Exogenously applied nitrate improves the photosynthetic performance and nitrogen metabolism in tomato (*Solanum lycopersicum* L. cv Pusa Rohini) under arsenic (V) toxicity

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Abstract Tomato (*Solanum lycopersicum* L.) being a widespread and most commonly consumed vegetable all over the world has an important economic value for its producers and related food industries. It is a serious matter of concern as its production is affected by arsenic present in soil. So, the present study, investigated the toxicity of As(V) on photosynthetic performance along with nitrogen metabolism and its alleviation by exogenous application of nitrate. Plants were grown under natural conditions using soil spiked with 25 mg and 20 mM, As(V) and nitrate, respectively. Our results revealed that plant growth indices, photosynthetic pigments, and other major photosynthetic parameters like net photosynthetic rate and maximum quantum efficiency (F_v/F_m) of photosystem II (PSII) were significantly ($P \leq 0.05$) reduced under As(V) stress. However, nitrate application significantly ($P \leq 0.05$) alleviated As(V) toxicity by improving the aforesaid plant responses and also restored the abnormal shape of guard cells. Nitrogen metabolism was assessed by studying the key nitrogen-metabolic enzymes. Exogenous nitrate revamped nitrogen metabolism through a major impact on activities of NR, NiR, GS and GOGAT enzymes and also enhanced the total nitrogen and NO content while malondialdehyde content, and membrane electrolytic leakage were remarkably reduced. Our study suggested that exogenous nitrate application could be considered as a cost effective approach in ameliorating As(V) toxicity.

Keywords Nitrate reductase · Nitrite reductase · Photosynthetic quantum yield · *Solanum lycopersicum* L. · Scanning electron microscope · Nitric oxide

Abbreviations

As(V)	Arsenic
MDA	Malondialdehyde
A	Net photosynthetic rate
AAS	Atomic absorption Spectroscopy
SEM	Scanning electron microscopy
NO	Nitric oxide

Introduction

A wide variety of heavy metals are released into the environment by unprecedented anthropogenic activities like agricultural practices, industrialization, urbanization and mining activities. In the recent decades, there has been a massive As(V) accumulation in the environment through series of consequences such as burning of coal, industrial metal smelting, mining, semiconductor manufacturing, fertilizers, pesticides and sewage (Rubinos et al. 2010; Marmiroli et al. 2014).

Arsenic, a metalloid is a lethal poison as its toxicity has been already proven on plants and animals and its low level exposure can cause cancer (Mandal and Suzuki 2002). In the environment, arsenic is found in both organic and inorganic forms where later forms are more toxic. Inorganic forms include arsenate [As(V)] and arsenite [As(III)] where As(V) taken up by plants *via* phosphate transporter is first reduced into As(III) that got chelated or effluxed out of the cell (Kumar et al. 2015) and/or poses toxicity to several metabolic processes by generating reactive oxygen species (ROS) like superoxide (O_2^-) radicals, hydroxyl

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(OH[−]) ions and hydrogen peroxide (H₂O₂). Reactive oxygen species cause irreparable cellular damage to nucleic acids, amino acids, and lipids, and thus leads to severe metabolic dysfunction (Gill and Tuteja 2010; Finnegan and Chen 2012). The toxicity occurred either through the generation of ROS or its direct interferences in many vital metabolic processes like photosynthesis, transpiration etc., and disruption of chloroplast membrane resulting in inhibition of photosynthetic activity (Li et al. 2006; Mascher et al. 2002), and decrease in soluble protein content (Stoeva et al. 2005).

It is evident that photosynthesis and N metabolism are interdependent and work in unison. The reduction in photosynthetic performance under As(V) toxicity is attributed to decrease in maximal velocity of Rubisco carboxylation that is reversed by nitrogen availability. It is reported that photosynthesis improves under nitrogen availability as efficient N-metabolism helps in channelizing photosynthates into organic-nitrogen molecules and prevents feedback inhibition of photosynthesis (Ripullone et al. 2003). Nitric oxide (NO), also regulates broad-spectrum functions during normal and abnormal conditions (Guo et al. 2014) and involved in the cross-talk of many traditional plant growth regulators and also plays a crucial role in defence mechanism of a plant. The production of NO largely depends on efficiency of nitrogen metabolism while effects could be either beneficial or harmful depending on its concentration (Rinalducci et al. 2008).

