Kinetics of Arsenic Accumulation and its Impact on Biochemical Responses of *Brassica juncea*

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**Abstract**

Arsenic (As) contamination of the environment is a widespread problem with the situation at its worst in the South Asian region of West Bengal, India, and Bangladesh. In order to cope up with the problem and to be able to engineer plants in near future, there is a need to thoroughly understand the kinetics of As uptake and its impact on responses of plants. In this work, *Brassica juncea* (L.) Czern was used as a model system to understand kinetic interaction between As uptake and transport to the shoot and corresponding biochemical responses. The seedlings of *B. juncea* were exposed to 100 μM arsenite [As(III)] in hydroponics for different time points of 1, 4, and 24 hours. The As concentration was found to show a gradual increase in different tissues with time and the level of As followed the order: lower root > upper root > shoot > top leaves at all the times points. However, the level of hydrogen peroxide (H₂O₂) and the activities of NADPH oxidase, superoxide dismutase (SOD) and ascorbate oxidase (AO) were increased even at 1 h in lower shoot and leaves. The expression of transporters genes, NIP1;1, NIP2;1, NIP5;1 and NIP6;1 was found significantly up-regulated at 24 hours. This work establishes a kinetic relationship between As accumulation and ensuing biochemical responses in *B. juncea*.

**Keywords:** Arsenic; Indian mustard, NADPH Oxidase, NIPs.

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**Introduction**

Arsenic (As) is a naturally occurring element widely distributed in the earth’s crust, having properties of both metal and a non-metal. Arsenic can be found in rock, soil, water, air, and the earth’s biosphere (Shukla et al., 2020). The sources include both anthropogenic inputs and natural biogeochemical processes (Awasthi et al., 2017). There are many forms, or species of As, which are broadly categorized as inorganic and organic (ATSDR, 2000). The major forms of As are inorganic arsenite [As(III)] and arsenate [As(V)]. Arsenite is an analog of inorganic phosphate, and it interferes with the essential cellular processes like oxidative phosphorylation and ATP synthesis. Arsenite binds to sulfhydryl groups of proteins and has detrimental effects on general protein functioning (Finnegnan and Chen, 2012; Awasthi et al., 2017; Majumdar and Bose, 2017, 2018). Arsenate is taken up and transported in plants by phosphate transporters (Catarecha et al., 2007; Cao et al., 2017; Ye et al., 2017). Arsenite is able to enter root cells mainly through nodulin26-like intrinsic proteins (NIPs; Lindsay and Maathuis, 2017; Awasthi et al., 2017; Majumdar and Bose, 2018) of aquaglyceroporins.

Roots are usually the first tissue to be exposed to As and therefore root response to As and changes in root architecture play a crucial role in As stress tolerance (Srivastava et al., 2019). With increasing exposure duration and translocation of As to shoot tissues, As severely inhibits plant growth by slowing or arresting cellular expansion and by reducing photosynthetic efficiency (Garg and Singla, 2011; Mishra et al., 2014). One of the major mechanisms of As toxicity is the increased production of reactive oxygen species (ROS) and consequent damage to lipids, proteins and DNA (Srivastava et al., 2011; Gupta et al., 2018; Gautam et al., 2020). An increase in the activity of pro-oxidant enzymes like NADPH oxidase and ascorbate oxidase (AO) occurs for regulated increase in ROS production to mediate As stress signaling (Srivastava et al., 2011; Gupta et al., 2013). The fine-tuning of scavenging of ROS is achieved through the role of enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and molecules like glutathione (GSH) and ascorbate (Srivastava et al., 2007). When the level of ROS increases uncontrollably, stress responses are evident (Gautam et al., 2020). Arsenic detoxification is achieved through its complexation via thiol (-SH) containing ligands including GSH, and phytochelatins (PCs) and subsequent transport of complexed As to the vacuoles (Song et al., 2014).

Considerable information has come to light about As induced stress responses in a number of plant systems like rice, Indian mustard, as well as aquatic plants like *Hydrilla verticillata* (Srivastava et al., 2007, 2009; Chauhan et al., 2017; Awasthi et al., 2018; Pandey and Gupta, 2018). Despite this knowledge resource, there is still paucity of information, especially on the temporal and spatial dynamics of As uptake. In this regard, it is interesting to study how kinetics of As uptake and translocation...
within plants is correlated to the responses of plants in various plant tissues. In the present study, time- and tissue-dependent responses of Indian mustard (*Brassica juncea* (L.) Czern.) were investigated. Among the plants of the *Brassic* species, *B. juncea* has become a candidate system in a number of metal related studies owing to its metal hyperaccumulator potential and significant tolerance to a number of metals like As, Cd, Zn and Pb (Srivastava et al., 2009; Rizwan et al., 2018).

