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Comparative Antioxidant Profiling of Tolerant and Sensitive Varieties of *Brassica juncea* L. to Arsenate and Arsenite Exposure

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Abstract Comparative antioxidant profiling of tolerant (TPM-1) and sensitive (TM-4) variety of *Brassica juncea* L. was performed after exposure to arsenate [As(V)] and arsenite [As(III)]. TPM-1 demonstrated higher accumulation of As upon exposure to both 500 μM As(V) and 250 μM As(III) (49 and 37 μg g^{-1} dw after 15 days) as compared with that observed in TM-4. The activities of various antioxidant enzymes and the level of glutathione and proline demonstrated, in general, a comparatively better response in TPM-1 than in TM-4 that presumably allowed TPM-1 to tolerate higher As concentrations as compared with that of TM-4.

Keywords Antioxidants · Arsenic · *Brassica juncea* · Glutathione

Arsenic (As) has been an element of considerable environmental concern in the recent past because of its toxicity and carcinogenic properties. Plants typically encounter As in the anionic forms of arsenate [As(V)] and arsenite [As(III)], which have different cytotoxic effects (Srivastava et al. 2007). Arsenic exposure to plants interrupts with several physiological, and biochemical processes (Ahsan et al. 2008). It also induces generation of reactive oxygen species (ROS) through intra-conversion from one ionic form to other (Srivastava et al. 2007). Various antioxidants of plants are broadly divided into two general classes; (1) molecular antioxidants, [e.g., glutathione (GSH) and proline]; and (2) enzymatic antioxidants. Among enzymatic antioxidants, superoxide dismutase (SOD) constitutes the first line of defense converting superoxide radicals ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2), which is further reduced to water and oxygen by ascorbate peroxidase (APX) and guaiacol peroxidase (GPX). Other enzymes, such as monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) are involved in the maintenance of ascorbate and GSH in reduced form (Noctor and Foyer 1998). It was hypothesized that differential antioxidant potential might constitute one of the important determinants of tolerance/sensitive characteristics of different varieties of a plant upon exposure to As. To visualize this hypothesis, antioxidant profiling of the contrasting genotypes of *Brassica juncea* was performed in the present study.

Materials and Methods

A total of 14 varieties of *B. juncea*, namely TPM-1, TM-2, TM-4, Rohini, Vardan, Vaibhav, Varuna, GM-1, GM-3, Ashirwad, RL-1359, RH-30, Maya, and Urvashi, were screened for tolerance to As on the basis of germination and seedling growth. Seeds were sterilized in 30% ethanol for 3 min and washed thoroughly with distilled water to remove any traces of ethanol. Twenty-five sterilized seeds were put on each Petri plate on a moist cotton bed and watered with 50% Hoagland nutrient medium with or

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without As(V) (0, 50, 500 and 1,000 μM; prepared using the salt Na_2HAsO_4). Petri plates were kept in dark for 1 day and then transferred to the light (a 12 h photoperiod) with a day/night temperature of 25 ± 2°C and relative humidity of 70%. After 7 days, the number of germinated seeds was counted and root and shoot lengths were measured using a metric scale. After preliminary screening, the variety TPM-1 was selected as the tolerant variety and TM-4 as the sensitive variety. The two selected varieties were grown in the field in plastic pots containing 1 kg of soil (clay loam containing 45 mg kg^{-1} available nitrogen, 30 mg kg^{-1} available potassium, 25 mg kg^{-1} available phosphate, 8 mg kg^{-1} available sulfate-sulfur at pH 6.6). Seeds were spread in the pot and allowed to grow until the appearance of the first photosynthetic leaves (15 days). Then, seedlings were subjected to As(V) (50 and 500 μM) and As(III) (25 and 250 μM) stress for a period of 7 or 15 days. At each harvesting period, shoots of seedlings were washed thoroughly with double-distilled water and were used for the analysis of various parameters.

