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Role of GSH homeostasis under Zn toxicity in plants with different Zn tolerance

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ABSTRACT

Tripepthide glutathione (GSH) is a pivotal molecule in tolerance to heavy metals, including Zinc (Zn). The aim of our work is to examine the role of GSH metabolism in two different horticultural plants under Zn toxicity in order to select and/or generate plants tolerant to Zn toxicity. We show a comparative analysis of the toxic effect of 0.5 mM Zn between *Lactuca sativa* cv. Phillipus and *Brassica oleracea* cv. Bronco. In *L. sativa* the accumulation of Zn resulted in an increase in reactive oxygen species (ROS), while enzymes of GSH metabolism and the activities of the antioxidant enzymes were negatively affected. On the contrary, *B. oleracea* showed the existence of a detoxification mechanism of these ROS. Moreover, while in *L. sativa* increased the oxidized GSH (GSSG) and phytochelatins (PCs) concentration with the reduction of leaves biomass, in *B. oleracea* the higher concentration of reduced GSH and its use in the detoxification of ROS seems to be a major mechanism to provide tolerance to Zn toxicity without reducing leaf biomass. Our results suggested that under Zn toxicity, *B. oleracea* is more efficient and tolerant than *L. sativa* through the detoxification of lipid peroxidation products due to the reduced GSH.

1. Introduction

Zinc (Zn) is an essential micronutrient for plants, animals, and microorganisms [1]. In plants, Zn is required at optimal concentrations, both for the normal functioning of cell metabolism as well as for plant growth [2]. As cofactor of several enzymes, Zn is involved in many physiological processes such as the metabolism of carbohydrates, lipids, and nucleic acids, gene expression and regulation, enzyme activation, protein synthesis, and reproductive development such as pollen formation [3]. However, in environmental situations of heavy-metal soil pollution, Zn constitutes a serious problem for worldwide agricultural production [4]. Under these conditions, Zn accumulates in excess in the plant tissues, reaching toxic concentrations (>300 $\mu g\, g^{-1}$) for plants, causing physiological alterations and growth inhibition [5]. Particularly, the deleterious effects of Zn on photosynthesis has been associated with Zn-induced oxidative damage to membranes [2]. In this situation, the primary response of plants is the generation of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet -}$), hidroxyl radical (OH), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), causing the lipid peroxidation [6,7]. The non-redox-active metal ions, Zn and Cd, have been reported to increase lipid peroxidation via ROS generation in plants. Increased lipid peroxidation in plants exposed to toxic Zn levels has been attributed to stronger activity of membrane-bound lipoxygenase (LOX), which is known to oxidize polyunsaturated fatty acids and produce free radicals with concomitant increased production of malondialdehyde (MDA) [8].

The main enzymes and antioxidant compound determining the tolerance to the oxidative stresses might dependent on the plant species and metal toxicity [9]. However, several studies have suggested that the tripeptide glutathione (GSH) is a pivotal molecule in tolerance to heavy metals, include Zn [6,10,11]. The amount of GSH in a given organism is the result of the combined action of biosynthesis, consumption, and degradation [12]. Data suggest that excess of Zn in plants induces the generation of GSH, which in turn accelerates the enzyme activities of GSH synthesis [10,13]. The enzyme

Abbreviations: AsA, ascorbate; APX, ascorbate peroxidase; CDNB, 1-chloro-2,4-dinitrobenzene; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; GSH, glutathione; GSSG, oxidized glutathione; Gly I, glyoxalase I; Gly II, glyoxalase II; GPX, glutathione peroxidase; GR, glutathione reductase; GS, glutathione synthetase; GST, glutathione-S-transferase; H_2O_2, hydrogen peroxide; LOX, lipoxygenase; MDA, malondialdehyde; MDHAR, monodehydoascorbate reductase; NBT, nitroblue tetrazolium; $^1O_2$, singlet oxygen; $O_2^{\bullet -}$, superoxide anion; OH, hidroxyl radical; PCs, phytochelatins; PPFD, photosynthetic photon flux density; PVP, polyvinylpyrrolidone; ROS, reactive oxygen species; SAT, serine acetyltransferase; SLG, S-D-lactoylglutathione; TBA, thiobarbituric acid; TCA, trichloroacetic acid; TF, transfer factor; TNP-SH, non-protein thiols; γ-ECS, γ-glutamylcysteine synthetase.