**MATERIALS AND METHODS**

**Plant material and Treatment**

The seeds of *B. juncea* var. TPM-1 were surface sterilized with 30% ethanol for 3 minutes and then washed thoroughly with distilled water to remove the traces of ethanol. The seeds were soaked in water for 6 hours and then kept overnight under dark for germination. The seeds were placed in theromcol float with holes filled with 0.4% agarose. Once the seeds were germinated, the floats were transferred to 500 ml beaker containing ½ strength Murashige and Skoog nutrient medium. The seedlings were grown in a Plant growth chamber (Sanyo, Japan) with a temperature of 25 ± 2°C, 125 μM photon m⁻² s⁻¹ of illumination, with a 12 hours photoperiod, a relative humidity of 70% and vigorous aeration to maintain good growth. The medium was changed every third day. After 14 d, seedlings subjected to 100 μM As(III) (NaAsO₂; Sigma) for three-time points (1, 4, and 24 hours). The sets without As were maintained for the control treatment. At harvesting, seedlings were cut into four segments – lower root (LR; 1 cm from tip), upper root (UR), shoot (Sh; young stem plus lower leaves), and topmost leaves (TL) (as depicted in Fig. 1). The harvested tissues were cut directly in pestle mortar in liquid nitrogen and were homogenized to a fine powder. The tissues were stored at -80°C until further analysis. For weight measurements, a separate set was used. The fresh weight of seedlings was recorded, and dry weight was taken after drying at 80°C for 2 to 3 days to reach a constant weight.

**Enzymatic Assays**

For enzymatic assays also, the plants were divided into four segments, as mentioned above, and each segment was ground in liquid nitrogen. The samples were used for protein extraction in 20 mM HEPES (pH 7.0) containing 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 2.5% polyvinylpyrrolidone (PVP) and 5% glycerol under chilled conditions. The homogenate was centrifuged at 10000 g for 10 minutes and the supernatant was used for enzyme assay. The protein content in the supernatant was assayed following Lowry et al. (1951). The activity of ascorbate oxidase (AO; EC 1.10.3.3) was determined by measuring the decrease in absorbance at 265 nm due to ascorbate oxidation (ε = 2.8 mM⁻¹cm⁻¹) as described previously (Yamamoto et al., 2005). NADPH-dependent O₂⁻ generation by the enzyme NADPH oxidase (NOX; EC 1.6.3.1) was measured using nitro blue tetrazolium (NBT) as an electron acceptor (Bielski et al., 1980), whose reduction was monitored at 530 nm. Monoformazan concentrations (and therefore O₂⁻ concentrations) were calculated using an extinction coefficient of 12.8 mM⁻¹cm⁻¹. The reaction mixture consisted of Tris buffer (50 mM Tris-HCl, pH 7.4), 5 mM NBT, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM NADPH, and a suitable aliquot of enzyme extract. The selective reduction of NBT by O₂⁻ was calculated from the difference in the NBT reduction rate in the presence and absence of SOD (50-100 units ml⁻¹; Sigma). No NBT reduction with NADPH was observed in the absence of protein fractions. The activity of

**Arsenic Estimation**

To determine the total content in the plants, plants were cut into four segments as mentioned above and oven-dried to constant weight (Srivastava and D’Souza, 2010). The samples were subjected to acid digestion by using concentrated HNO₃ on a heating block 180°C for 1 h and subsequently at 200°C for 45-60 min so as to evaporate the samples to dryness. Arsenic concentrations were determined on an atomic absorption spectrophotometer (GBC 906AA, Australia) coupled to a hydride generation system (HG3000). In each analytical batch, reagent blanks and spiked samples were included in the acid digestion to assess the accuracy of the chemical analysis. Analytical data quality was ensured through repeated analysis of standard reference materials. The translocation factor (TF) of As from root (LR + UR) to Sh (As concentration in Sh / As concentration in root) and from Sh to TL (As concentration in TL/As concentration in Sh) was calculated.