Total As in the shoots of seedlings was estimated in the digested plant material (100 mg) as described previously (Srivastava et al. 2007). Arsenic concentrations were determined on an atomic absorption spectrophotometer (GBC 906AA, Australia) coupled to a hydride generation system (HG 3000). For enzyme assays, extraction was done following Srivastava et al. (2006). Protein content of the supernatant was measured following Lowry et al. (1951) using bovine serum albumin (BSA) as standard. The activities of SOD (EC 1.15.1.1), APX (EC 1.11.1.11), GPX (EC 1.11.1.7), and GR (EC 1.6.4.2) were assayed following Beauchamp and Fridovich (1971), Nakano and Asada (1981), Hemeda and Klein (1990) and Smith et al. (1988), respectively, as described previously (Srivastava et al. 2006). DHAR (EC 1.8.5.1) activity was assayed by the formation of ascorbate at 265 nm ($\varepsilon = 14\ mM^{-1}\ cm^{-1}$) in a reaction mixture containing 0.1 M Na–phosphate buffer (pH 6.2), 2 mM GSH, 1 mM dehydroascorbic acid (DHA) and enzyme extract (De Tullio et al. 1998). MDHAR (EC 1.6.5.4) activity was assayed by monitoring NADPH oxidation at 340 nm ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$) in a reaction mixture containing 0.1 mM NADPH, 2.5 mM ascorbate, 50 mM Na-phosphate buffer (pH 7.6) and enzyme extract. The reaction was started by the addition of 4 units of ascorbate oxidase (Drążkiewicz et al. 2003). The estimation of reduced (GSH) and oxidized (GSSG) glutathione was done fluorometrically following the method of Hissin and Hilf (1976) as described previously (Srivastava et al. 2006). The level of proline was measured following Bates et al. (1973).

The experiments were carried out in a randomized block design. All analyses were performed in triplicate and each replicate contained shoots from twenty-five seedlings. Two-way analysis of variance (ANOVA) was done on all the data to confirm the variability of data and validity of results. Tukey test was performed to determine the significant difference between treatments using the statistical software Origin 7.5 (Origin Lab Corporation, Northampton, MA, USA) (Kaiser et al. 2009).

Results and Discussion

In the present study, a total of 14 varieties of *Brassica* were screened for tolerance to As(V) (1–1,000 μM exposure for 7 days; Supplementary Fig. 1). Screening of the varieties was performed on the basis of seedling growth and germination rate since these parameters are more sensitive to any stress and hence tolerance or sensitivity may be easily scored (Lee and Schroeder 2002). TPM-1 was selected as the most tolerant variety and TM-4 as the most sensitive variety. Both varieties were grown in field conditions and exposed to As(V) and As(III). Arsenic accumulation in the shoots of both varieties was found to correlate to exposure concentration and duration. The maximum As accumulation in response to As(V) was 49 μg g^{-1} dw in TPM-1 and 30 μg g^{-1} dw in TM-4 after 15 days. In response to As(III), the maximum As accumulation after 15 days was 37 μg g^{-1} dw in TPM-1 and 33 μg g^{-1} dw in TM-4 (Fig. 1). Earlier studies on *Brassica* have also demonstrated such a concentration duration dependent influx of As (Gupta et al. 2009; Khan et al. 2009). A higher level of As accumulation in TPM-1 as compared to that observed in TM-4 might be attributed to a greater As-tolerance of TPM-1 as compared to that of TM-4.

In the present study, TM-4 demonstrated a significant increase in malondialdehyde (MDA), an indicator of lipid peroxidation of membranes, on both durations. In contrast, in TPM-1, the level of MDA did not increase significantly (Supplementary Fig. 2). The levels of antioxidants were analyzed at all concentrations of As(V) (50 and 500 μM) and As(III) (25 and 250 μM), however for the sake of conciseness, the results of only the maximum concentrations are being given. The activity of SOD showed a similar profile in both varieties except the significant decline in TM-4 upon exposure to 250 μM As(III) for 15 days (Fig. 2a). TPM-1 showed a significant increase in GPX activity on both durations in response to As(III) but only after 7 days upon exposure to As(V) (Fig. 2b). The activity of APX in TPM-1 increased significantly in response to As(V) and As(III) at both 7 and 15 days (Fig. 2c). By contrast, TM-4 showed a significant decline in GPX and APX activities in response to both As(V) and As(III) after 15 days (Fig. 2b, c). The activity of MDHAR in TPM-1 showed a little increase upon exposure to 500 μM As(V) on both durations, whereas no significant

