

Response of adenine and pyridine metabolism during germination and early seedling growth under arsenic stress in *Brassica juncea*

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Abstract Adenine and pyridine nucleotides play vital roles in virtually all aspects of plant growth. This study analyzed the response of adenine and pyridine metabolism during germination and early seedling growth (ESG) of *Brassica juncea* exposed to two doses of arsenate (AsV), 100 and 250 μM , having non-significant or significant inhibitory effects, respectively, on germination and ESG. The ratio of NAD/NADP and NAD/NADH showed no significant change in control and 100 μM AsV, but increased significantly at 250 μM AsV during initial 24 h and also at 7th day. The activity of enzymes of NAD metabolism, viz. NAD kinase, NADP phosphatase, nicotinamidase and poly(ADP-ribose) polymerases showed significant change mostly at 250 μM AsV. Further, significant decrease was observed in the ratio of ATP/ADP and in the activities of adenylate kinase and apyrase at 250 μM AsV at 7th day. External supply of ATP (1 mM) to 100 and 250 μM AsV significantly improved germination percentage and germination strength of the seeds as compared to AsV treatments alone. The study concludes that with the increase in concentration of AsV, the balance of NAD/NADP, NAD/NADH and ATP/ADP and the

activities of enzymes of adenine and pyridine metabolism were significantly altered and that these changes may be responsible for inhibitory effects of AsV on germination and ESG.

Keywords Arsenic · ATP · Germination · Nicotinamidase · PARP · Pyridine nucleotides

Introduction

Arsenic (As) is a toxic metalloid whose exposure to plants disturbs nitrogen (Singh et al. 2009) and sugar (Jha and Dubey 2005; Choudhury et al. 2010) metabolism and induces oxidative stress reactions (Srivastava et al. 2011) leading to toxicity. However, with respect to toxicity, germination and early seedling growth (ESG) may play a determining role in tolerance at later stages of plant life. It is known that As exposure affects germination and seedling growth and that arsenite (AsIII) and arsenate (AsV) have differential effects (Abedin and Meharg 2002). However, the mechanism of toxicity at initial stages is still unknown.

Srivastava et al. (2011) established that redox state and energetic equilibrium of the plants are the major determinants of the extent of toxicity under As stress in *Hydrilla verticillata*. The redox state of a cell is influenced by the balance of various redox couples such as GSH/GSSG, ASC/DHA, NAD/NADH and NADP/NADPH. Among these, pyridine nucleotides, NAD(H) and NADP(H), are essential for electron transport and are known as the central metabolites orchestrating cellular redox homeostasis. The importance of NAD and NADP metabolism in plant development and stress tolerance is well known (Amor et al. 1998; Chai et al. 2005, 2006; Hashida et al. 2009). These nucleotides also play vital roles in signaling via the

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generation and scavenging of reactive oxygen species (ROS; Mittler et al. 2004). The multiple functions of NAD(H) and NADP(H) imply that the regulation of the NAD(H)/NADP(H) balance is critical for cell survival (Ziegler 2000). NAD kinase, NADP phosphatase, poly(ADP-ribose) polymerases (PARPs) and nicotinamidase are the important enzymes of NAD biosynthetic pathway (Hashida et al. 2009). Inter-conversion of NAD and NADP occurs through NAD(H) kinase and NADP phosphatase. Multiple NAD(H) kinase genes exist in eukaryotes. Depending on their preference for the oxidized (NAD) or the reduced (NADH) form, NAD(H) kinases have been classified into NAD kinases and NADH kinases, respectively (Berrin et al. 2005). NADP⁺ phosphatase activity, which converts NADP⁺ to NAD⁺, has also been reported in plants (Gallais et al. 2000). PARPs catalyze the transfer of ADP-ribose groups from NAD to nuclear protein acceptors, resulting in branched chains of ADP-ribose polymers. Nicotinamidase is involved in the degradation of nicotinamide to nicotinic acid (Anderson et al. 2003). Nicotinamide is an inhibitor of NAD degrading PARPs enzymes. Arabidopsis nicotinamidase mutant (*nic2-1*) seeds with reduced nicotinamidase activity have increased dormancy, due to inhibition of PARP activity, increased NAD and reduced poly(ADP-ribosyl)ation levels (Hunt et al. 2007). Energetic equilibrium of the cells depends on the ratio of ATP/ADP, and adenylate kinase (AK) and apyrase are the two important enzymes maintaining this equilibrium (Igamberdiev and Kleczkowski 2006). The present work was aimed to analyze the effect of As stress on adenine and pyridine metabolism in *Brassica juncea* during germination and ESG.

Materials and methods

Plant material and treatment

Brassica juncea (L.) Czern. var. TPM-1 was used as the plant material, which is an As-tolerant variety (Srivastava et al. 2009). Seeds were sterilized with ethanol (30 %) for 3 min and then rinsed with distilled water to remove any traces of ethanol. The seeds were soaked in six different solutions with As concentration ranging from 0 (control), 50, 100, 250, 500–1,000 μM AsV (prepared using the salt Na_2HAsO_4) for 6 h. The seeds were then put on fine cotton bed in small plastic bottles (5.5 cm height, 3.5 cm diameter) having holes at the bottom. These bottles were fitted in a plastic box (6 bottles in each box) having 150 ml of 50 % Hoagland nutrient medium or As solution up to a level where cotton bed in the bottles just touches the solution and remains wet. Plastic boxes were incubated in dark for 2 days to observe germination of seeds and then

transferred to a plant growth chamber (Sanyo, Japan) having a daily cycle of a 14-h photoperiod with a light intensity of $150 \mu\text{E}/\text{m}^2/\text{s}$, day/night temperature of $25 \pm 2 \text{ }^\circ\text{C}$ and relative humidity of 65–75 % for a week.