**Histochemical Staining by using 3, 3’-diaminobenzidine (DAB)**

For the detection of H₂O₂ in vivo, plant samples were stained with 3, 3’-diaminobenzidine (DAB) as detailed in Srivastava et al. (2011). DAB is oxidized by H₂O₂ in the presence of peroxidases, to generate a dark brown precipitate. To perform DAB staining, a solution of 0.5 mg ml⁻¹ DAB (pH 3.8) was prepared in 10 mM MES buffer (pH 6.5). Leaves (2nd leaves from top) and roots were dipped in 20 ml of DAB solution in scintillation vials and were vacuum infiltrated 3 times in dark with the interval of 5 min each. After infiltration, the plantlets were incubated in the dark overnight. The next day, leaves and roots were placed carefully on transparency sheets and scanned.
superoxide dismutase (SOD; EC 1.15.1.1) was assayed following the protocol of Beauchamp and Fridovich (1971).

**Real-Time PCR Expression Profiling of Transporter Genes**

For real-time RT-PCR analysis of NIP transporters, gene sequences of NIP1;1, NIP2;1, NIP5;1 and NIP6;1 were downloaded from *Brassica rapa* database (BRAD; www.brassicadb.org), and primers of intron-exon boundary were then prepared (Table 1). Primer optimal concentrations of target and reference genes were determined with serial dilutions of cDNA isolated from 10 µg of RNA isolated from *B. juncea*. Real-time quantitative RT-PCR was carried out using a Corbett rotor gene 3000 (Corbett Life Science, www.corbettlifescience.com; Srivastava et al., 2009). The actin gene was amplified in parallel with the target gene, allowing for gene-expression normalization and providing quantification. The procedure detailed in Srivastava et al. (2009) was followed for real-time PCR expression of selected genes. A relative expression ratio plot was generated using the software REST-MCS (Pfaffl et al., 2002).

**Statistical Analysis**

Statistical treatments included one way ANOVA and Duncan’s multiple range test (DMRT) to assess the variability of the data and to know significant differences between treatments. Statistical analysis was performed by using Statistical Package for the Social Sciences (SPSS) version 16.0.

**RESULTS**

**Arsenic Accumulation Occurred in Time-dependent Manner**

The As accumulation showed a gradual increase over different time points, from 1 h to 24 h in *Brassica* seedlings in all the studied tissues. As accumulation at 1 h was very high in LR (110.08 µg g⁻¹ dw) and UR (121.35 µg g⁻¹ dw) as compared to that of Sh (3.17 µg g⁻¹ dw) and TL (0.09 µg g⁻¹ dw) (Fig. 2). With an increase in duration, there was an enhanced translocation of As from root to shoot tissue. At 24 hours, the As concentration in LR, UR, Sh, and TL was 202.85 µg g⁻¹ dw, 187.5 µg g⁻¹ dw, 97.5 µg g⁻¹ dw, and 7.16 µg g⁻¹ dw, respectively (Fig. 2). The translocation factor of As was calculated at each time point. The TF for root to Sh increased from 0.01 at 1-hour to 0.12 at 4-hour and 0.25 at 24 hours. The TF values for Sh to TL were 0.03, 0.03, and 0.07 at 1, 4, and 24-hour, respectively. Hence, the translocation of As from roots to Sh showed a marked increase from 1 h to 4 h while As translocation to TL was very low.

**Arsenic Exposure Resulted in an increase in H₂O₂ and Induced Responses of Prooxidant and Antioxidant Defense**

H₂O₂ accumulation was histochemically detected in the root and leaf tissues by using DAB staining (Fig. 3A-H). The accumulation

![Fig. 2: Arsenic accumulation in LR, UR, Sh, and TL of *B. juncea* seedlings exposed to 100 µM As(III) for 1, 4, and 24-hour. All the values are means of three replicates ±SD. ANOVA was found significant at 95% significant level. Different symbols indicate significantly different values for a particular tissue (DMRT ≤0.05).](image1)

![Fig. 3: Effect of 100 µM As(III) exposure on hydrogen peroxide accumulation in 2nd leaves and roots of *B. juncea* seedlings at 1-hour (B, F), 4-hour (C, G) and 24-hour (D, H). Control leaf (A) and root (E) is also shown.](image2)
of $\text{H}_2\text{O}_2$ was observed from 1-hour onwards and was continued to increase until 24 hours. Thus, As exposure resulted in $\text{H}_2\text{O}_2$ accumulation even at 1-hour in 2nd leaves from top in seedlings, where As accumulation was very low. Among enzymes, SOD showed a highly significant increase in all tissues on all time points. At the early time point of 1-hour, SOD showed an increase of 36, 61, 58, and 40% in LR, UR, Sh, and TL, respectively, as compared to corresponding control for each tissue (Fig. 4A).