Tomato is a major vegetable crop, belonging to family Solanaceae (2n = 24) and occupies an outstanding place among vegetables for its lycopene richness and anti-oxidant properties. In recent decades, tomato production is drastically affected by As(V) contamination in soil. The effect of several nutrients present in soil have been tested in tomato (Carbonell et al. 2008) but little has been reported on nitrate assisted abatement of As(V) toxicity. Hence, present investigation was undertaken to study how exogenous nitrate and endogenous NO improves the photosynthetic performance and Nitrogen metabolism in Tomato (*Solanum lycopersicum* L. cv Pusa Rohini) under As(V) toxicity.

Materials and methods

Layout and design of the experiment

Seeds of tomato (*Solanum lycopersicum* L. cv Pusa Rohini) were obtained from National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute (IARI), New Delhi. Sodium hypochlorite (1 %) solution was used for sterilization of seeds and subsequently, seeds were sown in 12 plastic pots containing soilrite for growth

in open field conditions. Exposure of As(V) was in the form of disodium hydrogen arsenate (Na₂HAsO₄·7H₂O), nitrate was given as potassium nitrate (KNO₃). Only one concentration of As(V) and nitrate (25 mg and 20 mM, respectively) was selected for the experiment. All the four combinations comprising [Control, As(V), Control + Nitrate and As(V) + Nitrate] were prepared in triplicate manner and plants were grown for six weeks to assess the various growth and physiological parameters.

Determination of plant growth indices, gas exchange attributes and chlorophyll fluorescence

Impact of As(V) and nitrate were assessed by estimating changes in the morphological (root and shoot length, fresh and dry weight), and physiological (protein content, photosynthetic pigments, gaseous exchange, chlorophyll fluorescence, lipid peroxidation and electrolytic leakage) parameters. The plant material was thoroughly washed with distilled water up to four times and fresh weight determined. This was subsequently dried in an oven at 50–55 °C till weight loss became constant for estimating dry weight. The soluble protein content was estimated by Bradford (1976) assay. Chlorophyll extraction was done using the method of Hiscox and Israelstam (1979), where 100 mg of fresh leaves homogenized in 5 ml of 100 % DMSO. After centrifugation (10 min at 7500×g), absorbance of supernatant was taken at 452.5, 645 and 663 nm using UV–visible spectrophotometer (Beckman Coulter-DU 730).

Gaseous exchange analysis were performed by taking plant responses in terms of A, E and G_{H₂O} on fully expanded young leaves by using an portable open system Infra Red Gas Analyzer (IRGA, Gas Exchange Fluorescence System, GFS-3000) between 9.00 am and 12.00 noon. Before taking final measurements, instrument was calibrated for leaf surface area as 3.00 cm², standard ambient CO₂ concentration (C_a) as 398 ppm, flow rate as 750 μmol sec^{−1}, photosynthetic active radiations (PAR) as 500 μmol m^{−2} s^{−1}, impeller at 7 and relative humidity inside the cuvette as 35 %. Chlorophyll fluorescence was observed on leaves after 30 min of dark adaptation for determination of ratio F_v/F_m which indicates optimal photochemical efficiency of PS II where F_m is maximum fluorescence using a saturating light flash and F_v is variable fluorescence.

Arsenic quantification in plant

Dried and powdered plant tissue (150 mg each of roots and shoots) were digested in acid solution of HNO₃:H₂O₂ (3:1, v/v) using microwave digester (CEM-MDS 2000 Microwave digester) till the volume reached upto 3 ml (APHA

2005). The digested metalloid solution diluted up to a final volume of 50 ml by gradual addition of distilled water and finally, As(V) concentration was quantitatively analysed using atomic absorption spectrometer (Perkin-Elmer; Analyst 600) enabled with graphite furnace and translocation factor (TF) using the formula “metal concentration in shoots/metal concentration in roots”.