For time-dependent analysis of metabolic changes taking place from 1 to 24 h during soaking and germination initiation phase or at 7th day, seeds were subjected to selected doses of 100 and 250 μM AsV. The entire seedling was harvested, powdered in liquid nitrogen, filled in 1.5 ml pre-weighed microfuge tubes and stored till further use at $-80 \text{ }^\circ\text{C}$.

Estimation of NAD, NADP, NADH and NADPH following plate reader method

Extraction procedures

All extraction steps were performed at $4 \text{ }^\circ\text{C}$ or below using an extraction medium/fresh weight ratio of 1 ml/100 mg. For extraction of NAD and NADP, powdered samples were extracted into 1 ml of 0.2 N HCl and the homogenates were centrifuged at $16,000g$ for 10 min at $4 \text{ }^\circ\text{C}$. Supernatant (0.2 ml) was incubated in boiling water bath for 1 min, rapidly cooled and neutralized (for approx pH of 5–6). For neutralization, 20 μl of 0.2 M NaH_2PO_4 (pH 5.6) was added. Subsequently, stepwise addition of aliquots of 0.2 M NaOH was performed. After each addition, the sample was vortexed and pH was tested using a pH strip. The procedure for the extraction of NADH and NADPH remained the same as for NAD and NADP but the extraction medium was 0.2 M NaOH instead of 0.2 N HCl. Further, in the final neutralization step, 0.2 N HCl was added in stepwise manner to reach a pH value of 7–8.

Pyridine nucleotide assays

Pyridine nucleotides were assayed using the plate reader method of Queval and Noctor (2007). The assay involved phenazine methosulfate (PMS) catalyzed reduction of dichlorophenolindophenol (DCPIP) in the presence of ethanol and alcohol dehydrogenase (ADH) (for NAD⁺ and NADH) or glucose 6-phosphate and glucose-6-phosphate dehydrogenase (G6PDH) (for NADP⁺ and NADPH). Reduced and oxidized forms are distinguished by preferential destruction in acid or base. To assay NAD⁺ and NADH, ADH was freshly diluted in 0.1 M Hepes (pH 7.5) and 2 mM EDTA to a concentration of 2,500 U/ml. Duplicate aliquots of 20 μl neutralized supernatant were introduced into plate wells containing 0.1 ml of 0.1 M Hepes (pH 7.5), 2 mM EDTA, 20 μl of 1.2 mM DCPIP, 10 μl of 20 mM PMS, 25 μl water, and 10 μl ADH. The reaction was started by the addition of 15 μl absolute ethanol. Following automatic mixing by shaking, the

decrease in A_{600} was monitored for 5 min. Contents were calculated by reference to standards run (0–1 pmol NAD⁺ or NADH in the well), and unknowns and standards were corrected for absorbance decreases measured for duplicate blank assays (0 NAD⁺ or NADH).

To assay NADP⁺ and NADPH, G6PDH was freshly diluted to a final concentration of 200 U/ml in 0.1 M Hepes (pH 7.5) and 2 mM EDTA. Duplicate aliquots of 30 μ l neutralized supernatant were introduced into plate wells containing 0.1 ml of 0.1 M Hepes (pH 7.5), 2 mM EDTA, 20 μ l of 1.2 mM DCPIP, 10 μ l of 20 mM PMS, 10 μ l of 10 mM glucose 6-phosphate and 30 μ l water. The reaction was started by the addition of 10 μ l G6PDH. Following automatic mixing by shaking, the decrease in A_{600} was monitored for 5 min and rates were calculated over the first 2 min using relevant standards (0–1 pmol NADP⁺ or NADPH) and blank correction, as described above for NAD⁺.

Extraction and analysis of adenine nucleotides

For the analysis of adenine nucleotides by HPLC, samples (100 mg) were subjected to acid extraction using 0.6 M perchloric acid. The extract was centrifuged at 14,000 \times *g* at 4 °C for 10 min followed by neutralization with 0.5 M KOH and re-centrifugation at 14,000 \times *g* at 4 °C for 10 min to remove the precipitate. Supernatant was used for the assay after filtration through 0.22 μ m syringe filters. HPLC conditions were standardized following Caruso et al. (2004) with some modifications. The mobile phase consisted of 0.1 M KH₂PO₄ solution at pH 6.0 (Buffer A) and 0.1 M KH₂PO₄ solution at pH 6.0, containing 10 % (v/v) of CH₃OH (Buffer B). All buffer solutions, after preparation and pH adjustment, were filtered through a 0.22 μ m Millipore filter. The chromatographic conditions were as follows: 8 min at 100 % of Buffer A, 7 min at up to 25 % of Buffer B, 2.5 min at up to 90 % of Buffer B, 2.5 min at up to 100 % of Buffer B, held for 7 min at 100 % B, 5 min at up to 100 % Buffer A and held for 8 min at 100 % Buffer A to restore the initial condition. The flow rate was 1 ml/min and detection was performed at 254 nm (Waters 996, PDA detector). Separation was performed on a 10 μ m C18 analytical column (250 \times 4.6 mm) equipped with a guard column. The peaks were identified using the standard samples. The analytical recovery was tested by adding a known amount of standard prior to extraction and recovery was found to be 94–100 % for different compounds. The data were analyzed using Empower software.

Enzyme extraction

For enzymatic assays, samples were homogenized in potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 2.5 % glycerol and 0.1 mM PMSF under chilled

conditions. Homogenate was centrifuged at 12,000 \times *g* for 15 min at 4 °C. Protein content of the supernatant was measured following Lowry et al. (1951).