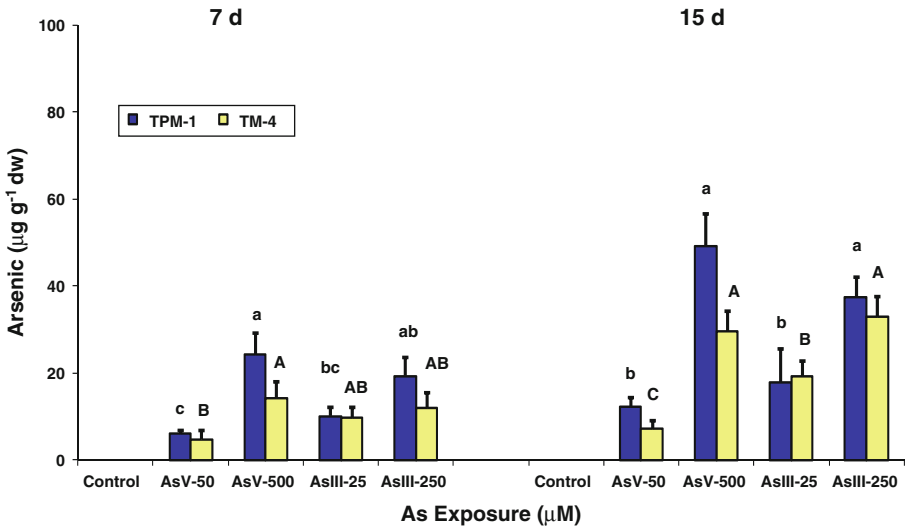

Fig. 1 Accumulation of arsenic by *Braccica juncea* varieties TPM-1 and TM-4 exposed to different concentrations of arsenate and arsenite for 7 and 15 days. All values are means of triplicates ±SD. ANOVA significant at $p \leq 0.01$. *Different letters* indicate significantly different values at a particular duration ($p \leq 0.05$)

effect in response to 250 μM As(III). In TM-4, MDHAR activity did not show a significant effect except the significant decline at 250 μM As(III) after 7 days (Fig. 2d). DHAR activity exhibited significant increase in response to As(V) and As(III) in TPM-1 at both 7 and 15 days. In TM-4, DHAR activity showed a significant increase in response to As(V) but a significant decline upon exposure to As(III) on both durations (Fig. 2e). The activity of GR did not exhibit significant modulations in TPM-1 except the decline in response to 250 μM As(III) after 15 days. In TM-4, GR activity showed a significant decrease in response to As(V) after 15 days and in response to As(III) at both 7 and 15 days (Fig. 2f). The level of GSH (Supplementary Fig. 3) and the ratio of reduced glutathione to oxidized glutathione (GSH/GSSG; Fig. 2g) also demonstrated an increase in TPM-1 after 15 days, whereas a decline in TM-4. The level of proline increased significantly in response to As(V) and As(III) at both 7 and 15 days in TPM-1. By contrast, TM-4 showed a significant increase in the level of proline only upon exposure to As(III) (Fig. 2h).

