International Journal of Advanced Research and Development
ISSN: 2455-4030, Impact Factor: RJIF 5.24
www.advancedjournal.com
Volume 2; Issue 4; July 2017; Page No. 341-344

Arsenic effect on morphology and rate limiting step of Nitrate assimilation pathway of *Phaseolus vulgaris*

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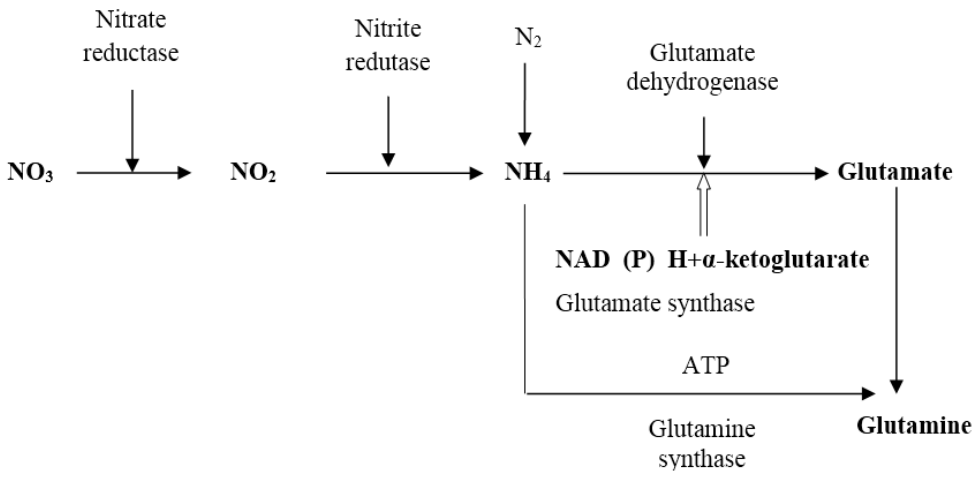
Abstract
Arsenic is one of the oldest and most toxic heavy metal in the earth's crust and biosphere. As exists in the environment in various organic and inorganic forms. Due to greater cellular uptake, inorganic As^{+3} is 60 times more soluble, mobile and toxic form then As^{+5}. In plants, As is accumulated according to tolerance. In general, with increasing As_{soil}, there was a concomitant increase in As_{plant}, results in growth inhibition, low fruit yield, physiological disorders and finally their death. Growth inhibition is one of the important manifestations of As-induced toxicity in plants. Most significant percent germination as well as seed germination retardation pronounced in 1mM when sand was treated with 0.001, 0.01, 0.1 and 1mM concentrations. Highest Nitrate activity and endogenous pool activity found more significant at 0.01mM. The effect of As on chlorophyll was increased with increased concentrations. Total chlorophyll concentration was inhibited ranged from 42.45% to 86.98%. Therefore the present study will definitely help in assessing/evaluating the effect of As on overall productivity of important crops and detoxification of As by phytochelatin synthesis in As enriched soil.

Keywords: arsenic, tolerance, seed germination, nitrate activity, endogenous pool

1. Introduction
Soil and water contamination with heavy metals has become a worldwide problem leading to losses in agricultural crops yield and causes hazardous health effects to human population [1, 3]. Arsenic (As) is one of the oldest and most toxic heavy metal [4] exists in various organic and inorganic forms. The most important inorganic species are arsenite (As^{+3}) and arsenate (As^{+5}). Its toxicity to plants depends on its valence state. Due to greater cellular uptake, As^{+3} is 60 times more soluble, mobile and toxic than As^{+5}. Bioavailability of As to plant is governed by biological, chemical and physical processes and their interactions altering metal speciation and behavior in soil-plant systems [5]. In general, with increasing As_{soil}, there was a concomitant increase in As plant, results in growth inhibition, low fruit yield, physiological disorders [6] and finally their death [7]. In plants, Arsenic is accumulated according to tolerance and enters into the food chain and bioaccumulation [8].

Nitrate reductase (NR, E.C 1.6.6.1), the rate-limiting enzyme in nitrogen assimilation [9], is well known to be sensitive to metal stress [10, 12]. It is a complex enzyme that has been characterized as sulfahydryl (-SH) group containing molybdo flavor haemo protein [13]. Sulfhydryl groups are required for NADH (nicotinamide adenine dinucleotide, reduced form) binding and catalytic activity of NR [14], As might affect the NR activity by binding to functional –SH groups present in the active sites of this enzyme [15, 19]. Supply of inorganic As inhibited substantially in vivo as well as in vitro nitrate reductase activity and endogenous nitrate pool in excised bean leaf segments. According to Singh *et al.* [20] NR and nitrite reductase activity decreased in *pteris ensiformis* in the presence of As.



