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Short Communication

Metabolic adaptation of *Pteris vittata* L. gametophyte to arsenic induced oxidative stress

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ABSTRACT

The sporophyte and gametophyte of *Pteris vittata* are arsenic hyperaccumulators, however, little is known about the mechanism by which the gametophyte deals with this toxic element. An *in vitro* system (spores grown in arsenic amended nutrient media) was used to investigate the impact of arsenic on growth of the gametophyte and the role of antioxidative systems in combating As-stress. When mature spores of *P. vittata* were grown in medium amended with 0–50 mg kg^{-1} of arsenic (as arsenate), the arsenic concentration in the gametophyte increased, with increasing arsenate in the media, but did not inhibit the spore germination and biomass development. Increases in the level of antioxidant enzymes, superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase, and glutathione-Stransferase) and of ascorbic acid and glutathione probably enabled the gametophyte to withstand the oxidative stress caused by arsenate.

1. Introduction

Arsenic (As) is ubiquitous in the environment, and is the twentieth most abundant element in the Earth crust. Environmental As contamination results from natural processes, such as rock weathering and volcanic emissions, as well as from human activities such as, the use of As-containing pesticides, herbicides, wood preservatives, feed additives etc. Arsenic present in soil and groundwater can enter the food chain through drinking water and contaminated vegetables/agriculture products (Meharg and Hartley-Whitaker, 2002).

Arsenic contaminated sites can be remediated through phytoremediation. *Pteris vittata* L. is the first reported As-hyperaccumulating fern (Ma et al., 2001) accumulating extremely high concentration of As in its above ground biomass. Arsenate is taken up by *P. vittata* via the phosphate transport system, immediately reduced to arsenite by arsenate reductase (Duan et al., 2005) and subsequently transported to the fronds (Ma et al., 2001). As also induces the formation of the thiols in *P. vittata* (Srivastava et al., 2005). The results also suggest that both enzymatic and non-enzymatic antioxidants play significant roles in As detoxification by *P. vittata* (Srivastava et al., 2005; Singh et al., 2006).

Arsenic-related studies, with *P. vittata* have mainly focused on the sporophyte, and the mechanisms by which the gametophyte copes with As exposure are largely unknown. The small autotrophic gametophyte has a simple structure without a vascular system and grows/develops independently of the sporophyte. In general, fern gametophytes are sensitive to stressful conditions as they are devoid of protective devices, such as a thick cuticle on their epidermal cells. Despite these features, gametophytes of *P. vittata* are tolerant to high doses of As in the growth medium and can accumulate As (Gumaelius et al., 2004).

One of the possible mechanisms that make a plant species tolerant to metal stress is the presence of a strong anti-oxidant defense system (Srivastava et al., 2005; Tripathi et al., 2007). Arsenic enhances the production of reactive oxygen species (ROS) (Srivastava et al., 2005; Singh et al., 2006, 2010; Kertulis-Tartar et al., 2009), causing significant injury to plant cells. The scavenging system controlling ROS comprises both non-enzymatic antioxidant (e.g., glutathione, ascorbate, and carotenoids) and an enzymatic anti-oxidative system (e.g., superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase) (Srivastava et al., 2005; Singh et al., 2006).

We hypothesize that such an anti-oxidant system also operates in the gametophyte of *P. vittata* and alleviates oxidative stress caused by arsenic. Hence, the objectives of this study were to examine (i) the extent of arsenic-induced oxidative stress, and (ii) the role of antioxidant system in combating oxidative stress in the gametophyte of *P. vittata*.

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2. Methods

2.1. Experimental procedure

Mature spores of *P. vittata* were collected from a sporophyte growing in the Fern Conservatory of National Botanical Research Institute, Lucknow, India, and surface sterilized by soaking in 2% NaClO for 5 min followed by washing four times in sterile water. These spores were plated on Parker and Thompson medium, (Llyod and Bawa, 1974) containing 2% agar at pH 5.4, supplemented with 5–50 mg kg^{-1} arsenate (in 5 mg kg^{-1} increments) (taking $Na_2HAsO_4{\cdot}7H_2O$ as arsenate source). The spores were allowed to germinate in a culture chamber with a Photo-synthetic photon flux density (PPFD) at 350 μ mol m^{-2} s^{-1} supplied by fluorescent tubes and tungsten lamps for 14 h daily. The culture room was maintained at 22–23 °C day/night temperature and 60–70% relative humidity. The gametophyte prothalli were transferred to fresh arsenic supplemented medium every 15 days. The gametophyte responses against arsenic were monitored on the 45th day of treatment. Five plates (20–25 gametophytes per plate) per treatment were taken and the experiment was repeated twice. The data are the average of the two experiments.

2.2. Spore germination %

The percentage of spore germination was determined by counting the number of germinated spores (a spore was considered to have germinated when a protonema was identified) under a light microscope (NIKON ECLIPSE 80I). The percentage of spore germination in the control was normalized to 100%.

2.3. Gametophyte biomass

Twenty full grown gametophytes from each plate were harvested on day 45 and desiccated in an oven at 70 °C for 24 h to obtain bulk dry weight. Biomass is expressed as dry weight (DW) per gametophyte.