Determination of electrolytic leakage

Electrolyte leakage (%) was measured following the method of (Mobin and Khan 2007). Plant leaves were cut and washed with distilled water to remove the adhered electrolytes and kept in boiling tubes containing 10 ml of double distilled water and incubated on a shaker at 25 °C for 6 h for measuring electrical conductivity (C_1). Samples were again kept at 90 °C for 2 h for measuring electrical conductivity (C_2) using the conductivity meter. Finally, percent electrolyte leakage was calculated using the formula:

$$\text{Electrolytic leakage (\%)} = \frac{C_1}{C_2} \times 100$$

Determination of lipid peroxidation

Degree of lipid peroxidation was measured by determining malondialdehyde (MDA) content using thio barbituric acid (TBA) as described by Heath and Packer(1968). Briefly, 1.0 gm fresh leaf sample from each treatment was homogenized and centrifuged at 19,000×g for 20 min. The obtained supernatant was mixed with 2.5 mL 20 % TCA containing 0.5 %TBA and further heated in boiling water bath for 30 min following its rapid cooling in ice bath. The supernatant was collected and again centrifuged at 10,000×g for 10 min and finally the resulting supernatant was used for determining the MDA content at 532 nm using molar extinction coefficient ($\epsilon = 155 \text{ mM}^{-1} \text{ cm}^{-1}$).

Analysis of nitrogen-metabolic enzyme activities

Nitrate reductase (NR)

Leaf tissues (100 mg) were grounded in chilled phosphate buffer (1 ml) having pH 7.5 and centrifuged at 12000×g for 10 min at 4 °C. Enzyme activity was determined from reaction mixture comprising of 0.1 M potassium phosphate (pH 7.5), 10 µM KNO₃, 0.27 µM DPNH (reduced diphosphopyridine nucleotide) and a suitable aliquot of enzyme extract. The entire aforesaid reaction ingredients were kept for 30 min at room temperature following termination of reaction by addition of 50 µL of

zinc acetate. The entire mixture was again centrifuged at 12000×g for 10 min and the supernatant was used for determining NR activity using a freshly prepared mixture solution containing both 1 % (w/v) sulfanilamide in 3 N HCl and 0.01 % (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride (NED). The absorbance of pink colour was recorded at 540 nm and nitrite content was determined using a standard of KNO₂ (Sanderson and Cocking 1964). The NR activity was expressed in units, one unit is defined as the amount of enzyme catalyzing the formation of 1 µmol NO₂[−] h^{−1} g^{−1} of fresh weight.

Nitrite reductase (NiR)

The activity of NiR was estimated following NADPH-dependent nitrite reductase method with minor modifications (Sengupta et al. 1996). The oxidation of coenzyme NADPH was determined at 340 nm by measuring the decrease in absorbance of reaction mixture containing 50 mM Tris–HCl buffer (pH 7.5), 10 mM NADPH, 1 mM KNO₂, 1 mM FAD and enzyme extract. The activity was expressed in units, one unit is defined as the amount of enzyme catalyzing the reduction of 1 µmol NO₂[−] h^{−1} g^{−1} of fresh weight and disappearance of NADPH and the estimation of nitrite was obtained by using a KNO₂ standard.

Glutamine synthetase (GS)

The activity of GS was measured using the method of O’Neal and Joy (1973) which measures the amount of γ-glutamyl hydroxamate. The standard assay mixture (1 mL) consisted of 100 mM Tris–HCl buffer (pH 7.9), 10 mmol glutamine, 60 mM hydroxylamine hydrochloride, 50 µM ATP, 20 µM disodium hydrogen arsenate, 1 µM MgCl₂, and 0.1 mL enzyme extract for transferase activities of GS. The reaction was initiated by the addition of glutamine as substrate following incubation at 37 °C for 30 min. The γ-glutamyl hydroxamate produced was determined by adding 2 mL of FeCl₃ reagent (equal volumes of 0.57 M FeCl₃, 1 M HCl, 0.5 M trichloroacetic acid) and taking absorbance at 540 nm. The activity was expressed in units, one unit is defined as the amount of enzyme catalyzing the formation of 1 µmol γ-glutamyl hydroxamate min^{−1} g^{−1} of fresh weight.