NAD kinase and NADP phosphatase assay

NAD kinase and NADP phosphatase assay was performed following the protocol given in Hunt and Gray (2009). For both assays, 250 μ l of extract was incubated at 20 °C for 30 min in a final volume of 500 μ l. Reaction mixture for NAD kinase assay contained 5 mM NAD, 5 mM ATP, 100 mM Tris HCl (pH 7.6), 2 mM MgCl₂ and 2 mM CaCl₂. For NADP phosphatase assay, reaction mixture comprises 2 mM NADP, 50 mM Tris HCl (pH 7.6) and 2 mM MgCl₂. Reaction was stopped by heating at 100 °C for 5 min and samples were neutralized. Then the levels of NADP and NAD for NAD kinase and NADP phosphatase, respectively, were quantified using the plate reader method of Queval and Noctor (2007) as described above.

Nicotinamidase assay

An appropriate amount of enzyme extract was incubated with 10 mM nicotinamide for 5 min at 30 °C in a final volume of 100 μ l containing 10 mM Tris, pH 7.5, 150 mM NaCl and 1 mM MgCl₂. The concentration of the product, ammonia, was determined using the Sigma ammonia diagnostic kit (Sigma Chemical Co., <http://www.sigmaaldrich.com/>) (Wang and Pichersky 2007).

PARP assay

The activity of PARP was assayed using a PARP universal colorimetric assay kit (R&D Systems Europe, Ltd., UK), which measures the incorporation of biotinylated poly (ADP-ribose) onto histone proteins in a 96-well plate. The manufacturer's protocol was followed for the assay.

Adenylate kinase and apyrase assay

AK was assayed in the direction of ATP and AMP formation. The reaction mixture (1 ml) contained 100 mM Tricine (pH 7.8), 2 mM ADP, 2 mM MgCl₂, 0.5 mM NAD⁺, 60 mM KCl, 5 mM D-glucose, and 5 U each of hexokinase and G6PDH. Reactions were initiated by the addition of enzyme extract and run at 25 °C. One unit of AK activity was defined as amount of the enzyme required to reduce 1 μ mol NAD/min under conditions of the assay (Kleczkowski and Randall 1986).

To measure apyrase activity, reaction buffer containing 20 mM Hepes, pH 7.4, 120 mM NaCl, 5 mM KCl, 0.2 mM EDTA, 1 mM NaN₃ and 0.5 mM Na₃VO₄, with or without 5 mM CaCl₂ was used. After preincubation of enzyme

extract for 5 min at 37 °C nucleotide phosphatase reactions were initiated by the addition of 5 ml of the same buffer containing 10 mM ATP to give a final concentration of 1 mM (Biederbick et al. 2000). Samples were incubated for 20 min at 37 °C and then stopped by adding 10 % (v/v) TCA. Apyrase activity was determined by measuring the inorganic phosphate released (Fiske and Subbarow 1925). Values obtained from samples without CaCl₂ were subtracted from those obtained with CaCl₂.

ATP supplementation experiment

To analyze the effect of ATP supplementation, seeds were soaked and germinated either in AsV alone (100 and 250 μM AsV) or in AsV + ATP (1 mM) and analyzed for germination for 3 days.

Germination percentage (GP) of seeds was calculated at 3rd day following the formula:

$$GP = \left(\frac{\text{number of germinated seeds}}{\text{total number of seeds}} \right) \times 100.$$

The germination strength (GS) of the seeds was calculated using the formula proposed by the International Seed Testing Association (ISTA) (Hampton and Tekrony 1995).

$$GS = \sum (\text{number of germinated seeds/day of first count}) + \dots (\text{number of germinated seeds/day of final count}).$$

Statistical analysis

The experiments were carried out in a randomized block design. One-way analysis of variance (ANOVA) was done on all the data to confirm the variability of data and validity of results. Duncan's multiple range test (DMRT) was performed to determine the significant difference between treatments using statistical software SPSS 10.0.

Results

AsV treatments (100 and 250 μM AsV) have contrasting effects on seed germination and ESG

Initial experiments were performed to find out two contrasting As doses: one at which germination and ESG were not significantly affected and other at which 50 % or higher reduction was achieved. The screening was based on the analysis of germination percentage and length of root and shoot. In addition, the level of malondialdehyde, a measure of lipid peroxidation, was also tested (Online Resource 1). In all tested parameters, there was significant difference in response at 100 and 250 μM. Hence, detailed experiments

were conducted at these two As doses. There was a decline of 10–15 % in germination, root and shoot length at 100 μM AsV at 7th day, while the decrease was 48–60 % in these parameters at 250 μM AsV (Fig. 1).

NAD/NADH and NAD/NADP ratios exhibit significantly different pattern under AsV treatments

In a time-dependent analysis of pyridine nucleotides from the start of soaking till 24 h (0.2, 1, 2, 3, 6, 24 h), NAD level declined progressively in control and 100 μM AsV, but not at 250 μM AsV (Fig. 2a). The NADH level showed an increasing trend in control, while it declined at 100 and 250 μM AsV with the maximum decline at 24 h being 31 and 62 %, respectively (Fig. 2b). NAD/NADH ratio did not change significantly at control and 100 μM AsV, while it increased significantly at 250 μM beyond 2 h (161 % increase at 24 h) (Fig. 2c). NADP demonstrated an increasing trend while NADPH showed no significant change in control during 24 h (Fig. 2e). Upon AsV exposure, both NADP and NADPH declined with an increase in time with the greater decline being at 250 μM (62 and 51 %, respectively at 24 h) than at 100 μM (26 and 34 %, respectively, at 24 h) (Fig. 2e, f). However, NADP/NADPH ratio was not changed significantly both in control and in AsV exposure conditions (Fig. 2g). NAD/NADP showed a profile similar to that of NAD/NADH with no significant effect at control and 100 μM AsV and an increasing trend at 250 μM (137 % increase at 24 h) (Fig. 2d). NADH/NADPH was also not significantly altered at control and 100 μM AsV but declined significantly at 250 μM AsV beyond 2 h (Fig. 2h).

