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Comparative analysis of antioxidant response by *Pteris vittata* and *Vetiveria zizanioides* towards arsenic stress

Sarita Tiwari, Bijaya Ketan Sarangi *

Environmental Biotechnology Division, National Environmental Engineering Research Institute (CSIR-NEERI), Nehru Marg, Nagpur, 440020, Maharashtra, India

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ABSTRACT

Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX) reportedly allow plants to combat toxic metal stress. Hydroponic experiment was conducted to study the response of antioxidant enzymes under arsenic (As) stress in the fronds and roots of an Indian ecotype of *Pteris vittata* (As hyperaccumulator) and *Vetiveria zizanioides* (As non hyperaccumulator). These plants were exposed to different As levels (0, 10, 20, 30 & 50 mg As L^{-1}) for 10 days in green house. In *P. vittata and V. zizanioides,* total As accumulation in biomass increases with increase in As concentrations. Arsenic accumulation was more in the fronds than in the roots of *P. vittata,* though not in case of *V. zizanioides.* Arsenic accumulation in total plant biomass of *P. vittata* and *V. zizanioides* was 879 mg and 785 mg As kg^{-1} respectively. Enzymatic antioxidants response in *P. vittata* under As stress showed increased activity in different parts in comparison to control. All enzyme activity was higher in fronds as compared to roots which corresponded to more As accumulation in fronds. Results indicate that enhanced activities of the antioxidant enzymes play a significant role in As tolerance and hyperaccumulation by *P. vittata* whilst changes in these enzyme activities did not show a specific trend in *V. zizanioides* under As stress. The results indicate that *P. vittata* has evolved mechanisms for As detoxification and hyperaccumulation while *V. zizanioides* does not, but sustains a moderate level of As stress presumably as excluder.

1. Introduction

Arsenic(As) is a serious environmental issue in many parts of the world because of different anthropogenic as well as geogenic reasons (Bhattacharya et al., 2007; Henke 2009). The unprecedented effect of As had been observed in many countries such as Bangladesh, India, Chile, America and China (Hossain 2006; Sun et al., 2011). At present, several technologies for As pollution remediation from soil and water are available (Khan et al., 2004; Bhargava et al., 2012). In recent time, R & D is more focused towards the development of sustainable processes based on 'green chemistry' approach taking into consideration human health, resources availability and energy input. Phytoremediation, a ecofriendly, cost effective and sustainable technology, employs plants for *in situ* remediation of contaminated site (Pilon-Smits and Pilon, 2002; Bhargava et al., 2012). It has become apparent as an attractive, economical, aesthetically popular and socially acceptable option for cleanup of contaminants from different matrices. Phytoextraction using plants having extraordinarily high metal accumulation ability known as hyperaccumulators, will augment the quality of As contaminated site(s). So far, many plants belonging to fern species within *Pteridaceae* family have been reported to be As hyperaccumulators (Meharg, 2003; Zhao et al., 2009). The first identified As hyperaccumulator plant within them was brake fern *P. vittata* (Ma et al., 2001). Subsequently some ecotypes of this plant have been identified having potential for As remediation (Visoottiviseth et al., 2002; Xiao et al., 2008; Sarangi et al., 2010). However some plants such as *Pseudotsuga menziesie*, *Equisetum* species and *Isatis capadocica* form different groups are classified between accumulators and excluders due to their intermediatary ability to accumulate As (Meharg, 2003; Haug et al., 2004; Karimi et al., 2009).

In plants, the onset of inorganic stress initiates generation of reactive oxygen species (ROS) inside the cellular milieu which causes injury to the major biomolecules such as proteins, DNAs and lipids of the cell system (Mittler et al., 2004; Miller et al., 2008). After exposure to As, both inorganic forms of As i.e. arsenite [As(III)] and arsenate [As(V)] generate ROS which cause toxicity and dam-

* Corresponding author.
E-mail addresses: bk_sarangi@neeri.res.in, bijayaketan@yahoo.com (B.K. Sarangi).

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age to cell system (Meharg and Hartley-Whitaker, 2002). To avoid such condition, inbuilt defense mechanisms are activated which lead to the commencement of adaptive biochemical responses such as synthesis of phytochelatins and increased production & activity of enzymatic and nonenzymatic antioxidant to antagonize heavy metals & ROS, and prevent degradation of biomolecules (Schmoger et al., 2000; Shri et al., 2009). Various enzymatic and non enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), ascorbate, glutathione, phenolic compounds have been reported to be involved in neutralizing ROS (Dat et al., 2000; Wang et al., 2010). Reactive oxygen species produced during metal stress are eliminated by SOD by virtue of converting it into H_2O_2 which can further be removed by CAT, APX and GPX enzymes (Sharma and Dietz, 2008). Enhanced activity of these enzymes had been reported in plants exposed to metal stress (Sun et al., 2007; Shri et al., 2009; Gusman et al., 2013).