Glutamate synthase (GOGAT)

The reaction buffer for determining the GOGAT activity (Glutamate:oxoglutarate aminotransferase, E.C. 1.4.1.14) comprised of 5 mM α-oxoglutaric acid, 10 mM glutamine and 0.15 mM NADH in 50 mM phosphate buffer (pH 7.5). The reaction was initiated by adding 0.1 mL of enzyme

extract and finally oxidation of NADH was estimated by reading the absorbance at 340 nm (Rachina and Nicholas 1985). The results were expressed in units, one unit is defined as amount of enzyme catalyzing the oxidation of 1 μmol NADH $\text{min}^{-1} \text{g}^{-1}$ of fresh weight.

Analysis of structural changes in guard cells using scanning electron microscope

The control and treated leaves trimmed into sections of about 0.5 mm, transferred in 2.5 % glutaraldehyde and 2 % p-formaldehyde fixative prepared in 0.1 M phosphate buffer (pH 7.0). Subsequently, samples were dehydrated in graded ethanol series: 30 min each in 25, 50, 75 and 100 % ethanol and after critical point drying, sections were mounted on carbon stubs (Yuan et al. 2012). Afterwards, each sample was coated to a layer of gold–palladium, in a rotating vacuum coating apparatus and finally analyzed for structural changes in guard cells using a scanning electron microscope (JEOL- JSM-6610LV).

Nitrogen and Nitric oxide content estimation in leaves

Nitrogen content in leaves was estimated according to the method of Lindner (1944). 10 mL aliquot of the digested material (by H_2SO_4) was mixed with 2.5 N sodium hydroxide and 1 mL 10 % sodium silicate solutions to neutralize excess acid and to check turbidity, respectively. 5 mL aliquot of this solution was added with 0.5 mL Nessler's reagent and allowed to stand for 5 min so as to can develop maximum colour. The absorbance (520 nm) of each sample was compared with the standard curve of ammonium sulphate and finally, nitrogen was calculated as percent dry weight.

For the measurement of NO, leaf samples (1 g) of control and treated plant were extracted in 100 mM sodium phosphate buffer (pH7) in pre-chilled mortar and pestle, following centrifugation at 12,500 g for 25 min (4°C). The

NO concentration was determined using NO measuring system (inNO, Innovative instruments) having amiNO-700 sensor (Ciszewski and Milczarek 2003).

Statistical analysis

Results are prepared as mean $\pm$ SD of three independent experiments having three replicates each and the level of significant differences between the results was tested by one-way ANOVA followed by Duncan's multiple range test. Comparison between control and means of treatments were done by Duncan's test at significance level $P \leq 0.05$.

Results and discussion

Physiological responses of plant under As(V) toxicity and nitrate supplementation

Exposure of tomato plants to As(V) reduced important growth parameters such as root length, shoot length, fresh biomass and protein content by 60, 33, 57 and 53 % respectively. Toxicity effect of As(V) was more pronounced on root than shoot because of As(V) sequestration mainly in root's vacuoles resulting in stunted growth while reduction in biomass is probably due to water loss and decreased water uptake (Li et al. 2006). Also, protein degradation occurs because of carbohydrates deficiency, an adaptation of cells which resulted in the reduction of soluble protein content (Journet et al. 1986).

Addition of nitrate was observed to impart ameliorating effects on toxicity by a notable improvement in all the aforesaid growth indices (Table 1). This might be due to the low extent of As(V) accumulation in root and shoot as nitrate being a nutrient got preference over As(V). Also, nitrate helps in increasing cell wall thickness of root epidermis which checks uptake of As(V) in roots and its further translocation to shoots (Probst et al. 2009).