At 7th day, NADP demonstrated a marginal decline of 4–5 % in AsV treatment, while NADPH increased by 20 and 53 % at 100 and 250 μM, respectively (Fig. 3a). The level of NADH did not change significantly upon AsV exposure but that of NAD showed a significant increase of 75 % at 250 μM AsV (Fig. 3b). The ratios of NAD/NADH and NAD/NADP were not significantly affected at 100 μM AsV but increased significantly by 32 and 83 %, respectively, at 250 μM AsV. NADH/NADPH was not significantly affected, while NADP/NADPH ratio declined at both 100 and 250 μM (22 and 38 %, respectively) (Fig. 3c, d).

AsV stress affects activity of the enzymes of NAD metabolism

During initial 24 h, only the activities of NAD kinase and NADP phosphatase were analyzed. The activity of NAD kinase was not significantly affected at control, and showed significant increase at 100 μM, while a decline was noted beyond 2 h at 250 μM (the maximum decline being 35 % at 24 h) (Fig. 4a). The activity of NADP phosphatase

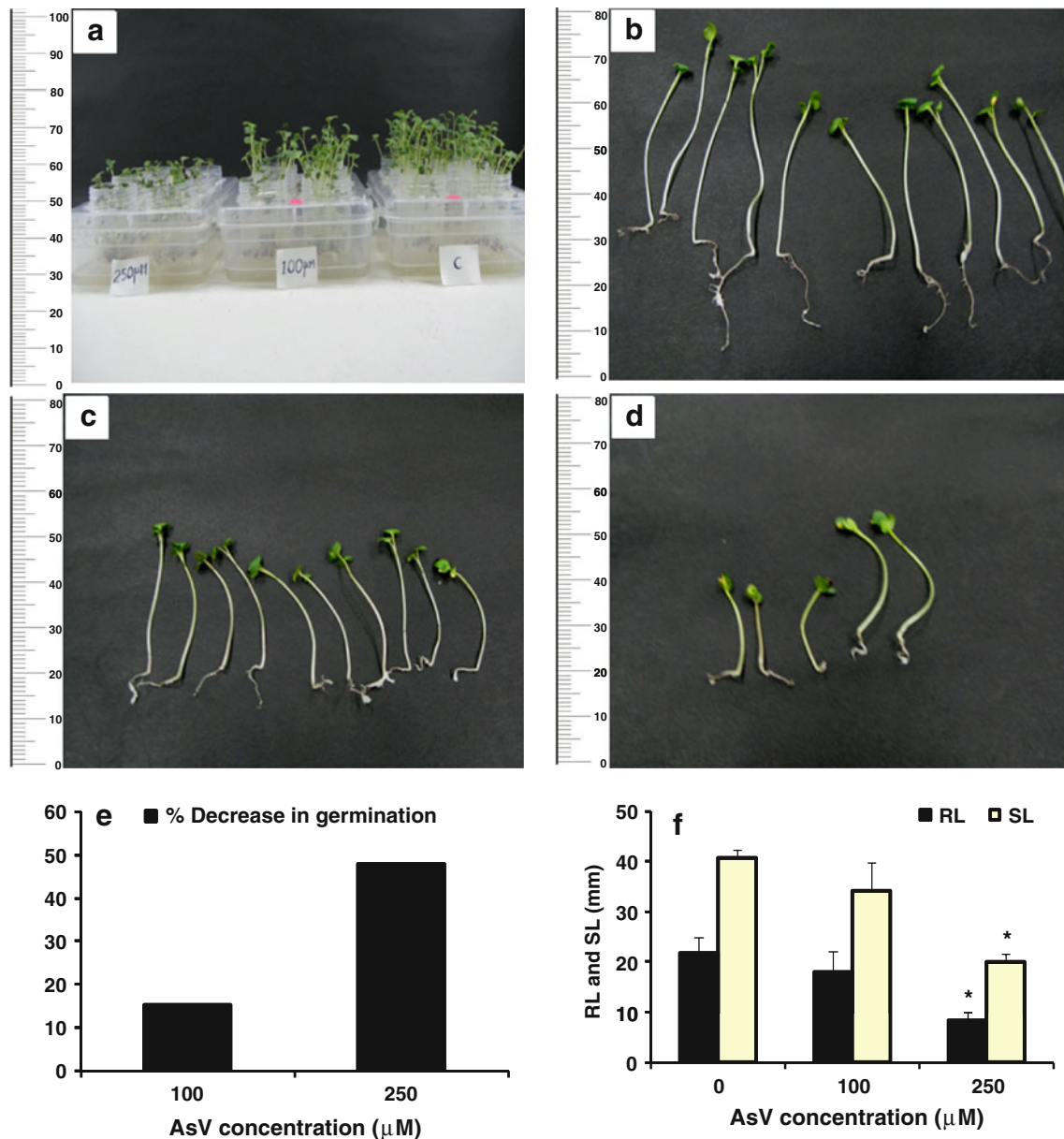


Fig. 1 Germination and growth analysis of *Brassica juncea* exposed to 100 and 250 μM arsenate: growth of seedlings in trays (a), seedlings grown in control (b), 100 μM AsV (c) and 250 μM AsV (d). Scale bars are in millimeters. Percent decrease in germination in comparison to control was calculated and found to be differential at

showed no significant effect at control and 100 μM AsV at any time point and at 250 μM up to 6 h. However, at 24 h NADP phosphatase activity showed significant increase of 35 % at 250 μM AsV (Fig. 4b).

At 7th day, in addition to the activities of NAD kinase and NADP phosphate, PARP and nicotinamidase activities were also assayed. At 7th day, NAD kinase and NADP phosphatase showed decline and increase, respectively, under As stress with the changes being significantly higher at 250 μM (69 and 80 %, respectively) than at 100 μM (19

and 31 %, respectively) (Fig. 4c, d). PARP activity declined significantly at both 100 and 250 μM (26 and 45 %, respectively) (Fig. 4e). Nicotinamidase activity was not affected significantly at 100 μM but increased significantly at 250 μM (Fig. 4f).