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serine acetyltransferase (SAT), is the key enzyme in cysteine synthesis, thereby providing one of the substrates of GSH synthesis [14]. GSH is synthesized in two ATP-dependent steps catalyzed by γ-glutamylcysteine synthetase (γ-ECS), a rate-limiting enzyme, and glutathione synthetase (GS) [12,15]. First, γ-ECS catalyzes the formation of a peptide bond between the γ-carboxyl group of glutamate and the α-amino group of cysteine. Following, GS catalyzes the formation of a peptide bond between the cysteinyl carboxyl group of γ-glutamylcysteine and the α-amino group of glycine [16]. Several works have demonstrated that the overexpression of SAT or γ-ECS in various plants has triggered higher tolerance and accumulation of heavy metals such as Zn [6].

On the other hand, GSH is consumed in a number of redox reactions to combat oxidative stress, resulting in its oxidation to GSSG [17]. In this sense, in the ascorbate–glutathione cycle, the enzyme ascorbate peroxidase (APX) reduces H_2O_2 into water using ascorbate (AsA) as the electron donor and the resulting dehydroascorbate (DHA) is cycled back to ascorbate using reduced GSH as the electron donor, while the GSSG formed is converted back to reduced GSH by NAD(P)H-dependent glutathione reductase (GR) [11]. Some authors have demonstrated that the ascorbate–glutathione cycle plays a major role in tolerance to excess of heavy metals [9]. Recently, some studies have shown an increase in these enzymes in plants tolerant of Zn-toxicity conditions [18,19]. Regarding to Zn, Cuypers et al. [20] have suggested that an improvement in the activity of these enzymes could alleviate the toxic effects of 50 μM of $ZnSO_4$ in primary leaves of *Phaseolus vulgaris*.

Another highly reactive compound that is detoxified by the use of glutathione is the methylglyoxal. This compound is formed spontaneously in plants by non-enzymatic mechanisms under physiological conditions from glycolysis and from photosynthesis intermediates. However, under stress conditions, the rate of glycolysis increases, leading to an imbalance in the pathway [21]. Several reports indicate the overproduction of methylglyoxal under heavy-metals toxicity, including Zn [22,23]. Methylglyoxal is detoxified mainly by the maintenance of GSH homeostasis via glyoxalase system, which consists of two enzymes: glyoxalase I (Gly I) and glyoxalase II (Gly II). Gly I uses one molecule of reduced GSH to convert methylglyoxal to S-D-lactoylglutathione (SLG). Then Gly II converts SLG to D-lactate and one molecule of reduced glutathione is recycled back into the system [23]. Singla-Pareek et al. [22] demonstrated as both enzymes promoted stress tolerance in tobacco plants (*Nicotiana tabacum*) subjected to Zn stress (5 mM $ZnCl_2$).

In addition, the enzymes glutathione-S-transferase (GST) and glutathione peroxidase (GPX) use the pool of GSH as substrate to detoxify H_2O_2 and sequestration of heavy metals by acting as a precursor for phytochelatins (PCs) synthesis [8,24]. In this sense, overexpression of GST and GPX in transgenic tobacco enhanced seedling growth under a variety of stressful conditions [25]. With respect Zn, in *Hydrilla verticillata* exposed to a higher Zn dosage, GST activity increased with respect the control with the enhanced Zn-stress tolerance [26].

Finally, many works suggest that in stress situations involving trace elements, the plants produce low-molecular-weight compounds involved in the long-distance transport in the xylem and their subsequent detoxification in the cytoplasm and/or vacuole [27–29]. Notable among these compounds are phytochelatins (PCs), organic anions, histidine, nictotinamine, and phytosiderophores [30]. With respect to PCs, due to their ability to bind metals, generally these have been considered to be important cellular chelating agents, which function as heavy-metal detoxification and/or homeostasis agents [31]. GSH is a key compound for the synthesis of PCs. Gasic and Korban [32] observed that PCs function as important chelators of Zn excess due to slightly elevated Zn tolerance in *Brassica juncea* plants transformed with *AtPCS1*. However, recently, many studies have questioned the role of this compound as a tolerance mechanism in *Sedum alfredii* submitted Cd stress [33–35] or *Arabis paniculata* under different Zn stress [36].