Table 1 Different morphological and physiological responses of *Solanum lycopersicum* L. under As(V) stress and exogenous nitrate application

Parameters	Treatments			
	Control	As(V)	Control + nitrate	As(V) + nitrate
Fresh weight (g)	12.81 $\pm$ 0.453 ^d	5.55 $\pm$ 0.091 ^a	11.26 $\pm$ 0.749 ^c	7.78 $\pm$ 0.302 ^b
Dry weight (g)	1.23 $\pm$ 0.046 ^d	0.53 $\pm$ 0.01 ^a	1.02 $\pm$ 0.068 ^c	0.75 $\pm$ 0.025 ^b
Shoot length (cm.)	46.4 $\pm$ 2.706 ^b	30.93 $\pm$ 3.495 ^a	35.87 $\pm$ 3.873 ^a	35.83 $\pm$ 3.153 ^a
Root length (cm.)	12.6 $\pm$ 3.6 ^b	5.0 $\pm$ 1.353 ^a	12.0 $\pm$ 0.361 ^b	7.67 $\pm$ 0.351 ^a
Protein (mg g ⁻¹ FW)	5.296 $\pm$ 0.020 ^c	2.495 $\pm$ 0.028 ^a	7.379 $\pm$ 0.044 ^d	2.989 $\pm$ 0.045 ^b
Electrolytic leakage (%)	11.41 $\pm$ 1.189 ^a	75.88 $\pm$ 0.673 ^d	17.6 $\pm$ 0.749 ^b	45.57 $\pm$ 3.121 ^c
MDA (μmol g ⁻¹ FW)	0.020 $\pm$ 0.0009 ^a	0.278 $\pm$ 0.0026 ^c	0.020 $\pm$ 0.0009 ^a	0.248 $\pm$ 0.0029 ^b

Data are mean $\pm$ SD of three independent experiments; one way ANOVA was run to check the significance ($P \leq 0.05$) and different lower case letters indicate significant differences as per Duncan's test

Table 2 Quantitative estimation of photosynthetic pigments under As(V) toxicity and their alleviation by exogenous nitrate

Parameters	Treatments			
	Control	As(V)	Control + nitrate	As(V) + nitrate
Chlorophyll <i>a</i> (mg g ⁻¹ FW)	1.90 ± 0.011 ^c	0.72 ± 0.007 ^a	1.91 ± 0.010 ^d	0.98 ± 0.007 ^b
Chlorophyll <i>b</i> (mg g ⁻¹ FW)	0.92 ± 0.019 ^c	0.23 ± 0.016 ^b	0.90 ± 0.015 ^c	0.08 ± 0.014 ^a
Total Chlorophyll (mg g ⁻¹ FW)	2.81 ± 0.008 ^c	0.95 ± 0.018 ^a	2.81 ± 0.005 ^c	1.05 ± 0.020 ^b
Carotenoids (mg g ⁻¹ FW)	3.92 ± 0.015 ^c	1.39 ± 0.027 ^a	3.93 ± 0.021 ^c	1.71 ± 0.020 ^b

Data are mean ± SD of three independent experiments; one way ANOVA was run to check the significance ($P \leq 0.05$) and different lower case letters indicate significant differences as per Duncan's test

Effects on photosynthetic pigments

Plants showed decline in photosynthetic pigments (Chlorophyll *a*, Chlorophyll *b*, Total Chlorophyll or Chl-T and Carotenoids) upon As(V) exposure; significant ($P \leq 0.05$) reduction in Chl-*a* (62 %), Chl-*b* (75 %), Chl-T (66 %), and carotenoids (64 %), respectively was observed as compared to control (Table 2). This reflects hampering of photosynthetic machinery, possibly due to peroxidation of chloroplast, alteration in membrane ultra structure, or interference with dark and light reaction of photosynthesis (Probst et al. 2009).

Application of 20 mM nitrate along with As(V) reduced the decline of photosynthetic pigments (Table 2). It is possibly due to up-regulation of genes for δ -amino levulinic acid dehydratase entailed in chlorophyll biosynthesis (Cornah et al. 2003).