NADPH oxidase activity showed a significant decrease in LR at 1-hour while an increase at 4-hours as compared to control (Fig. 4B). In Sh, NADPH oxidase did not show a significant change while in TL, NADPH oxidase activity showed a significant decline at 4-hour (27%) and 24 h (40%) in comparison to control. Only UR depicted a significant increase in NADPH oxidase activity on all-time points in comparison to control, which were 187, 197, and 138% at 1, 4, and 24 hours, respectively (Fig. 4B). Similar to SOD, AO also showed a significant increase in all tissues on all time points except in UR at 1-hour (Fig. 4C). The maximum increase in AO activity in LR (147%) and Sh (204%) was observed at 1-hour in comparison to control while the maximum increase was seen at 24 h in UR (65%) and TL (88%).

**Transporter Genes Showed Significant Arsenic-induced Change in Expression**

The expression level of four transporter genes ($\text{NIP1;1}$, $\text{NIP2;1}$, $\text{NIP5;1}$ and $\text{NIP6;1}$) depicted significant changes in tissue- and time-dependent manner. The gene expression was analyzed in root and shoot tissues as a whole with no further separation. An expression change of $>1\log2$-fold was considered to be significant. At 4-hours, only $\text{NIP1;1}$ showed significant down-regulation in roots while other genes were not altered significantly (Fig. 5). At 24 h, $\text{NIP1;1}$ expression was up-regulated significantly (4.88-fold) while $\text{NIP2;1}$ was down-regulated (-1.51-fold). $\text{NIP5;1}$ and $\text{NIP6;1}$ were not significantly regulated in roots. In shoot, the expression of $\text{NIP1;1}$ was not detected while other three genes ($\text{NIP2;1}$, $\text{NIP5;1}$ and $\text{NIP6;1}$) showed significant up-regulation at both 4, and 24 hours. The up-regulation in $\text{NIP2;1}$, $\text{NIP5;1}$ and $\text{NIP6;1}$ was 4.6-, 6.7- and 6.0-fold, respectively at 24 h (Fig. 5).

**Discussion**

Arsenic accumulation in plants is determined by several factors related to soil and plant; the latter is related to As uptake by roots, its translocation, and distribution and relocation in different plant tissues (Zheng et al., 2011). As induced physiological, biochemical, and molecular responses have received great attention (Srivastava et al., 2009; 2016; Du et al., 2020). Insights into temporal and spatial dynamics of As stress responses will provide valuable information about the transport and
accumulation. This study demonstrated that stress responses occur in tissue- and time-dependent manner in association with As accumulation changes. As accumulation in roots was found to occur rapidly, reaching to high level even at 1-hour. However, the translocation of As to Sh and TL was rather slow. The increase in As translocation from roots to Sh occurred with an increase in time and therefore, the fold-difference in root (LR / UR) and shoot As decreased from about 22-fold at 1 h to 2-fold at 24 h. Although As translocation also increased from Sh to TL with time, it could be seen that As translocation to TL was restricted to a great extent. This appears to be due to the fact that plants attempted to protect the most sensitive and young tissues, i.e., TL from As damage. A graded level of As concentration from roots to grains is known to be a phenomenon in rice plants (Shrivastava et al., 2020). In B. juncea also, a significant difference in As concentration in root and shoot has been observed (Pathare et al., 2013). Thus, roots are presumably more resistant to As accumulation than leaves. The uptake and transport of As are mediated by various transporters. The As in As(III) form is mostly taken up and transported through nodulin26-type intrinsic proteins (NIPs) of the aquaglyceroporin family. The role of a number of NIPs transporters in As(III) uptake and transport has been elucidated. NIP1;1 from A. thaliana is an important transporter involved in As(III) uptake, and its mutation has been found to enhance As tolerance of plants (Kamiya and Fujiwara, 2009; Ji et al., 2017). Another homolog of NIP1;1, i.e., NIP5;1, was also found to mediate As transport as mutant lines of nip5;1 had reduced As content in Arabidopsis (Kamiya and Fujiwara, 2009). Bienert et al. (2008) expressed a number of genes of NIPs in yeast and examined their As(III) transport potential. In this study, AtNIP5;1 and AtNIP6;1 were found to mediate bidirectional As(III) transport in yeast; however AtNIP2;1 was not found to mediate As(III) transport (Bienert et al., 2008). However, in rice, OsNIP2;1 (Ls11) is a major transporter involved in As(III) uptake and transport (Ma et al., 2008; Awasthi et al., 2017). In this study, the studied transporters showed a significant change in expression. At 24 h, NIP1;1 in roots and NIP2;1, NIP5;1 and NIP6;1 in shoots showed significant up-regulation; >=4.6-fold for each gene. As accumulation data shows a significant increase in uptake as well as translocation of As from root to shoot, which was correlated to the up-regulation observed in transporter genes. Thus, in B. juncea, NIP1;1, NIP2;1, NIP5;1 and NIP6;1 might have a role in As(III) uptake and transport, which needs to be studied in future.