Many studies have shown that there are great differences between the capacity to accumulate and tolerance mechanisms in different plants species under metal-toxicity conditions [1,2,5,7,21]. Therefore, in this work we performed a comparative study of two species of horticultural plants of great agricultural interest, i.e. *Lactuca sativa* and *Brassica oleracea*, determining the effect Zn toxicity on: (i) Zn concentration in root and leaves, Zn accumulation and transfer factor; (ii) oxidative stress in terms of lipid peroxidation; (iii) the response of GSH and its metabolism as a possible key role in tolerance to Zn toxicity in both species. These information will be useful to understand Zn-tolerance and to establish physiological markers of plants suited for phytoremediation of Zn-contaminated soils. Moreover, and considering that both horticultural plants used in this research are widely used in biofortification programs trace elements, comparing the toxic effect of Zn between them allow us to select which plant is more suited to agricultural biofortification programmes with Zn, consisting of the application of a supraoptimal amount of this element.

2. Materials and methods

2.1. Plant materials and experimental design

Seeds of *L. sativa* cv. Phillipus and *B. oleracea* cv. Bronco were germinated and grown for 35 days in cell flats of 3 cm × 3 cm × 10 cm filled with a perlite mixture substratum. The flats were place on benches in an experimental greenhouse located in southern Spain (Granada, Motril, Saliplant S.L.). After 35 days, the seedlings were transferred to a growth chamber under the following controlled environmental conditions, with relative humidity of 50%, day/night temperature of 25/15 °C, a photoperiod of 16/8 h at a photosynthetic photon flux density (PPFD) of 350 μmol m^{-2} s^{-1} (measured at the top of the seedlings with a 190 SB quantum sensor, LI-COR Inc., Lincoln, NE, USA). Under these conditions the plants were grown in hydroponic culture in lightweight polypropylene trays (60 cm diameter top, bottom diameter 60 cm and 7 cm in height) in a volume of 3 l. At 35 days after germination and throughout the experiment, control plants received a growth solution, which was composed of 4 mM KNO_3, 3 mM $Ca(NO_3)_2{\cdot}4\,H_2O$, 2 mM $MgSO_4{\cdot}7\,H_2O$, 6 mM KH_2PO_4, 1 mM $NaH_2PO_4{\cdot}2\,H_2O$, 2 μM $MnCl_2{\cdot}4\,H_2O$, 10 μM $ZnSO_4{\cdot}7\,H_2O$, 0.25 μM $CuSO_4{\cdot}5\,H_2O$, 0.1 μM $Na_2MoO_4{\cdot}2\,H_2O$, 5 ppm Fe-chelate (Sequestrene; 138 FeG100) and 10 μM H_3BO_3. At the same time, treatments were applied with the same growth solution amended with 0.5 mM of $ZnSO_4$. This solution, with a pH of 5.5–6.0, was changed every three days.

The experimental design was a randomized complete block with 4 treatments (*L. sativa*-control, *B. oleracea*-control, *L. sativa*-0.5 mM, *B. oleracea*-0.5 mM) arranged in individual trays with eight plants per treatment and three replications each, so that the total number of plants was 96.

2.2. Plant sampling

L. sativa and *B. oleracea* plants were sampled after 21 days further growth under these conditions. Plants of each treatment were divided into roots and leaves, washed with distilled water, dried on filter paper and weighed, thereby obtaining fresh weight (FW). Half of the roots and leaves from each treatment were frozen at −30 °C for later performance of biochemical assays and the other half of the plant material sampled dried in a forced air oven at 70 °C for 24 h to obtain the dry weight (DW) and the subsequent analysis of the concentration of Zn.