Effects on gaseous exchange and chlorophyll fluorescence

The gaseous exchange parameters (A, E, G_{H_2O}) and chlorophyll fluorescence were examined for validating the photosynthetic behaviour of plants. Exposure of plants to As(V) notably declined net assimilation rate (A) by 30 % (8.0 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) and stomatal conductance (G_{H_2O})

by 36 % (333.80 $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), while increased transpiration rate (E) by 28 % as compared to control. The results indicate that As(V) primarily targets photosynthesis through several ways like inhibition of chlorophyll biosynthesis and alternation in the shape of stomata manifested in its rounding and shortening of its longitudinal axis.

Chlorophyll fluorescence study provides a clear understanding of leaf photosynthesis efficiency in terms of F_v/F_m ratio and a decline in this ratio provides an indication of damage to photosynthetic machinery and subsequent decrease in the yield of the plant. A significant ($P \leq 0.05$) reduction in maximum quantum yield and F_v/F_m was observed up to 13 and 12 % respectively, as a consequence of As(V) (Table 3). Arsenic affects PSII efficiency by inducing ROS production that disturbs cellular redox state and causes lipid peroxidation (Sharma and Dietz 2008). On the other hand, nitrate increased photosynthetic pigments and minimized ROS production (Iqbal et al. 2015) which further boosts A, G_{H_2O} , F_v/F_m and ΦPSII . It might be possible that nitrate up-regulates anti-oxidative genes which stimulates the production of anti-oxidative enzymes, thereby improving PSII efficiency. Nitrate also up regulates the overall nitrogen metabolism and provides uninterrupted nitrogen for chlorophyll biosynthesis along with other photosynthetic enzymes like, Rubisco.

Table 3 Effect of As(V) and exogenous nitrate on gaseous exchange (A- net photosynthetic rate, E-transpiration rate, G_{H_2O} -stomatal conductance) and Chlorophyll fluorescence (F_v/F_m - maximum quantum yield of PSII, qP- photochemical quench, qN- non-photochemical fluorescence quench, NPQ- maximal variable fluorescence) parameters in *Solanum lycopersicum* L

Parameters	Treatments			
	Control	As(V)	Control + nitrate	As(V) + nitrate
A ($\mu\text{mol m}^{-2} \text{ s}^{-1}$)	11.43 ± 0.002 ^b	8.00 ± 0.774 ^a	18.27 ± 0.008 ^c	11.28 ± 0.011 ^b
E ($\text{mmol m}^{-2} \text{ s}^{-1}$)	0.73 ± 0.001 ^b	0.94 ± 0.050 ^c	0.13 ± 0.009 ^a	0.70 ± 0.003 ^b
G_{H_2O} ($\text{mmol m}^{-2} \text{ s}^{-1}$)	522.68 ± 0.077 ^d	333.80 ± 1.299 ^a	504.35 ± 0.311 ^c	421.09 ± 0.162 ^b
F_v/F_m	0.75 ± 0.0006 ^c	0.66 ± 0.0005 ^a	0.81 ± 0.0002 ^d	0.73 ± 0.0003 ^b
Yield	0.75 ± 0.003 ^c	0.65 ± 0.0009 ^a	0.81 ± 0.002 ^d	0.73 ± 0.007 ^b
qP	0.98 ± 0.011 ^a	0.99 ± 0.005 ^a	1.03 ± 0.002 ^c	1.01 ± 0.009 ^b
qN	0.006 ± 0.001 ^a	0.007 ± 0.002 ^a	0.031 ± 0.002 ^b	0.008 ± 0.002 ^a
NPQ	0.005 ± 0.0008 ^b	0.006 ± 0.0007 ^b	0.002 ± 0.0008 ^a	0.005 ± 0.002 ^b

Data are mean ± SD of three independent experiments; one way ANOVA was run to check the significance ($P \leq 0.05$) and different lower case letters indicate significant differences as per Duncan's test