2.4. Digestion and arsenic determination

About 15 g of gametophyte [fresh weight (FW)] were frozen in liquid nitrogen and stored at −80 °C for enzyme analysis and biochemical parameters. The remainder of the plant material was dried at 65 °C in oven for 48 h, and 0.1 g [Dry weight (DW)] were mixed with 5 ml of 60% nitric acid and 1 ml of 40% hydrofluoric acid, and digested in microwave digester (Speed wave Digester MSW + 3 BERGHOF) for one hour. Arsenic was determined on a flame atomic spectrometer (GBC Avanta ∑, Australia) which was coupled to a GBC Hydride Generation System (HG 3000).

2.5. Quality control and quality assurance

The standard reference material of arsenic (consisting of 998 ± 4 mg l^{-1} As-NIST and BAM-CRM traceable) (E-Merck, Germany) was used for each analytical batch. Analytical data quality was ensured with repeated analysis of quality control samples ($n = 3$) and the results were within (±2.82 mg l^{-1}) limit of the certified values. Standard AA03N-3 (Accustandard, USA) was used as a matrix reference material which was spiked with known concentration (0–50 μg L^{-1} As) of standard reference material, and the recovery of total As were within 85.3% (±2.8; $n = 5$) to 89.5% (±3.1; $n = 5$).

2.6. Protein determination

Protein estimation was carried out by the method of Bradford (1976) with bovine serum albumin (BSA Sigma) as standard, and absorbance measurement at 600 nm.

2.7. Determination of lipid peroxidation

The level of lipid peroxidation in plant tissues was determined as 2-thiobarbituric acid (TBA) reactive metabolites mainly malondialdehyde (MDA) (Heath and Packer, 1968). Plant tissues (0.5 g) were extracted in 2.5 ml of 5% trichloroacetic acid (TCA) and centrifuged at 10,000*g* for 15 min. To 1 ml of aliquot of the supernatant, 1 ml of 0.5% TBA in 20% TCA were added and incubated at 95 °C for 25 min and then quickly cooled on ice. The solutions were centrifuged at 10,000*g* for 5 min and the absorbance was measured at 532 nm, using a UV–Vis Spectrophotometer (Thermo-Helios-ß UVB-131312). The value for non-specific turbidity was made by subtracting the absorption value taken at 600 nm from the one measured at 532 nm. The level of lipid peroxidation was expressed as nmol of MDA formed using an extinction coefficient of 155 mM cm^{-1} at 532 nm.

2.8. Estimation of antioxidant enzymes

Superoxide dismutase (SOD) activity was measured with the reduction of nitroblue tetrazolium (NBT) to form formazon (Beyer and Fridovich, 1987). The samples were homogenized in 5 ml extraction buffer consisting of 50 mM phosphate, pH 7.5 containing 1 mM dithiothreitol (DTT) and 1 mM ethylenediamine tetra acetic acid (EDTA), and centrifuged at 20,000*g* for 15 min at 4 °C. The assay mixture contained 50 mM phosphate (pH 7.8), 1% (w/v) Triton X-100, 0.0044% (w/v) riboflavin, 57 μM NBT and 9.9 mM L-methionine. The photoreduction of NBT (formation of purple formazon) was measured at 560 nm. One unit of SOD activity is defined as the amount of enzyme that gave 50% inhibition of NBT reduction in one minute.

For the estimation of catalase (CAT) activity, 0.5 g of plant samples were homogenized in 5 ml extraction solution, containing 50 mM phosphate buffer (pH 7.0) and 1 mM DTT. CAT activity was assayed in 50 mM phosphate buffer (pH 7.0) by monitoring the production of dioxygen from hydrogen peroxide (1%) at 240 nm (del Río et al., 1977).

Glutathione Reductase (GR) was assayed from 0.5 g plant tissues extracted in 0.1 M phosphate buffer (pH 7.5) containing 0.5 mM EDTA, the extract was centrifuged at 20,000*g* for 15 min at 4 °C and GR activity was monitored by following the increase in absorbance at 412 nm, when DTNB (5,5′-dithiobis-2-nitrobenzoic acid) was reduced by glutathione (GSH) to form TNB (5-thio-2-nitrobenzoic acid) (Smith et al., 1988). The reaction mixture was 0.2 M phosphate buffer (pH 7.5) containing 1 mM EDTA, 3 mM DTNB in 0.01 M phosphate buffer (pH 7.5), 2 mM NADPH and 20 mM GSSG (oxidized glutathione).

Glutathione-*S*-transferase (GST) activity was determined with 1 g plant material extracted in 5 ml extraction solution consisting of 50 mM phosphate buffer (pH 7.5), 1 mM EDTA and 1 mM DTT. The enzyme activity was assayed in a reaction mixture containing 50 mM phosphate buffer (pH 7.5), 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and extract equivalent to 100 μg of protein. The reaction was initiated by the addition of 1 mM GSH and formation of *S*-(2,4-dinitrophenyl) glutathione (DNP-GS) was monitored as an increase in absorbance at 334 nm to calculate the GST specific activity (Li et al., 1995).

Ascorbate peroxidase (APX) activity was determined with 0.5 g plant samples extracted with 2.5 ml of 100 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 0.1 mM ascorbate and 2% (v/v)