Literature review reveals that heavy metal tolerance by plants is the results of enhanced production of antioxidant enzymes (Gratao et al., 2005) and complexation of the metal with peptides called phytochelatins (Andra et al., 2009; Andra et al., 2010) or other low molecular mass thiol compounds. The present investigation was carried out to assess As stress response in two different plant species i.e. *P. vittata* and *V. zizanioides*. Out of the two, *P. vittata* is known having exceptional tolerance to heavy metals such as cadium, nickel, lead, zinc, selenium with effective As hyperaccumulation capacity and also viable for remediation of metals co-contaminated soil and water matrix (Salido et al., 2003; An et al., 2006; Xiyuan et al., 2008; Srivastava et al., 2009; Feng et al., 2009, 2011). The antioxidant enzymes assay in *P. vittata* grown in As contaminated soil has shown significant importance of such enzymes in imparting tolerance to As stress (Srivastava et al., 2005). Similarly, *V. zizanioides* plant is known to poses many characteristics required for phytoremediation application such as; fast growth rate, high biomass, extensive root system, ease of harvest and also reported for extensive environment application (Pichai et al., 2001). However, its potentiality for As phytoextraction as an accumulator is a matter of debate (Singh et al., 2007; Datta et al., 2011). The previous studies revealed the potentiality of *V. zizanioides* grass to tolerate a high range of heavy metals concentrations other than As without hindering its growth and development (Roongtanakiat and Chairoj 2001; Andra et al., 2011). The antioxidant response following exposure to different metals such as, lead (Andra et al., 2011), zinc & cadmium (Weihong et al., 2009) etc. in *V. zizanioides* had been studied but, to the best of our knowledge, the antioxidant response of the plant to As remains less explored. This study is focused on (i) performance evaluation of two plants for As phytoextraction reflecting tolerance capacity (ii) assay of anti-oxidant enzymes activities i.e. SOD, CAT, APX and GPX in the fronds and roots of *P. vittata* and *V. zizanioides* plants after treatment with different concentration of As (V).

2. Materials and methods

2.1. Experimental setup

The experimental plants *P. vittata* and *V. zizanioides* were maintained in glass house under $30 \pm 5\,^{\circ}C$ under day night photo period. Plants of same age group about 4–5 months old having 5–7 fronds were taken for the experiment. The plants were acclimatized for 4 days in 300 ml of 20% Hoagland nutrient solution kept in glass house. These plants were then transferred to different flask amended with As ($Na_2HAsO_4{\cdot}7H_2O$) ranging from 0 to 50 mg L^{-1} of nutrient solution (control being grown without As). The plants were harvested after 10 days of As treatment, fresh weight was determined and plants were separated into shoot and root. Plant parts were washed three times with ice-cold distilled water and then were pat dried. A portion of each root and shoot samples were used for further biochemical analysis. Remaining samples of root and shoot were oven dried at 60 °C for As estimation.

2.2. Lipid peroxidation assay

The level of oxidative stress caused by As in studied plants parts was determined by lipid peroxidation assay. The protocol for estimating Thiobarbituric acid reacting substances (TBARS) indicator of lipid peroxidation, was done according to de Oliveira et al. (2014). About 0.3 g of plants tissue i.e. both root and shoot of each plant was freeze and grinded into fine powder after adding liquid nitrogen followed by addition of 1.5 ml of 5% (wt/v) trichloroacetic acid (TCA). The above reaction was carried out in ice bath. The homogenate was taken in eppendorff and centrifuged at 10,000*g* for 10 min (R.T). 1 ml of supernatatnt was mixed with 1 ml of 20% TCA containing 0.5% Thiobarbituric acid (TBA) and 100 μl 4% butylated hydroxytoluene in ethanol and mixed properly. The reaction mixture was then heated in hot water bath at 95 °C for 30 min and after completion immediately cooled in ice bath. The TBARS was quantified using spectrophotometer (Hitachi, Japan) at A_{532nm} using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.3. Enzyme extraction

About 2 g fresh tissues of root and leaflets of *P. vittata* and *V. zizanioides* were homogenized in an ice-cold mortar with 10 ml of ice-cold extraction buffer solution (pH7.5). The extraction buffer comprised of 50 mM N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES), 0.4 mM EDTA, 5 mM $MgCl_2$, 1% polyvinylpolypyrrolidone, 2 mM dithiothreitol, 10% glycerol and 1 mM Phenylmethylsulfonylfluoride (PMSF). The homogenate was filtered through four layers of autoclaved muslin cloth and centrifuged at 14000*g* at 4 °C for 20 min. All the above steps were carried out at 4 °C. The supernatant i.e. enzyme extract was stored in separate aliquots at −80 °C prior to enzyme analysis.

2.4. Determination of protein concentrations

Protein concentration was determined as described by Bradford (1976). The enzyme extracts were mixed with 5 ml of protein reagent containing 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (v/v) ethanol and 8.5% (v/v) phosphoric acid. After mixing thoroughly for 2 min, the absorbance was measured at 595 nm using Bovine V albumin as standard.

2.5. Estimation of anti-oxidant enzymes

2.5.1. Superoxide dismutase (SOD) EC 1.15.1.1

All four enzymatic antioxidant assays was conducted according to Cao et al. (2004) protocol by spectrophotometer method. The SOD activity was determined by measuring the inhibition of the auto-oxidation of pyrogallol. Reaction mixture (10 ml) contained 3.6 ml Milli Q water, 0.1 ml enzymes extract from *P. vittata* and *V. zizanioides*, 5.5 ml of 50 mM phosphate buffer (pH 7.8) and 0.8 ml of 3 mM pyrogallol (dissolved in 10 mM HCl). Pyrogallol reduction was measured at 325 nm wavelength. One unit of enzyme activity was equivalent to amount of enzyme responsible for 50% inhibition of the auto-oxidation rate of pyrogallol.

2.5.2. Catalase (CAT) EC 1.11.1.6

For quantifying CAT enzyme by spectrophotometer, reaction mixture comprised of 40 μl enzyme extracts and 9.96 ml of H_2O_2 phosphate buffer (pH 7.0). The principle of quantification is based