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Insights into citric acid-induced cadmium tolerance and phytoremediation in *Brassica juncea* L.: Coordinated functions of metal chelation, antioxidant defense and glyoxalase systems

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ABSTRACT

Cadmium (Cd) is a serious environmental threat because it accumulates in plants from soil and is subsequently transported into the food cycle. Increased Cd uptake in plants disrupts plant metabolism and hampers crop growth and development. Therefore, remediation of Cd from soil and enhancing plant tolerance to metal toxicity is vital. In the present study, we investigated the function of different doses of citric acid (CA) on Cd toxicity in terms of metal accumulation and stress tolerance in mustard (*Brassica juncea* L.). *Brassica juncea* seedlings (12-day-old) were treated with Cd (0.5 mM Cd and 1.0 mM $CdCl_2$) alone and in combination with CA (0.5 mM and 1.0 mM) in a semi-hydroponic medium for three days. Cadmium accumulation in the roots and shoots of the mustard seedlings increased in a dose-dependent manner and was higher in the roots. Increasing the Cd concentration led to reduced growth, biomass, water status, and chlorophyll (chl) content resulting from increased oxidative damage (elevated malondialdehyde, MDA content; hydrogen peroxide, H_2O_2 level; superoxide, $O_2^{\bullet-}$ generation; lipoxygenase, LOX activity; and methylglyoxal, MG content) and downregulating of the major enzymes of the antioxidant defense and glyoxalase systems. Under Cd stress, both doses of CA improved the growth of the plants by enhancing leaf relative water content (RWC) and chl content; reducing oxidative damage; enhancing the pool of ascorbate (AsA) and glutathione (GSH) and the activities of the antioxidant enzymes (ascorbate peroxidase, APX; monodehydroascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; glutathione reductase, GR; glutathione peroxidase, GPX; superoxide dismutase, SOD; catalase, CAT); improving the performance of the glyoxalase system (glyoxalase I, Gly I and glyoxalase II, Gly II activity); and increasing the phytochelatin (PC) content. Exogenous CA also increased the root and shoot Cd content and Cd translocation from the roots to the shoots in a dose-dependent manner. Our findings suggest that CA plays a dual role in mustard seedlings by increasing phytoremediation and enhancing stress tolerance through upregulating the antioxidant defense and glyoxalase systems.

1. Introduction

Ever-increasing anthropogenic activities including rapid industrialization, urbanization, excessive use of chemical fertilizers, and sewage sludge are hastening the release of diverse toxic heavy metals, such as cadmium (Cd), into the environment (Prasad et al., 2001; Prasad, 2004; Liu et al., 2016). Cadmium is widespread and a dangerous toxic environmental pollutant, which severely reduces crop productivity by hampering plant growth and development (Abbas et al., 2017; Hasanuzzaman et al., 2017a; Pereira de Araújo et al., 2017).

Abbreviations: AO, ascorbate oxidase; APX, ascorbate peroxidase; AsA, ascorbic acid; CA, citric acid; CAT, catalase; CDNB, 1-chloro-2,4-dinitrobenzene; DAB, diaminobenzidine; DHA, dehydroascorbic acid; DHAR, dehydroascorbate reductase; DTNB, 5,5-dithio-bis-(2-nitrobenzoic) acid; EDTA, ethylenediaminetetraacetic acid; Gly, glyoxalase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GPX, glutathione peroxidase; GST, glutathione *S*-transferase; LOX, lipoxygenase; MA, maleic acid; MDHAR, monodehydroascorbate reductase; MG, methylglyoxal; NBT, nitroblue tetrazolium chloride; Pro, proline; ROS, reactive oxygen species; SLG, *S*-D-lactoyl-glutathione; SOD, superoxide dismutase; TBA, thiobarbituric acid; TCA, trichloroacetic acid

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Cadmium negatively affects the phenotype and physiology of plants. For example, leaf chlorosis, leaf and root necrosis, leaf epinasty, and root growth inhibition are common morphological changes in plants resulting from Cd exposure. Moreover, Cd hampers plant metabolism by reducing nutrient uptake, inhibiting photosynthesis, changing nitrogen and sulfur metabolism, and altering the activities of several key enzymes (Benavides et al., 2005; Gill and Tuteja, 2011; Hasanuzzaman et al., 2012a; Zong et al., 2017; Pereira de Araújo et al., 2017).

Since Cd is a redox inactive metal it cannot generate reactive oxygen species (ROS) through the Haber-Weiss reaction. However, by disrupting the electron transport chain, interacting with the antioxidant defense system, or disturbing the metabolism of essential mineral nutrients, Cd can cause the overproduction of toxic ROS, including singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$), which can oxidize important cellular ultrastructures (for example, proteins, lipids, and nucleic acids), leading to cellular destruction (Dong et al., 2006; Qadir et al., 2004; Srivastava et al., 2004; Hasanuzzaman et al., 2012a). To combat Cd toxicity, plants have developed numerous avoidance techniques to lessen the deleterious effects. Of these techniques, metal binding to the cell wall, decreasing transport across the cell membrane, active efflux, active excretion, compartmentalization, and metal chelation are common strategies (Hall, 2002; Gratão et al., 2005).

Although plants avoid metal toxicity but suffer from oxidative stress with the increase in stress intensity. Plant cells are equipped with the antioxidant defense system to minimize oxidative stress. The non-enzymatic components (ascorbic acid, AsA; glutathione, GSH; phenolic compounds; alkaloids; α-tocopherol; and non-protein amino acids) and enzymatic components (superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; glutathione reductase, GR; monodehydroascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; glutathione peroxidase, GPX; and glutathione *S*-transferase, GST) of the antioxidant defense system work together to scavenge ROS under stress conditions (Gill and Tuteja, 2010; Hasanuzzaman et al., 2012a).

