

Arsenic Induced Changes in Growth and Metabolism of Black Gram Seedlings (*Vigna Mungo* L.) and the Role of Phosphate as an Ameliorating Agent

Saumya Srivastava · Yogesh Kumar Sharma

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Abstract The response of black gram (*Vigna mungo* L.) seedlings to different levels of arsenate with or without phosphate was investigated. Arsenic exposure significantly hampered the growth of black gram seedlings showing reduction in root and shoot lengths together with reduction in fresh and dry weight. The level of total chlorophyll, chlorophyll_a, chlorophyll_b and carotenoids were decreased in arsenic treated seedlings. Arsenic restricted the concentration of nucleic acids (DNA and RNA) and soluble protein content in black gram seedlings. Arsenic exposure resulted in accumulation of sugars and starch. Activity of amylase decreased in presence of arsenic. When phosphate was applied with arsenic, it caused positive changes in all studied parameters. Thus, phosphate application ameliorated to a large extent the detrimental effects caused by arsenic, and hence, proved to be a good ameliorating agent for arsenic toxicity.

Keywords Arsenate · Photosynthetic pigments · Nucleic acids · Protein · Sugars · Amylase · Phosphate · Amelioration

1 Introduction

Arsenic, a known carcinogen and a toxic metalloid, is slowly engulfing the entire world under its influence. Not only humans, but plants too are suffering from its toxic effects. There are numerous reports where arsenic exhibited toxicity symptoms and hampered normal growth, metabolism and productivity of plants. According to Miteva et al. (2005), arsenic had caused profound negative effects on root and shoot of tomato. Changes were more significant in roots than stems especially at 100 mg/kg As. However, in another case, a positive response in tomato plants was observed at lower doses of arsenic, which was reflected in increase in shoot and root length, fresh weight, and other indicators, but these parameters also showed a severe negative change at higher arsenic doses (Miteva 2002).

Among the various plants studied for arsenic phytotoxicity, Woolson (1973) showed that green and lima beans were more sensitive towards As toxicity than radish and cabbage. Mung

S. Srivastava (✉) · Y. K. Sharma
Department of Botany, University of Lucknow, Lucknow 226007 Uttar Pradesh, India
e-mail: sonata906@gmail.com

bean or bean plants have been quite extensively studied for arsenic phytotoxicity and there are many reports confirming its sensitivity towards arsenic (Carbonell-Barrachina et al. 1997; Stoeva et al. 2005; Singh et al. 2007). Bean plants have shown reduction in their shoot and root length, fresh and dry weight, and relative water content in presence of arsenic. Another crop that is often used as a model for studying arsenic toxicity is rice; this may partly be due to its sensitivity towards arsenic and partly because rice is considered to be an important staple food for the majority of the world. Many workers have reported ill effects of arsenic on rice. Shri et al. (2009) have reported that rice seedlings have shown reduction in their germination percentage, biomass and root/ shoot elongation when exposed to arsenic. They have also emphasized on different mechanism of metabolism of As(III) and As(V). Increase in arsenic doses resulted in increase in accumulation of arsenic in rice, which is reflected in reduction of height, biomass and yield (Abedin et al. 2002).

High concentrations of arsenic severely hampered the growth and metabolism of wheat. Seed germination, root and shoot length, and biomass showed a negative trend on arsenic exposure (Li et al. 2007). Arsenic uptake in wheat increased with increase in concentration of both As(III) and As(V) (Liu et al. 2007). Arsenic phytotoxicity have been observed in other plant species also, like maize (Stoeva et al. 2003), mustard (Chaturvedi 2006), black gram (Srivastava and Sharma 2013a, b) etc. Leguminous crops like cowpea, *Prosopis* and alfalfa have shown alarming levels of arsenic in their plant parts (Huq et al. 2009; Maclauchlan et al. 1988; Mokgalaka-Matlala et al. 2008). Higher accumulations of arsenic are also reported in non-leguminous crops like arum, marigold, ornamental arum, turnip, rice, lodgepole pines and wheat etc. (Parvin et al. 2006; Carbonell-Barrachina et al. 1998; Huq et al. 2005; Huq and Naidu 2005; Kapustka et al. 1995; Yamare 1989).

Being a toxic metalloid, arsenic is likely to cause disturbances in plant metabolism. In maize, the rate of CO₂ fixation in young plants treated with arsenic has been reported to decrease by 20 % and functional activity of PS II was also significantly reduced (Stoeva et al. 2005). Reports showed that with increase in arsenic concentration in rice, non-reducing sugars, enzymes like α amylase and sucrose phosphate synthase decreased, and amount of reducing sugars and enzymes like starch phosphorylase increased. Starch content, however, increased to a marginal amount (Choudhary et al. 2010b). Another report concerning rice showed an increasing trend in reducing, non-reducing and total sugars in the presence of arsenic (Jha and Dubey 2004). Analysis of wheat seedlings under high arsenic concentrations showed increase in the soluble sugar content (Li et al. 2007). Wheat under As(III) and As(V) toxicity showed increased total amylolytic activity together with increment in α and β amylase activities (Liu et al. 2005).

Arsenic toxicity is very likely to affect photosynthetic machinery of plants. Likewise, there are numerous reports validating the above fact. Tomato plants showed reduction in their pigment content like chlorophyll_a, chlorophyll_b and carotenoids when treated with arsenic (Miteva et al. 2005). Choudhary et al. (2010a) reported a similar decrease in rice seedlings when exposed to higher doses of arsenic. He showed reduction in total chlorophyll, chlorophyll_a, chlorophyll_b and fluorescence activity under arsenic toxicity. In previous works, Miteva and Merakchiyska (2002) reported changes in the chloroplast shape and variation in the buildup and flow of assimilates due to increased arsenic concentrations that led to the reduction of chlorophyll contents in rice leaf. Rahman et al. (2007) observed marked relationship between rice chlorophyll levels, its growth and yield signifying that arsenic toxicity disturbs photosynthesis that leads to decline in rice yield. Net photosynthetic rate together with chlorophyll and carotenoids decreased in bean plants on arsenic exposure (Stoeva et al. 2005). Similar decline in pigment content were observed in other plant species

under arsenic toxicity, e.g., red clover plants (Mascher et al. 2002) and black gram (Srivastava and Sharma 2013a).