The observed increases in SOD activity (Fig. 2a) upon exposure to As(V) suggests significant accumulation of $O_2^{\bullet -}$. It seems that essentiality of conversion of As(V) to As(III), for the purpose of detoxification via thiol-mediated complexation, led to a higher increase in reactive oxygen species (Srivastava et al. 2007) that was reflected by a greater increase in SOD activity. No significant modulation in SOD activity in response to As(III) suggests its efficient chelation at a primary level via thiols in TPM-1. However, a decline in TM-4 indicates towards a mechanism of sensitivity. The present results indicated an enhancement of GPX activity upon exposure to both As(V) and As(III) in TPM-1 (Fig. 2b), suggesting that this enzyme served as an intrinsic defense tool to resist As-induced oxidative damage in TPM-1. An induction of GPX activity in plants has been previously reported under As stress in other plants (Srivastava et al. 2007; Mishra et al. 2008). On the other hand, TM-4 demonstrated a decline in GPX activity after 15 days in response to both As(V) and As(III) suggesting that downstream processing of H_2O_2 was not being efficiently taken care of. However, the major pathway of H_2O_2 degradation is considered to be ascorbate–glutathione cycle. The present results indicated that enzymes of ascorbate–glutathione cycle were positively modulated in TPM-1 in contrast to that of TM-4 (Fig. 2c–f). A positive enhancement of ascorbate–glutathione cycle has been recorded previously under As stress in *B. juncea* as well as other plants like *Hydrilla* and *Ceratophyllum* (Srivastava et al. 2007; Mishra et al. 2008; Khan et al. 2009). APX along with GPX and SOD are considered to be the key enzymes within the anti-oxidative defense mechanism, which directly determine the cellular concentration of oxygen radicals and H_2O_2 (Srivastava et al. 2007). Thus, various enzymes functioned well in coordination in TPM-1 as compared with their response in TM-4. In addition, the activity of GR did not show a significant increase with As treatment in TM-4 while it was maintained at par to control level in TPM-1. The lack of responsiveness of GR to As stress is in agreement with the transcriptomic results of Norton et al. (2008) in rice plants. Foyer et al. (1994) have shown that GR is a key enzyme that helps in reduction of GSSG to GSH and suggested its crucial role in combating oxidative stress in leaves. Thus, the two major enzymes of the ascorbate–glutathione pathway viz., APX and GR showed a declining trend in TM-4 particularly on longer durations implying that the antioxidants were not modulated in an integrated manner in the sensitive variety. In addition, the level of GSH (Supplementary Fig. 3) and the ratio of GSH/GSSG (Fig. 2g) showed a significant increase