Effect of AsV stress on ATP metabolism at 7th day

The levels of ATP and ADP did not change significantly at 100 μM AsV as compared to control. However, at 250 μM

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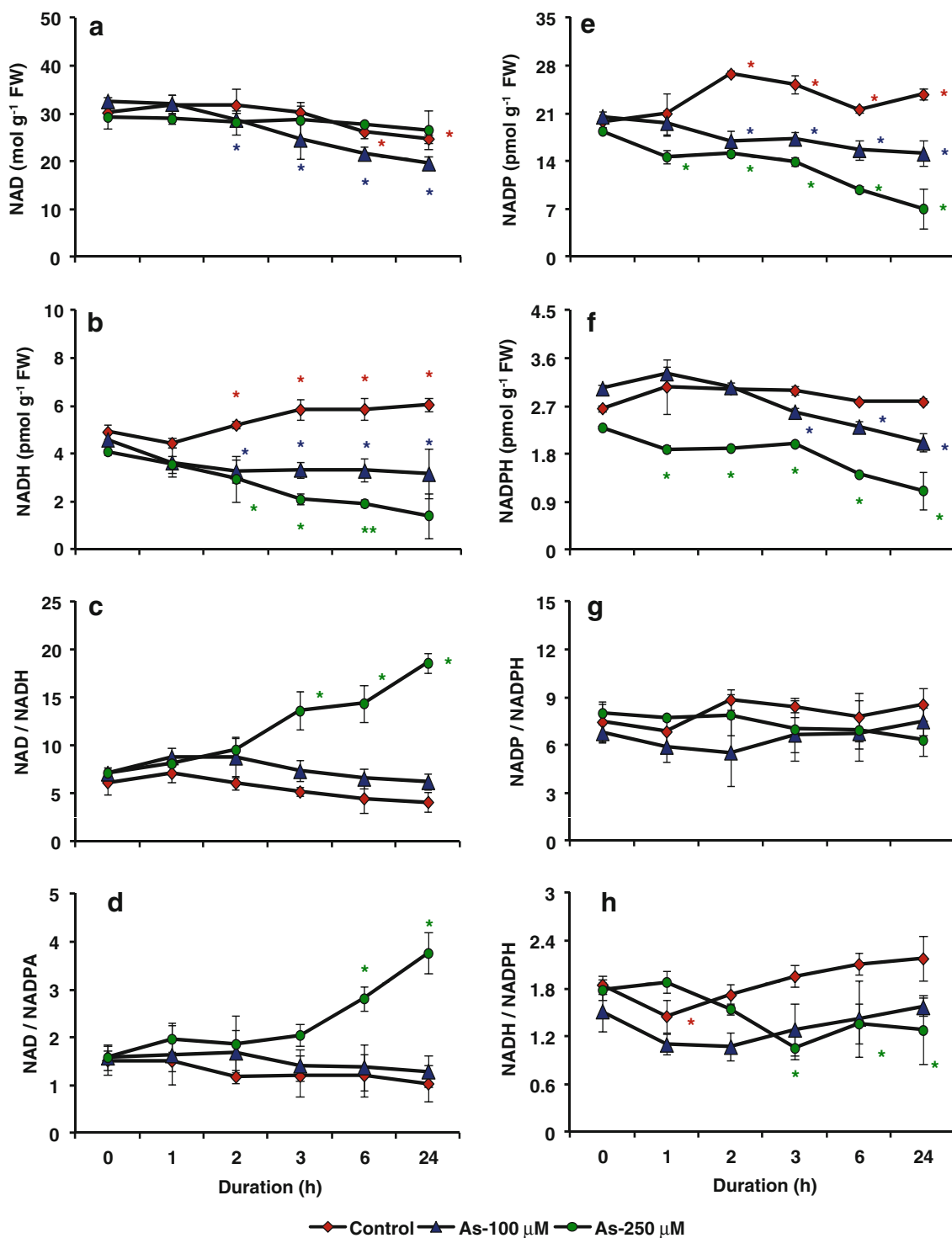


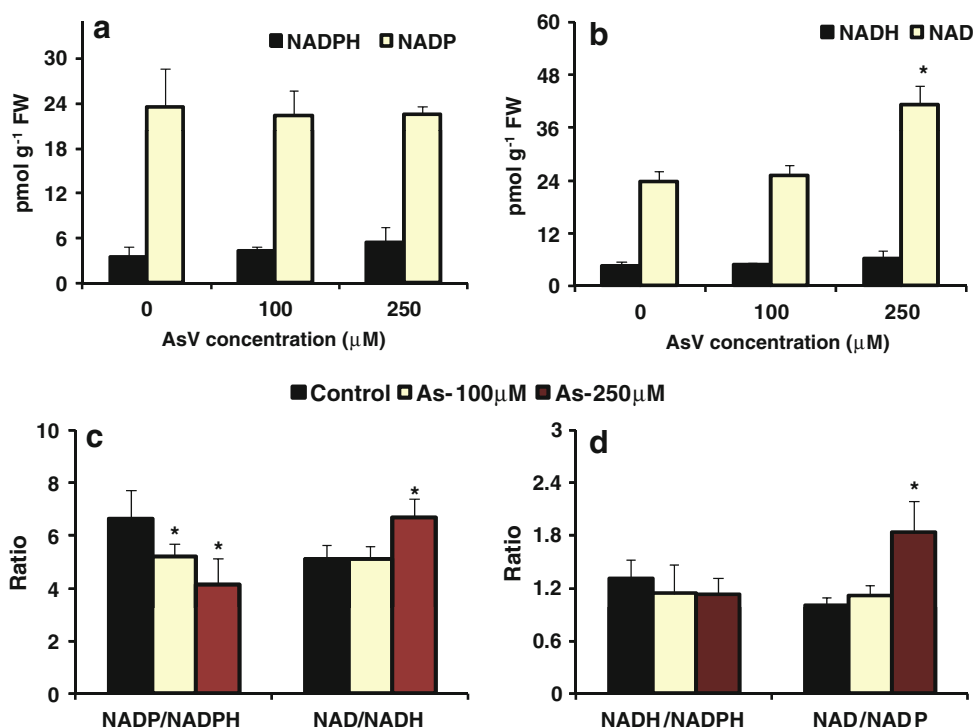
Fig. 2 Effect of different arsenate treatments on the level of metabolites of NAD metabolism was analyzed for 24 h: NAD (a), NADH (b), NAD/NADH (c), NAD/NADP (d), NADP (e), NADPH (f), NADP/NADPH (g), NADH/NADPH (h). Data represent the mean of