Sharma and Subhadra [21].

Phytochelatins (PCs) in plants are heavy metal-inducible, heavy metal-binding, cysteine rich polypeptides have a general structure [(γ-Glu–Cys)n–Gly) (n = 2–11) and synthesized non-translationally by PC synthase [17]. For the detoxification purpose, As^{+3} must be compartmentalized in vacuoles that may be achieved by shuttling from the cytoplasm, probably as As-PC complexes.

According to Marchetti [22] in phyto- (green plant based technology)-remediation plants are exploited as bio-pumps that use the sun's energy to remove water and contaminants from the soil to the above-ground portions or to immobilize pollutants. As effect on morphological and physiological level of *P. vulgaris* (Rajmah, an important legume crop and a great source of nutrition to millions of people) has not been studied yet. The objectives of the present study were: (1) to evaluate the intra-specific comparisons for As tolerance at the seed germination stage (including shoot, root length) which is considered more vulnerable to any stress than latter growth stages of most plant species (2) to evaluate the effects of As on nitrate activity (in vivo) (3) to calculate the effect on chlorophyll. Study should help to determined resistance to As contamination.

2. Material and methods

- River sand was washed and sterilized properly. 1 kg sand transferred into each pot and sand of these pots were treated with different concentrations (0, 0.001, 0.01, 0.1 and 1 mM) of Sodium Arsenite. The pot contained 0mM $NaAsO_2$ were served as a control.
- Seeds of *Phaseolus vulgaris* (Chitra) were surface sterilized with HgCl2 (0.1%) and 20 seed per pot were raised for 7-8 days in continuous light of 30 wm-2 intensity supplied by fluorescent tubes at 26±2 ºC. They were watered with ½ strength Hoagland's solution (pH 6.0) containing no nitrogen [23].
- Primary excised leaves of seedlings were used for analysis. 250mg leaves cut into about 0.5x0.5 cm segments and floated on 1/4th strength Hoagland's solution for 24 hrs at continuous light intensity of $40wm^{-2}$ at a temperature of 26±2ºC inside BOD.

2.1 Morphological analysis

For morphological assay, acid wash sand treated with different concentration of As for 24hrs than transfer of 20 properly sterilized seeds into each pot. After a week seedlings were harvested then root and shoot were separated for measurements. Seed germination was also counted. All values counted in three replicates of experiment [24-25].

2.2 Physiological and Biochemical assay

2.2.1 Enzymes of nitrogen assimilation pathway will be assayed as fellows

The activity of nitrate reductase in the treated material was estimated by in vivo [26] method with slight modification. About 0.25 g of leaf material were incubated with 10 ml of incubation medium consisting of 0.1 M sodium phosphate buffer (pH 7.2), 0.2 M KNO_3, and 25% isopropanol in dark vial of 20 ml capacity. The whole set was incubated in dark for 30 min at 30ºC. Nitrite released in the incubation mixture due to enzyme activity was measured by color development by the formation of diazo compound with sulfanilamide and nitrate coupled with NED to give a red dye. The absorbance was read at 540 nm. In intact seedlings nitrate reductase activity was measured only in the leaves as they are believed to be major nitrate reducing organic in most plants. Endogenous nitrate pool in the leaf segments were estimated according to Aslam [27].

2.2.2 Pigment estimation-

For chlorophyll estimation primary treated leaves were extracted with 80% acetone. The extract was centrifuged at 4000 rpm for 15 min. Absorbance was calculated at 663 and 645 nm. Total chlorophyll contained was calculated by method of Strain and Svec [28].

3. Results and discussion

Growth inhibition is one of the important manifestations of As-induced toxicity in plants [29, 32]. Significant inhibition of plant growth in the present *Phaseolus vulgaris* seedlings subjected to As treatment for 7 days was in agreement with these reports. The reduction of growth parameters were more pronounced in the roots compared to shoots. In 1mM there was only 7.53% germination found. Obviously, accumulation of As had more inhibitory effect on the roots of present *P. vulgaris* seedlings than on the shoots, agreeing well with earlier reports [31, 34]. The highest percentage of seed germination was observed in 0.01mM concentration compare to control (without treatment) then continuously decreased (Table 1).