Arsenic accumulation in *Solanum lycopersicum* L

The accumulation of arsenic was analyzed in root and shoot along with translocation factor (TF). As(V) accumulation in shoot was found to be 168.60 mg/kg DW in case of As(V) and increased to 212.7 mg/kg DW when nitrate was added. This was probably due to the enhanced synthesis of metallothionein-like proteins that may further bind simultaneously to nitrate ions and As(V) and facilitate their translocation (Abercrombie et al. 2008). On the other hand, As(V) concentration in root was found to be 317.02 mg/kg DW and declined to 268.23 mg/kg DW when nitrate was added because of preferential absorption of nitrate ions over As(V) ions. Differential accumulation in root and shoot may also be due to translocation hindrance and distinctive biosynthesis of phytochelatins (Barabasz et al. 2010). Translocation factor outlines the translocation efficiency of As(V) (from root to shoot) in plants. A significant increase of 49 % was noticed in TF (0.79) after nitrate application compared to the plants without nitrate (0.53). The increase in TF was probably due to the involvement of nitrate specific transporters in As(V) transport or nitrate mediated upregulation of As(V) specific Lsi2 transporters (Ma et al. 2008) (Table 4). Based on the TF value, *Solanum lycopersicum* L can be placed under a non accumulator plant.

Oxidative stress: MDA and electrolytic leakage

The oxidative stress was calculated in terms of increase in the levels of malondialdehyde (MDA), a cytotoxic aldehyde product of lipid peroxidation and electrolytic leakage. Significant ($P \leq 0.05$) increase of 39 % in MDA content and 64 % in electrolytic leakage was observed upon As(V) exposure; a marked elevation indicating ample ROS generation (Table 1). Excessive production of superoxide radicals following lipid peroxidation and electrolytic leakage; may further potentially damage photosynthetic pigment apparatus. Addition of nitrate to plants lowered As(V) induced lipid peroxidation by 11 % (in terms of MDA content) and electrolytic leakage by 30 %. It could be due to nitrate facilitate the enhanced NR-dependent NO biosynthesis which further exerts its protective role against

As(V) toxicity by minimizing electrolytic leakage and lipid peroxidation (Talukdar 2013).

Ultra structural changes in guard cells analysed by scanning electron microscope

Leaf surface scanning electron micrographs reveal that As(V) induced stomatal closure along with abnormal shrinkage of guard and epidermal cells (Fig. 1a–d). The results confirmed the negative effect of As(V) on the morphological and physiological parameters of epidermal cells of tomato. However, exogenously applied nitrate resulted in opening of stomata along with restoration of proper shape of guard and epidermal cells. Its impact on stomatal movement attained due to reduction in the production of Absciscic acid (ABA) which triggers closing of stomata for water conservation (Desikan et al. 2002).

Effects on nitrogen metabolism

Nitrate reductase and nitrite reductase

The performance of nitrogen metabolism was assessed by estimating the activities of some of the key enzymes encompassing NR, NiR, GS, and GOGAT along with total nitrogen content in leaves. The nitrogen metabolism depends on nitrate availability and has great implication on the functionality of photosynthesis and NO signalling.

Plant responded to a decrease in activities of NR and NiR by 42 and 63 % upon As(V) exposure (Fig. 2a–b) either due to lesser availability of nitrate in cells or damage in active sites of enzymes by As(V). However, exogenous nitrate improved nitrate availability thereby enhancing NiR activity by 16 %; less enhancement than NR activity (24 %) because nitrate availability directly affects NR activity.

Glutamine synthetase and glutamate synthase

Biochemical, genetic and molecular evidences have shown that the major pathway for assimilation of ammonium ion into organic molecules/amino acids is the GS/GOGAT cycle (glutamate synthase cycle), which involves the

Table 4 Accumulation of As(V) in root and shoot of *Solanum lycopersicum* L. and its translocation efficiency under exogenous nitrate application

Parameters	Treatments			
	Control	As(V)	Control + nitrate	As(V) + nitrate
Root (mg kg ⁻¹ DW)	ND	317.02 ± 2.803 ^c	ND	268.23 ± 2.686 ^b
Shoot (mg kg ⁻¹ DW)	ND	168.60 ± 2.819 ^b	ND	212.7 ± 2.364 ^c
Translocation Factor (TF)	ND	0.53 ± 0.008 ^b	ND	0.79 ± 0.008 ^c

Data are mean ± SD of three independent experiments; one way ANOVA was run to check the significance ($P \leq 0.05$) and different lower case letters indicate significant differences as per Duncan's test

Fig. 1 Scanning electron micrographs of abaxial leaf surface (focusing stomata) of *Solanum lycopersicum* L. under As(V) treatment and exogenous nitrate application

sequential actions of both GS and GOGAT enzymes (Stitt et al. 2002).