triplicates from two independent experiments. *Asterisk* represents significant difference for a particular parameter and duration (DMRT, $P < 0.05$)

AsV, ATP and ADP significantly declined and increased, respectively (35 and 48 %, respectively, in comparison to control) (Fig. 5a). Consequently, ATP/ADP ratio declined

significantly at 250 μM AsV (56 %) but not at 100 μM (Fig. 5b). The activities of both adenylate kinase and apyrase declined non-significantly at 100 μM (10 and

Fig. 3 Effect of different arsenate treatments on the level of metabolites of NAD metabolism was analyzed at 7th day: NADP and NADPH (a), NAD and NADH (b), NADP/NADPH and NAD/NADH (c), NADH/NADPH and NAD/NADP (d). Data represent the mean of triplicates from two independent experiments. Asterisk represents significant difference for a particular parameter (DMRT, $P < 0.05$)



15 %, respectively) but significantly at 250 μM (37 and 54 %, respectively) (Fig. 5c, d).

Effect of ATP supplementation on seed germination

External supply of 1 mM ATP to AsV was found to significantly improve germination percentage (GP; Fig. 6a) and germination strength (GS; Fig. 6b) of seeds as compared to AsV treatment alone. GP and GS were declined by 24 and 27 %, respectively, at 100 μM and by 71 and 75 %, respectively, at 250 μM. Upon ATP supplementation, GP came close to control at both AsV levels with only 7 and 14 % decline at 100 and 250 μM AsV, respectively. GS was also improved and became at par to control in 100 μM + ATP, but remained 30 % lower than control in 250 μM + ATP treatment.

Discussion

Germination and ESG are important parameters to determine relative stress tolerance in crop plants. Although As toxicity mechanisms at whole plant level (Srivastava et al. 2011) or even callus level (Rai et al. 2011) have been demonstrated, crucial parameters determining the extent of toxicity at germination and ESG are little understood. If the mechanisms of stress-induced damage at germination and ESG are understood, a large number of plants can be screened on the basis of an identified critical parameter that

would consume less time. In earlier studies a link between seed germination and NAD metabolism has been established (Hunt et al. 2004, 2007; Hunt and Gray 2009). To determine the relationship of NAD as well as ATP metabolism with germination and ESG under As stress, the levels of enzymes and metabolites of NAD and ATP metabolism were analyzed in *B. juncea* under two As doses selected after preliminary experiments (Online Resource 1).

At 250 μM, seeds and growing seedlings were found to be unable to mobilize NAD, which showed no significant change during initial 24 h as compared to control. Our results suggest that the level of NAD and ratio of NAD/NADP and NAD/NADH were correlated to the effects on studied enzymes, NAD kinase and NADP phosphatase. At 24 h and 7th day, NAD kinase and NADP phosphatase showed significant decline and increase, respectively, at 250 μM AsV stress. Hence, the conversion of NAD to NADP was probably reduced due to low NAD kinase activity, while NADP to NAD conversion increased due to high NADP phosphatase activity at 250 μM. Further, PARP activity declined significantly at both 100 and 250 μM (26 and 45 %, respectively). PARP is an enzyme involved in NAD degradation and decline in its activity would have further added restrictions on NAD consumption as compared to that of control conditions. Nicotinamidase activity increased significantly at 250 μM (27 %). Nicotinamidase acts on nicotinamide, a degradation product of NAD, and there are evidences that this