It is known that photosynthesis is affected to a great extent upon As exposure and As-induced loss of pigments and photosynthetic efficiency have been suggested as the major mechanism of As stress in plants (Mishra et al., 2014). The loss of photosynthetic efficiency is also related to the outburst of ROS and associated toxicity (Srivastava et al., 2011). A study by Rai et al. (2014) demonstrated that enhanced photosynthesis plays a role in As tolerance in Artemisia annua. Hence, As accumulation kinetics was clearly indicative of plant response through restriction of most of the As in root tissues allowing very little As reaching to TL. However, the biochemical responses, well known to be associated to stressed conditions (i.e., increased H$_2$O$_2$ production and SOD activity) (Hartley-Whitaker et al., 2001; Karimi et al., 2019) were observed even at 1 hour in TL when As concentration in TL was very low (0.09 µg g$^{-1}$ DW). The increase in SOD was 36% in LR, while 39% in TL. At the same time, As concentration was 1223-fold higher in LR (110.08 µg g$^{-1}$ DW) than that in TL (0.09 µg g$^{-1}$ DW). Hence, the response of plants appeared to be connected to the signaling of As stress to TL at a very early time. Plants appeared to have sensed As entry into roots and transferred this information to the TL to prepare it for tackling the incoming As stress (Huber and Bauerle, 2016; Ko and Helariuut, 2017). Hydrogen peroxide is a well-known messenger molecule that transfers stress signals to long distances under stress conditions (Cuypers et al., 2016; Sies, 2017). Therefore, the increased level of H$_2$O$_2$ observed in TL at 1 hour may either be attributable to increased production in TL itself or to transport of H$_2$O$_2$ from roots and Sh tissues.

A significant increase in SOD, which is responsible for dismutation of superoxide radicals to H$_2$O$_2$ might have also contributed to H$_2$O$_2$ accumulation (Culotta et al., 2006). Tissue-specific responsive nature of B. juncea was also evident through variable increases in NADPH oxidase and AO on different time points (Fig. 4). NADPH oxidase enzyme has also been suggested as a source of extracellular H$_2$O$_2$ generation (Qin et al., 2004; Steinite et al., 2004). NADPH oxidase uses cytoplasmic NADPH to transfer an electron to molecular O$_2$ to form superoxide radicals, which are then dismutated to H$_2$O$_2$ (Yang et al., 2007). In this study, NADPH oxidase, as well as SOD activities, were significantly increased in UR, and these two might have acted in a concerted manner to enhance H$_2$O$_2$ production. AO catalyzes the reduction of oxygen to water with the consumption of ascorbate. This affects ascorbate pool of cells and affects H$_2$O$_2$ quenching through the ascorbate-glutathione cycle (De Tullio et al., 2013). The activity of AO was also significantly increased in all tissues on all-time points; specific mention to be made of LR at 1 h. Hence, various enzymatic responses and H$_2$O$_2$ accumulation visualized at 1 h in TL indicate that plants induced H$_2$O$_2$ production through concerted changes in response to As stress. This appeared to be a signaling mechanism of plants for tolerance to As stress. It has also been proposed that AO induced ascorbate oxidation and consequent accumulation of dehydroascorbate plays a role in the signaling of stress (De Tullio et al., 2013). An active involvement of SOD, NADPH oxidase, and AO in As stress responses have been demonstrated in Hydrilla verticillata (Srivastava et al., 2007, 2011). Besides, the role of a pro-oxidant enzyme, glycolate oxidase, which is involved in increased production of H$_2$O$_2$, has been shown to play a crucial role in As stress responses of Arabidopsis thaliana (Gupta et al., 2013). Hence, with the beginning of As infiltration in roots, plants regulate both pro-oxidant and antioxidant enzymes so as to finely regulate H$_2$O$_2$ levels for the purpose of signaling the stress to distant tissues (TL).

In conclusion, the present study shows that spatial and temporal profiling of As in Brassica provides new insights into As accumulation. The data on the differential pattern overtime points and different tissues indicate towards the identification of important early check-points. Expression profiling of transporter genes showed a good correlation with As accumulation and tissue distribution.

References


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