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Original article

Arsenic-induced stress activates sulfur metabolism in different organs of garlic (*Allium sativum* L.) plants accompanied by a general decline of the NADPH-generating systems in roots

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ABSTRACT

Arsenic (As) contamination is a major environmental problem which affects most living organisms from plants to animals. This metalloid poses a health risk for humans through its accumulation in crops and water. Using garlic (*Allium sativum* L.) plants as model crop exposed to 200 μM arsenate, a comparative study among their main organs (roots and shoots) was made. The analysis of arsenic, glutathione (GSH), phytochelatins (PCs) and lipid peroxidation contents with the activities of antioxidant enzymes (catalase, superoxide dismutase, ascorbate-glutathione cycle), and the main components of the NADPH-generating system, including glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), NADP-malic enzyme (NADP-ME) and NADP-isocitrate dehydrogenase (NADP-ICDH) was carried out. Data showed a correlation among arsenic accumulation in the different organs, PCs content and the antioxidative response, with a general decline of the NADPH-generating systems in roots. Overall, our results demonstrate that there are clear connections between arsenic uptake, increase of their As-chelating capacity in roots and a decline of antioxidative enzyme activities (catalase and the ascorbate peroxidase) whose alteration provoked As-induced oxidative stress. Thus, the data suggest that roots act as barrier of arsenic mediated by a prominent sulfur metabolism which is characterized by the biosynthesis of high amount of PCs.

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1. Introduction

Arsenic (As) represents an important environmental pollutant which can affect living organisms throughout the trophic chain (Zhao et al., 2010; Sarkar and Paul, 2016; Clemens and Ma, 2016). In animals, the arsenic exerts its toxic action by interrupting mitochondrial ATP production in several ways and also influences voltage-gated potassium channels (VGKCs) by disrupting cellular electrolytic function whose consequences are neurological disturbances, high blood pressure, cardiovascular episodes, anemia and death (Ventura-Lima et al., 2011; Abdul et al., 2015; Escudero-Lourdes, 2016). In plants, arsenic has been studied under different aspects including metabolism, transport, accumulation, toxicity and detoxification strategies such as phytochelatin (PC) and hyper-accumulator plant species (Zhao et al., 2010; Schmöger et al., 2000; Li et al., 2004; Bienert and Jahn, 2010; Leterrier et al., 2012a,b; Finnegan and Chen, 2012). Nevertheless, the biochemical and molecular mechanisms involved in plant toxicity are less advanced than in animal cells. Among the different plant species with agronomical relevance, rice (*Oryza sativa* L) can be considered one of the most studied under arsenic stress (Duan et al., 2013; Dave et al., 2013a,b; Batista et al., 2014) because it represents a basic food in many countries and it is the cereal grain most widely consumed. On the other hand, research in plant models such as *Arabidopsis thaliana* have also provided significant knowledge at biochemical and molecular level (Schmöger et al., 2000; Li et al., 2004; Bienert and Jahn, 2010; Leterrier et al., 2012a,b; Lee et al., 2003; Quaghebeur and Rengel, 2004; Park et al., 2016). Plants can face arsenic contamination mainly in the anionic forms of arsenate (AsV) and arsenite (AsIII), both of which have different cytotoxic effects. Thus, AsIII reacts with the sulfhydryl (-SH) groups of proteins provoking cellular dysfunction which could

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involve cell death. On the other hand, AsV is an analog of the macronutrient phosphate, and it competes with phosphate for uptake in the roots, as well as in the cytoplasm where it might disrupt metabolism by replacing phosphate in ATP to form unstable ADP-As (adenosine-5′-diphosphate-arsenate). When root cells take up AsV, it is reduced by the enzyme arsenate reductase to a more highly toxic species, AsIII, with the concomitant conversion of glutathione (GSH) to its oxidized form (GSSG). AsIII can be detoxified via soluble thiols such as GSH and/or phytochelatins (PCs), and transported for vacuolar sequestration by the activity of the ABC (ATP-binding cassette) transporters (Song et al., 2014).

Garlic (*Allium sativum* L.) is a member of the Alliaceae family which is widely cultivated worldwide. Although, garlic cloves are the most popular way for human consumption (raw or cooked), "green garlic" can be also used when garlic is harvested and eaten fresh (not cured) from almost new shoots. This plant has been used for long time for both culinary and medical purposes since garlic has antioxidant, anti-inflammatory, antimicrobial, antifungal, antitumor, and cardioprotective properties (Nicastro et al., 2015). These beneficial effects seem to be associated to the content of sulfur and phenolic compounds (Bozin et al., 2008; Borlinghaus et al., 2014; Trio et al., 2014). To our knowledge, there is very few studies analyzing the behavior of garlic plants under heavy metals such as lead or cadmium (Jiang and Liu, 2001; Zhang et al., 2005; Liu et al., 2009: Ogra et al., 2015). It has been proposed that certain antioxidant enzymes of garlic could be used as biomarkers of the presence of heavy metals in wastewater (Fatima and Ahmad, 2005). However, little biochemical information is available about garlic plants under arsenic stress (Farmer and Mueller, 2013). Therefore, the goal of this work is to use garlic as a model plant with agronomical relevance to study the differential mechanism of response against arsenic stress in its main organs (roots and shoots). The data show that under our experimental conditions (200 μM arsenate), the redox status is clearly altered being the roots of As-exposed garlic plants the most affected organ with a drastic increase in the content of PCs (PC2 and PC3) and with a drop of the enzyme activities (catalase and the ascorbate peroxidase, APX) that control the H_2O_2 content. Moreover, arsenic stress provokes a general decline of the NADPH-regenerating systems in roots.