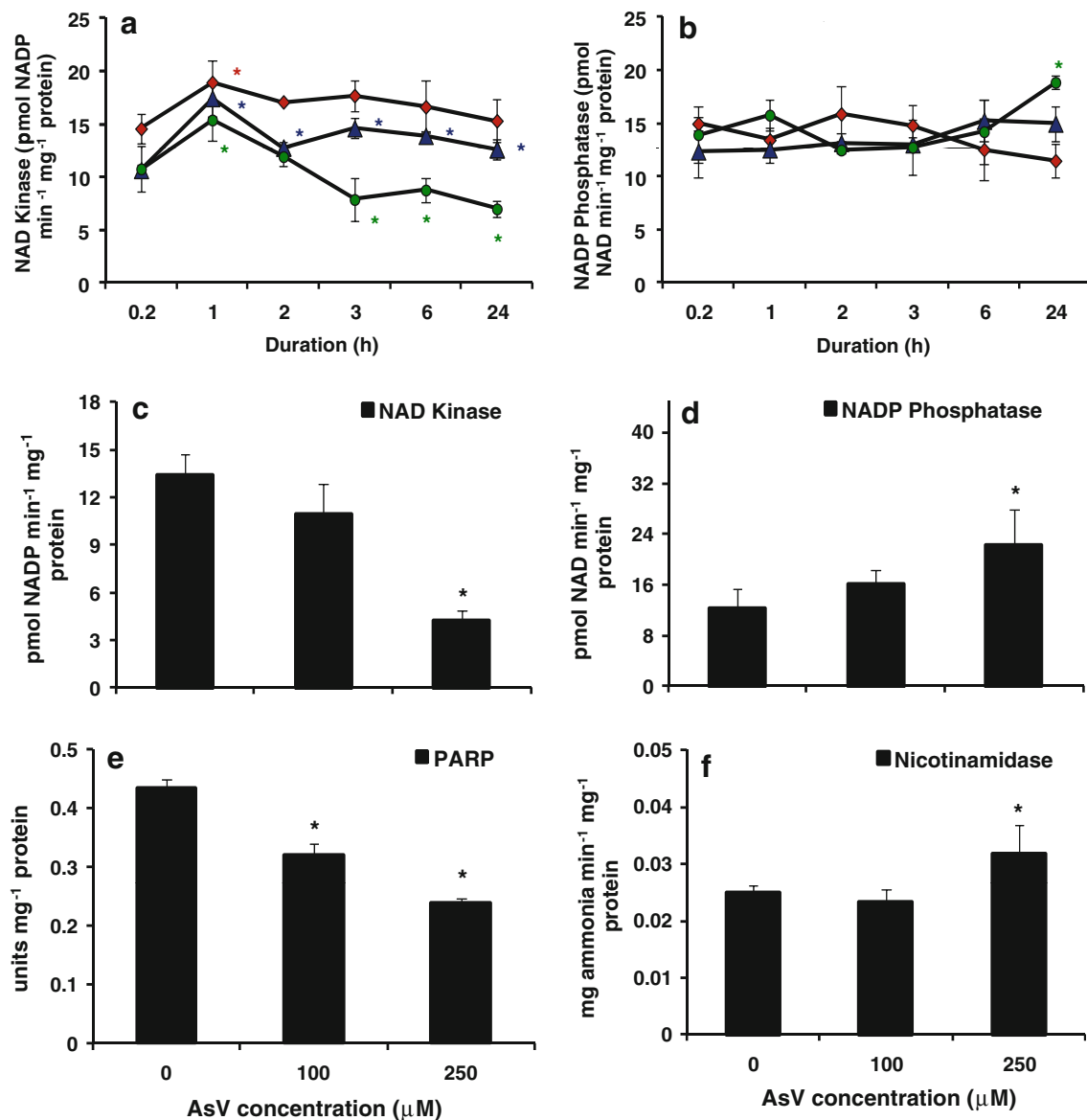


Fig. 4 Effect of different arsenate treatments on the activities of enzymes of NAD metabolism was analyzed for 24 h and at 7th day: NAD kinase (a, c), NADP phosphatase (b, d), PARP (e),

nicotinamidase (f). Data represent the mean of triplicates from two independent experiments. Asterisk represents significant difference for a particular parameter and duration (DMRT, $P < 0.05$)

reaction can again lead to NAD generation through a salvage pathway (Ashihara et al. 2005). Wang and Pichersky (2007) have confirmed the role of nicotinamidase in NAD generation by the analysis of nicotinamidase mutant (*nic1-1*) Arabidopsis plants. Due to observed effects of AsV on enzymatic changes, the mobilization of NAD at 250 μM AsV was affected, which presumably inhibited germination and ESG. The present results are in confirmation with another observation that seed dormancy and hence the germination response in different ecotypes of Arabidopsis seeds correlate with the activity level of enzymes of NAD metabolism and that germination is associated with a reduction in NAD levels (Hunt and Gray

2009). In addition, exogenously applied NAD has been found to act as an inhibitor of seed germination (Hunt et al. 2007). In our study also, NAD levels declined under control and 100 μM As treatments but not in 250 μM As treatments. Thus, the failure to balance consumption and synthesis of NAD could be an important step in controlling seed germination as well as ESG.

The capacity to generate sufficient energy for biosynthetic reactions must be important to ensure successful germination and ESG. The ratio of ATP/ADP showed a significant decline at 250 μM AsV and therefore, energy utilization might have been affected. This would have probably contributed to observed negative effects of