As the concentration of $NaAsO_2$ increases there was retardation in growth period of beans after 0.01mM. There was significant increase in growth days of 0.1 and 1 mM concentration in comparison to control (without treatment). There were inhibition shown in root length and shoot length. Initially shoot and root length increases up to 0.01mM concentration but after that these retarded comparatively to control (Table 1). Heavy metal-induced structural changes in the plant parts were also reported in mung bean [31], pea [35] and in radish [36], although exact mechanism of such effect is still unknown.

The effect of As on nitrate depends on concentration or whether the experiment was in vivo or endogenous. In vivo NR the activity of enzyme were increased in 0.001 and 0.01mM. The highest significant activity found at 0.01mM (143.12%), after that activity was decreased at 0.1 and 1mM but still activity was higher at 0.1mM (117.37%) while compared to control. In endogenous nitrate pool there was a continuous inhibition found from 0.1 to 1mM concentrations. As shown significant inhibition in endogenous pool with 0.01mM and 1mM concentrations which is similar significant in in-vivo nitrate assay (Table 2). The inhibition of NR and endogenous nitrate pool activity is decreased up to 62% and 56% respectively which was rather to NR activity inhibition found in As hyper-accumulator *P. vittata* and *P. ensiformis* ranged from 10-40% and 25- 85% respectively (Singh *et al.* 2009) [20].

The effect of As on chlorophyll was increased with increased concentrations, found most significant among all above parameters (Table 2). There still plenty of unknown aspects regarding arsenic's genotoxicity, namely, the mechanistic, target and extent of its effects in plants. Plants play a major role in trophy chains and economy hence, there is need to find process about uptake mechanism of As and protect our valuable ecosystem.

Table 1: Effect of $NaAsO_2$ on germination of *P. vulgaris* seed

Concentration of $NaAsO_2$ (mM)	Germination (%)	Growth in days Mean ± SD	Shoot lengths (cm)	Root lengths (cm)
Control (without treatment)	88.35	6.67 ± 0.58	8.89 ± 2.48	5.67 ± 2.28
0.001	90.25	6.33±0.58	9.47 ± 2.07	7.06 ± 1.85
0.01	90.02	6 ± 0.01	10.12 ± 2.09	7.79 ± 2.33
0.1	71	9.33 ± 0.58**	7.28 ± 0.98	3.78 ± 1.2
1	6.65*	13.33 ± 1.53**	3.25 ± 1.94	0.75 ± 1.17

Table 2: Effect of $NaAsO_2$ on nitrate *in vivo*, endogenous pool and chlorophyll in excised leaves

Concentration of $NaAsO_2$ (mM)	Nitrate reductase inhibition (in vivo) (µ mol NO2/hr/g fresh weight) Mean ± SD	Endogenous nitrate pool (µ mol NO2/hr/g) fresh weight) Mean ± SD)	Chlorophyll (mg/g fresh weight) Mean ± SD
Control (without treatment)	44.62± 2.35	28.4 ± 2.14	14.44 ± 0.61
0.001	53.74± 3.48*	31.22 ± 2.69	12.56 ± 0.9*
0.01	63.86± 4.6**	36.36 ± 1.93**	11.16 ± 0.52*
0.1	52.58± 2.74*	28.06 ± 1.23	8.07 ± 1.17**
1	27.85± 1.9**	15.86± 1.07**	6.13 ± 1.03**

**p<0.01 and *p<0.05

4. Conclusion

The present research shows As is a critical heavy metal causing toxic effect both at morphological and physiological level. It can be concluded that inorganic arsenite is non-essential to plants and higher concentrations of posed a stress over all germination parameters observed during seed germination. Low concentrations of arsenite caused increase in shoot (113.84%) and root (137.39%) lengths of *P. vulgaris* seedlings, as compared to control. Study showing moderated determined resistance to As contamination. Therefore the present study will definitely help in assessing/evaluating the effect of As on overall productivity of important crops and detoxification by phytochelatin synthesis in As enriched soil. It help to explore the possible mechanism(s) of toxicity and detoxification of other heavy metal pollutants on nitrate assimilation in *P. vulgaris*, because this present investigation is of fundamental importance as it reveals potential aspects of As toxicity on nitrate, an enzyme of nitrogen assimilation pathway.

5. Acknowledgement

The author highly obliged to Dr (Mrs.) Jot Sharma, vice principal of Vijyaraje Institute of Science and Management, Gwalior and Dr Archana Srivastava, Director of College of Life Sciences, CHRI Campus, Gwalior, for continuous support, encouragement and providing necessary facilities.

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