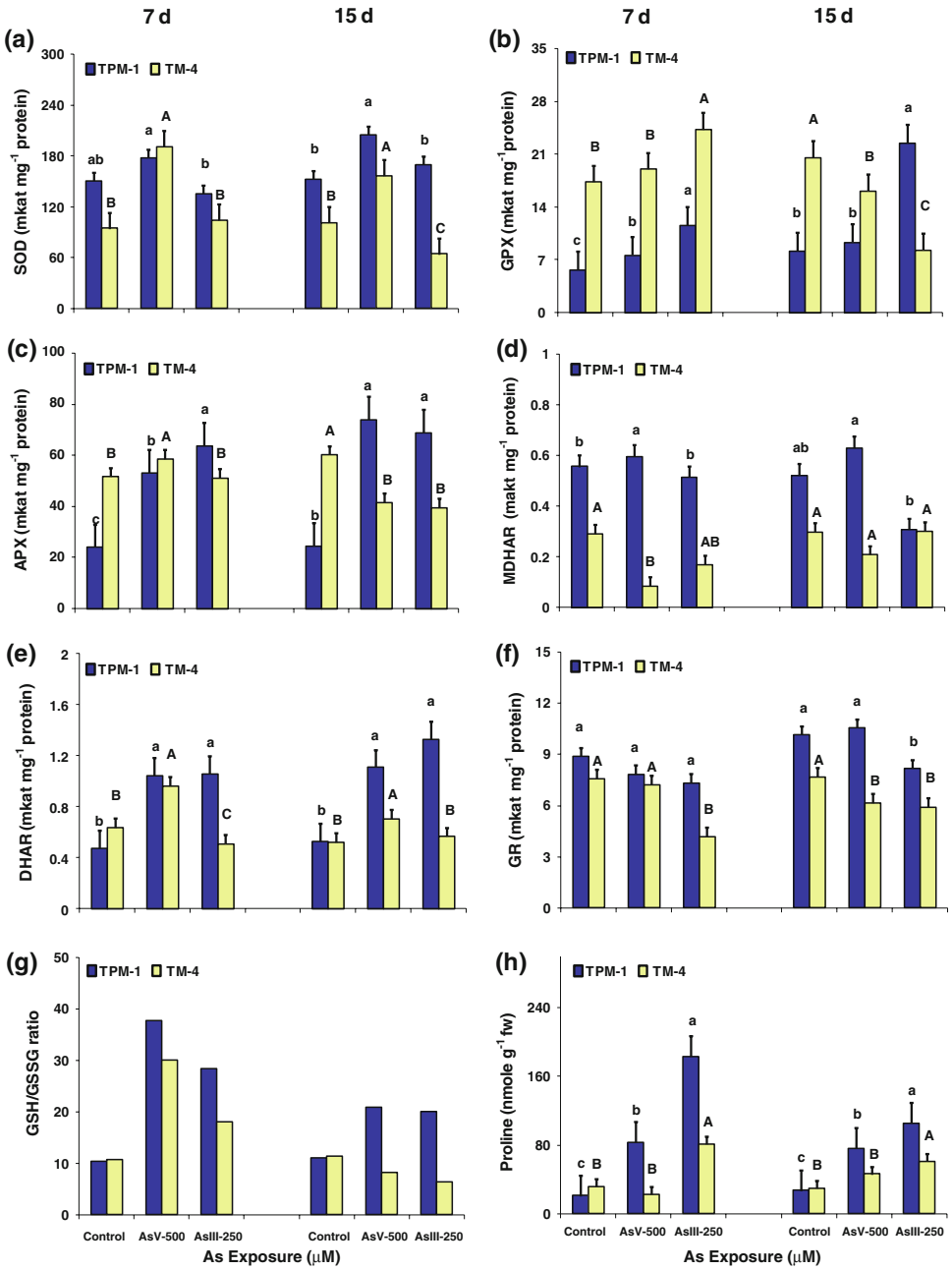

Fig. 2 Effect of As(V) and As(III) exposure on the activity of SOD (**a**), GPX (**b**), APX (**c**), MDHAR (**d**), DHAR (**e**), and GR (**f**), the ratio of GSH/GSSG (**g**) and the level of proline (**h**) in *Brassica juncea* varieties TPM-1 and TM-4 after 7 and 15 days. All values are means of triplicates ± SD. ANOVA significant at $p \leq 0.01$. *Different letters* indicate significantly different values at a particular duration ($p \leq 0.05$)

at both durations in TPM-1 but only until 7 days in TM-4. Thus, it seems that stimulated GSH synthesis played a major role in maintaining a high GSH/GSSG ratio in TPM-1 (Mishra et al. 2008). Similarly, Hartley-Whitaker et al. (2001) reported that GSH levels increased significantly in As-tolerant *H. lanatus* upon As exposure. A greater increase in GSH and GSH/GSSG ratio would have further led to a better antioxidant potential in TPM-1 as compared to that of TM-4 (Noctor and Foyer 1998). In addition, the level of proline increased to a higher level in TPM-1 than that of TM-4 (Fig. 2h). Proline is known to function as radical scavenger and cellular redox-potential buffer (Sharma and Dietz 2009). Hence, the capacity of proline to quench reactive oxygen species was available to a lower extent for TM-4 plants, whereas in TPM-1, proline accumulation would have acted as a supporting molecule to assist the enzyme mediated dismutation of reactive oxygen species.

In conclusion, the results suggest that modified levels of various antioxidants represented a well coordinated defense mechanism against oxidative stress in TPM-1 that was presumably responsible for its greater As tolerance. By contrast, antioxidant system failed to modulate in an integrated manner that resulted in leakage of reactive oxygen species to potential targets to cause toxicity and thus attributed to the sensitive characteristics of TM-4. The

study also implies that screening of a plant should make a primary step of any phytoremediation effort as there are significant varietal differences with respect to As tolerance. On the basis of screening, a higher As accumulation and tolerance ability, and integrated antioxidant responses, the variety TPM-1 appears to be a suitable candidate for the phytoremediation approaches.

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