Heavy metal stress is also responsible for spontaneous overproduction of methylglyoxal (MG), a cytotoxic compound, which destroys the cellular ultra-structure and causes mutations and even cell death by generating oxidative stress. However, plants also have an MG detoxification system consisting of two important enzymes, glyoxalase I (Gly I) and glyoxalase II (Gly II) (Yadav et al., 2008; Hasanuzzaman et al., 2017b). Generally, these two enzymes maintain a balance between MG manufacture and detoxification with the help of GSH (Yadav et al., 2008; Nahar et al., 2016a; Hasanuzzaman et al., 2017b). Therefore, their upregulation is essential in detoxifying elevated levels of MG under stress conditions. Effective functioning of the antioxidant defense and glyoxalase systems against ROS and MG under stress conditions determines the tolerance capability of plants. However, the efficiency of the antioxidant defense and glyoxalase systems varies greatly with plant genotypes and stress intensity.

Citric acid (CA) is a common organic acid that works as an intermediate of the trichloroacetic acid (TCA) cycle. It also supplies energy for cells, which is used in respiration and different biochemical mechanisms (Hu et al., 2016). The role of CA in enhancing phytoremediation of different heavy metals (by increasing metal solubility and mobilization) and working against metal-induced oxidative stress (through increasing key enzymes activity of the antioxidant defense system) has been established in many studies (Gao et al., 2010; Yeh et al., 2012; Freitas et al., 2013). However, the rate of metal accumulation and efficiency in plant stress tolerance depends on the dose of CA. In many studies, CA was used as an agent of phytoremediation and a mediator of plant stress tolerance, but the function of different doses of CA on different levels of Cd toxicity in the same study is rarely investigated. Moreover, the CA-induced coordinated action of the antioxidant defense and glyoxalase systems of plants under Cd stress has not yet been investigated. On the other hand, *Brassica juncea* L. is a well-known hyper accumulator of Cd (Mahmud et al., 2017a). Therefore, the main purpose of this study was to appraise the Cd accumulation capability of *B. juncea* and to analyze its physiological and biochemical responses under Cd stress in combination with exogenous CA.

The effect of CA on metal accumulation, metal chelation, growth and biomass, water status, photosynthetic efficiency, cellular damage, and performance of the antioxidant and glyoxalase systems of *B. juncea* was observed under Cd stress during the early seedling stage. To the best of our knowledge, this report is the first on the role of different doses of CA in *B. juncea* seedlings under Cd toxicity, in which the combined actions of the antioxidant defense and glyoxalase systems, and metal chelation have been studied.

2. Materials and methods

2.1. Plant materials, growing conditions, and stress treatments

Healthy uniform mustard (*Brassica juncea* L. cv. BARI Sharisha-11) seeds were thoroughly washed with distilled water after sterilizing with 70% ethanol. Seeds were then sown in Petri dishes (9 cm) lined with six layers of filter paper moistened with 10 mL of distilled water and kept in the dark in a germinator for 2 d. All Petri dishes contained 60 morphologically uniform germinated seedlings. The seedlings were then grown in a growth chamber (Iwaki; Asahi Techno Glass, Japan) under controlled conditions (light: 350 μmol photons m^{-1} s^{-2}; temperature: 25 ± 2 °C; relative humidity: 65–70%); 5000-fold diluted Hyponex solution (Hyponex, Japan) was used as a nutrient. Mustard seedlings (12 d old) were exposed to CA (0.5 and 1.0 mM) and Cd (0.5 and 1.0 mM $CdCl_2$) separately and in combination. We considered 0.5 mM as mild stress and 1.0 mM $CdCl_2$ as severe stress. Control seedlings were grown in Hyponex solution only. After 3 d of treatment, the leaves and roots were harvested and used to study various growth and physiological parameters following a completely randomized design (CRD) with nine treatments and repeated three times under similar conditions.

2.2. Measurement of Cd content, biological concentration factor (BCF), biological accumulation coefficient (BAC), and translocation factor (TF)

An atomic absorption spectrophotometer (Z-5000; Hitachi, Japan) was used to determine the Cd content of the shoots and roots of the mustard seedlings. Plant samples were oven dried at 80 °C for 72 h and then digested separately with acid mixture (HNO_3:$HClO_4$ = 5:1 v/v) for 48 h at 80 °C. The absorbance of the samples was recorded by the spectrophotometer, and the Cd content of the shoots and roots was calculated using a standard curve of known concentration. BCF, BAC, and TF were calculated according to Nahar et al. (2016a) using the following formula:

$$\text{BCF} = \text{Metal content (roots)/metal content (growing media)}$$

$$\text{BAC} = \text{Metal content (shoots)/metal content (growing media)}$$

$$\text{TF} = \text{Metal content (shoots)/metal content (roots)}$$

2.3. Estimation of phytochelatin (PC) content

Phytochelatin content was estimated after subtracting the total GSH content from the total non-protein thiols content. Ellman's reaction mixture was used to determine non-protein thiol content after homogenizing leaves in 3% (w/v) sulfosalicylic acid and read spectrophotometrically at 412 nm (Ellman, 1959).

2.4. Determination of plant growth parameters

Plant height was measured from each treatment and recorded in