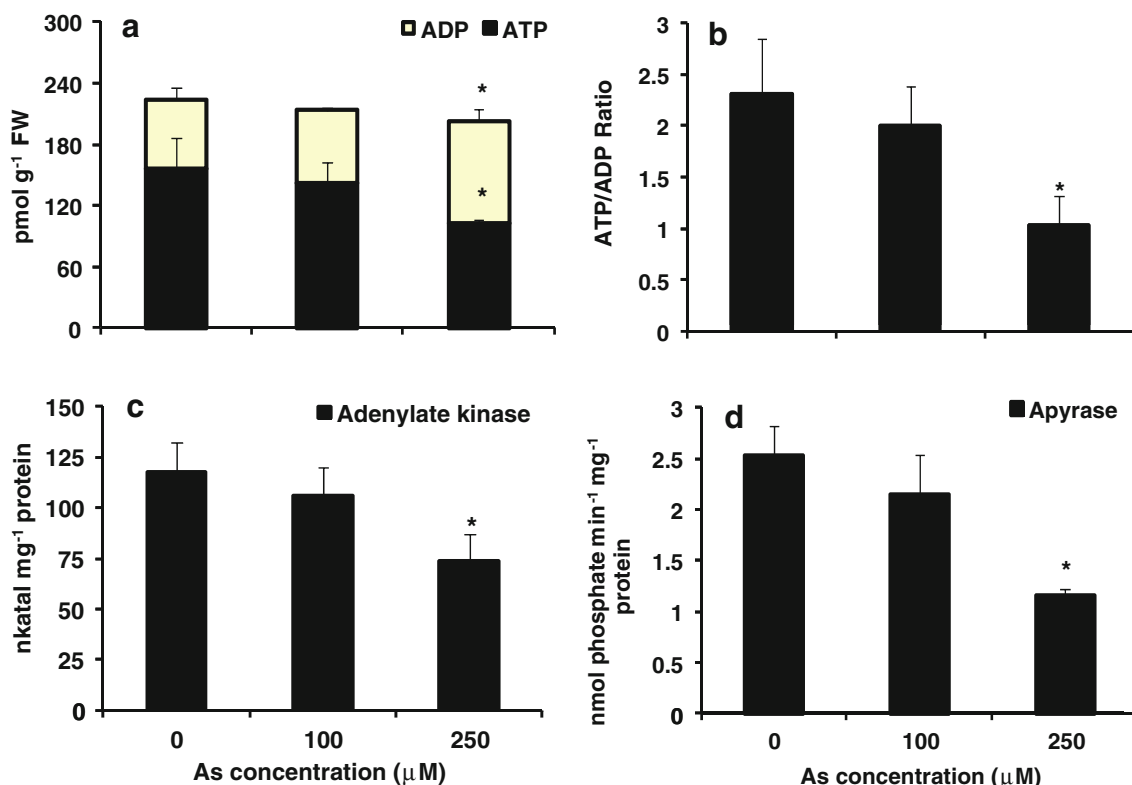


Fig. 5 Effect of different arsenate treatments on the level of metabolites and on the activities of enzymes of ATP metabolism was analyzed at 7th day: ATP and ADP (a), ATP/ADP (b), adenylate

kinase (c), apyrase (d). Data represent the mean of triplicates from two independent experiments. Asterisk represents significant difference for a particular parameter (DMRT, $P < 0.05$)

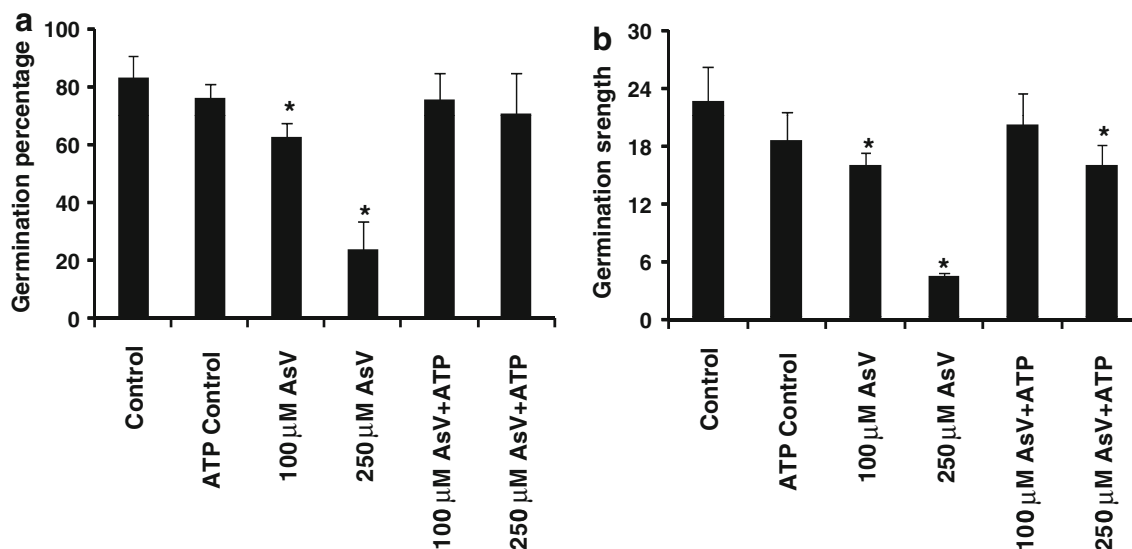


Fig. 6 Effect of 1 mM ATP supplementation on the germination percentage (a) and germination strength (b) of *Brassica* seeds in control and upon exposure to AsV. Data represent the mean of

triplicates from two independent experiments. Asterisk represents significant difference for a particular parameter (DMRT, $P < 0.05$)

250 μM AsV on germination and ESG. The importance of ATP in AsV tolerance could be realized by the observation that ATP supplementation to 100 and 250 μM AsV was able to significantly improve GP and GS to close to control

values. The activity of both adenylate kinase (AK) and apyrase also declined significantly at 250 μM. Adenylate kinase catalyzes a reversible reaction using MgADP and free ADP to generate ATP and AMP. However, depending

on the relative concentration of reaction components, AK reaction equilibrium may shift to ADP generation (Igamberdiev and Kleczkowski 2006). Apyrase catalyzes the production of AMP from ATP and/or ADP. AK and apyrase cycle maintains a dynamic equilibrium of adenylates in plants. The reduced activities of both AK and apyrase apparently affected the dynamics of equilibrium of adenylates leading to reduced ATP/ADP ratio (Igamberdiev and Kleczkowski 2006).

ATP levels are also affected by PARP and NAD kinase. Plant lines with low PARP activity have been found to maintain their energy homeostasis under stress conditions due to reduced NAD breakdown and energy consumption (De Block et al. 2005). On the other hand, Arabidopsis mutant lines for NAD kinase (*nadk2*) have decreased efficiency of photosynthetic electron transport and hence reduced ATP generation (Takahashi et al. 2006). Therefore, while decline in PARP activity might lead to less ATP consumption, decrease in NAD kinase activity would reduce ATP generation. The observed results of decline in ATP/ADP ratio may thus be attributed, at least to some extent, to the disturbed balance of PARP and NAD kinase.

In conclusion, we observed that AsV stress negatively affected the balance of NAD and ATP consumption and synthesis through its effects on the biosynthetic enzymes. These negative effects were possibly responsible for inhibitory effects of AsV on germination and ESG.

Author contribution S. Srivastava and J.J. Akkarakaran contributed to all experimental analysis and data calculation. S. Srivastava planned the experiments and interpreted the results and also wrote the manuscript. P. Suprasanna and S.F. D'Souza supervised the study and contributed in preparing the final manuscript.

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