2. Materials and methods

2.1. Plant material and growth conditions

Garlic (*Allium sativum* L) plants were grown from individual cloves taken out from the whole bulbs. Healthy cloves were selected and planted with the pointed end up and the blunt end down, and grown in a growth chamber in an aerated hydroponic culture (1 L tank) and grown in a nutrient solution under dark conditions for 5 days. Then, garlic seedlings were grown at 22/18 °C during 16 h photoperiod and irradiance of 100–120 μmol m^{-2} s^{-1}. The composition of the nutrient solution was as follows: 1 mM $Ca(NO_3)_2$, 1 mM KH_2PO_4, 1 mM KNO_3,1 mM $MgSO_4$, 50 μM Na–Fe–EDTA, 46 μM H_3BO_3, 10 μM $MnSO_4$, 0.77 μM $ZnSO_4$, 0.32 μM $CuSO_4$, 0.58 μM Na_2MoO_4, 0.01 μM $CoCl_2$ and pH adjusted to 6, with KOH if required. During the final 11 days, the hydroponic solutions were supplemented with 50, 100 or, 200 μM potassium dihydrogen arsenate (KH_2AsO_4) which corresponds to AsV. Plant biomass was determined in 22-days-old garlic plants grown under hydroponic conditions supplemented with μM 200 μM AsV in the final 11 days. In all cases, roots and shoots (bulb plus leaves) of 22-day old garlic plants were collected independently.

2.2. Arsenic content

Garlic material was washed with Milli-Q water containing 0.1 mM EDTA, then separated in roots and shoots, dried during 48 h at 80 °C, pulverized to powder and digested in a concentrated HNO_3:$HClO_4$ (2:1 v/v) solution. Mineral arsenic was analyzed by inductively coupled plasma spectrometry (Varian 720-ES ICP-OES spectrometer).

2.3. Crude extracts of plant tissues

Garlic samples were frozen in liquid N_2 and ground in a mortar with a pestle. The powder was suspended in a homogenizing medium containing 100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1 (v/v) Triton X-100 and 10% (v/v) glycerol (1:4, w/v) and 5 mM DTT. For the ascorbate peroxidase (APX) activity assay, 2 mM ascorbate was added to the homogenizing medium to preserve this activity (Miyake and Asada, 1996). Homogenates were centrifuged at 17,000*g* for 20 min, and the supernatants were used for the enzymatic assays.

2.4. Detection and quantification of GSH and GSSG by liquid chromatography-electrospray mass spectrometry (LC-ES/MS)

Garlic samples (300 mg) were frozen in liquid N_2 and ground using a mortar and pestle in the presence of 1 mL of 0.1 M HCl. Homogenates were centrifuged at 15,000*g* for 20 min at 4 °C. The supernatants were collected and filtered through 0.22-μm polyvinylidene fluoride filters and immediately analyzed. All procedures were carried out at 4 °C and protected from light to avoid potential degradation of the analytes (GSH, GSSG). The LC-ES/MS system consisted of a Waters Allience 2695 HPLC system connected to a Micromass Quattro micro API triple quadrupole mass spectrometer, both obtained from the Waters Corporation. HPLC was carried out using an Atlantis® T3 3 μm 2.1 × 100 mm Column obtained from the Waters Corporation. The Micromass Quattro Micro API masss pectrometer was used in positive electrospray ionization mode for simultaneous detection and quantification of GSH, and GSSG (Airaki et al., 2011).

2.5. Detection and quantification of phytochelatins (PCs) by liquid chromatography-electrospray mass spectrometry (LC-ES/MS)

Phytochelatin 2 (PC2: H-γ- Glu- Cys-γ- Glu – Cys – Gly- OH) and phytochelatin 3 (PC3: H-γ- Glu-Cys-γ- Glu-Cys-γ-Glu-Cys-Gly-OH) were synthesized (Pepmic Co., Ltd, Suzhou, China) and used as standard for quantification. Garlic samples were processed as it has been described for GSH/GSSG. Samples were assayed by LC-ES/MS using an Allience 2695 Separation module connected to a Quattro Micro triple quadrupole mass spectrometer detector (Waters, Milford, MA). Instrument control, data collection, analysis and management were controlled by the MassLynx 4.1 software package and Quanlynx V4.1 software packages. Separation was performed using a column XSelect CSH 130 C18 2.5 μm 2.1 × 100 mm (Waters) connected to a precolumn XSelect CSH C18 2.5 μm Vanguard 2.1 × 5 mm (Waters). The mobile phase consisted in acetonitrile and Milli-Q water, both added with formic acid at 0.1% (v/v). The gradient started at 2% (v/v) of acetonitrile, changed to 20% in 5 min, and to 40% in the next 5 min. Then the gradient changed to the initial condition in 5 min and these conditions were held for 10 min. With these conditions, retention times of the compounds were: PC2 (6.32 min) and PC3 (9.15 min). After three sample injections, one injection of acetonitrile: tetrahydrofuran (7:3) was done to preserve column pressure. This injection was performed using a HPLC method which starts at 2% of acetonitrile and changed to 50% in 10 min.

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