently incubated for 30 min with a