The activity of Glutamine synthetase (GS) under As(V) stress decreased by 56 % but decreased only by 40 % when nitrate is given and GS activity enhanced by 27 % under exogenous nitrate application in absence of As(V).

Similarly, the activity of Glutamate synthase (GOGAT) under As(V) stress decreased by 69 % and decreased by 64 % when nitrate is given and GOGAT activity enhanced by 19 % under exogenous nitrate application in absence of As(V) which clearly shows that nitrate has a major impact on GS as compared to GOGAT activity in control while its reverse pattern is true in presence of As(V) (Fig. 2c–d).

Nitrogen and nitric oxide content

Application of 20 mM nitrate was effective in increasing the total nitrogen content of the leaves in both absence and presence of As(V). Leaf nitrogen content was found to be 7.941 % in control and 9.983 % in control treated with nitrate showing an increment of 2.042 % in nitrogen content whereas it was 5.691 % in As(V) treated plants and 6.65 % in As(V) treated with nitrate showing a 0.959 % increase in leaf nitrogen content (Fig. 3). The total leaf nitrogen content is also reflected in the increased nitrogen metabolism which is strongly supported by the activity pattern of major enzymes like NR, NiR, GS and GOGAT.

Exogenous application of nitrate also triggered NO content in leaves of tomato. Nitric oxide content in control set was recorded 132.33 nM while 182.53 nM in control treated with nitrate indicating 27.5 % increase in NO content. On the other hand, NO content in As(V) treated plant was 177.7 nM while it was found to be 207.4 nM in plants treated with nitrate and As(V) both, indicating 14.32 % increment in NO content. The present finding is in conformity to Stohr and Stremlau (2005) reporting that enzymatic and non-enzymatic reduction of NO_3^- leads to NO synthesis and its further contribution towards alleviating As(V) toxicity in plants. It has been clearly established earlier that NO acts as a key molecule in the resistant response of plants against heavy metals, including As(V). The alleviation effect of NO might be due to the improvement in photosynthesis by increasing chlorophyll content, counteracted oxidative damage by decreasing the production of ROS and increased the antioxidants synthesis as well as uptake of beneficial nutrient elements (Dong et al. 2014; Singh et al. 2016).

Conclusion

Toxicity caused by As(V) is compensated primarily by up-regulation of photosynthetic attributes and key nitrogen metabolizing enzymes like NR, NiR, GS and GOGAT by

Fig. 2 Effect of exogenous nitrate on **a** nitrate reductase, **b** nitrite reductase, **c** glutamine synthetase (GS), **d** glutamate synthase (GOGAT), against As(V) in *Solanum lycopersicum* L. Data are mean $\pm$ SD of three independent experiments and one way ANOVA was run to check the significance at $P < 0.05$. The lower line indicates As(V) and upper line indicates As(V) + NO_3^-

Fig. 3 Nitrogen (%) and nitric oxide (nM) content in leaves of tomato. Data are mean $\pm$ SD of three independent experiments; one way ANOVA was run to check the significance ($P \leq 0.05$) and different lower case letters indicate significant differences

exogenous nitrate which might be due to enhanced synthesis of NO. Nitric oxide is an important bioactive signalling molecule which plays a crucial role in combating

abiotic and biotic stress and also activates the various defense pathways in plants. Present findings lay a ground for future work aimed at studying application of exogenous nitrate and stress indicators in coping the stress produced by As(V) in tomato and will provide further insight into metal-nitrate interactions in natural environments which will help in elucidation of N nutrition and metabolism-related alterations.

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