In rice seedlings, As₂O₃ exposure (25 and 50 μM) resulted in increase in RNA and protein content whereas ribonuclease and protease activity were reported to be inhibited (Mishra and Dubey 2006). Also, soluble protein content in wheat increased on arsenic application (Li et al. 2007). On the contrary, Stoeva et al. (2003, 2005) showed reduction in the levels of protein on arsenic application in maize and bean plants, respectively. A reduction in amount of RNA and DNA content was noticed in tomato leaves when they were treated with heavy metal cobalt (Gopal et al. 2003). Arsenic results in generation of reactive oxygen species (ROS) which has the capacity to harm proteins, as well as, nucleic acids inside plants (Panda et al. 2010). As(III) binds to proteins with sulfhydryl groups and interferes with their functions.

Plants absorb arsenate, through phosphate transporters in aerobic soils (Tripathi et al. 2007). As(V), being identical to phosphate, eventually results in production of very unstable 'ADP-As' compound inside the cytoplasm. Energy flow in cells, is thus badly affected (Meharg 1994). Competition by As(V) with phosphate for entry into the cell has been reported in numerous plants, comprising monocots, dicots, As-hyperaccumulators and non-hyperaccumulators (Finnegan and Chen 2012). Since both As(V) and phosphate compete for the same uptake carriers, it is seen that As(V) toxicity is lower under high P conditions, whereas, As(V) may outcompete P for uptake under low P conditions, increasing P deficiency (Tu and Ma 2003).

Swarnakar and Mukherji (2005) stated that amelioration of As(V) toxicity was possible when mungbean seeds were pre-treated with phosphate salts for 24 h. According to Macnair and Cumbe (1987), at low levels of arsenate, the high phosphate treatment reduced the toxicity of arsenate in non-tolerant but not in tolerant *Holcus lanatus* sp. Pigna et al. (2009) reported that application of P fertilizers, not only restricts the transfer of arsenic to above ground parts, but also, increases P content in plants. In another experiment with rice, phosphate application increased overall growth of rice seedlings together with carbohydrate metabolism (Choudhary et al. 2010a, b).

In the present study, black gram was especially chosen as there appears to be very little information regarding its sensitivity / tolerance to arsenic.

2 Materials and Methods

Black gram (*Vigna mungo* L. Hepper var. T9) seeds, were properly washed with mercuric chloride and then with distilled water to remove any surface contamination. 50 seeds were taken for each treatment. These were kept over in filter paper lined petri-dishes (ϕ 10 cm) having nutrient solution, replaced every other day. Seven days after the emergence, seedlings were subjected to different levels of sodium arsenate (Na₂HAsO₄·7H₂O) solutions – 0 μM (control), 100 and 200 μM with or without 40 ppm dipotassium hydrogen orthophosphate (K₂HPO₄) solution. Completely randomized design (CRD) was chosen for the experiment with 3 replications. Black gram seedlings were harvested after 15 days for the subsequent studies.

2.1 Composition of Nutrient Solution

Long Ashton formula of nutrient solution (Hewitt 1966) was used for growing plants. The composition of complete nutrient solution was: 4 mM of KNO₃ and 4 mM CaNO₃; 1.5 mM NaH₂PO₄ and 2 mM MgSO₄ in macronutrients, and in micronutrients: 100 μM Fe-EDTA;

10 μM MnSO_4 ; 30 μM H_3BO_3 ; 1.0 μM of CuSO_4 and 1.0 μM ZnSO_4 ; 0.2 μM Na_2MoO_4 ; 0.1 μM of NiSO_4 and 0.1 μM CoSO_4 ; and finally, 0.1 mM NaCl. Iron (Fe-EDTA) was prepared by the Jacobson (1951) method. The stock solutions of various nutrients were prepared in advance and kept in refrigerator.

2.2 External Morphology

Length of shoot and root of seedlings were measured using scale. For determination of fresh weight, seedlings were collected, properly washed and rinsed with distilled water. They were gently blotted to wipe out the absorbed water and weighed quickly on balance to avoid excessive loss of water by evaporation. The dry weight was determined after drying these fresh samples in pre-heated oven at 70 °C for 48 h. The oven-dried material was transferred to desiccator and weighed after cooling at room temperature.

2.3 Pigment Contents (Chlorophyll_a, Chlorophyll_b, Total Chlorophyll and Carotenoids)

The concentration of chlorophyll_a and chlorophyll_b was estimated by the method of Arnon (1949). Fresh leaf lamina, after removing the midrib, was chopped and 100 mg of this fresh tissue was ground in a clean pestle and mortar in 10 mL 80 % (v/v) acetone. To prevent denaturation of chlorophyll, a pinch of CaCO_3 was added prior to grinding. The homogenate was centrifuged. The optical density of supernatant was observed in spectrophotometer at the wavelength 480, 510, 645, 652 and 663 nm for estimation of chlorophyll_a, chlorophyll_b, total chlorophyll and carotenoids. The pigment contents were expressed in mg/g fresh weight of tissue. Carotenoids content were calculated on leaf fresh weight basis according to a formula given by Duxbury and Yentsch (1956).

2.4 Carbohydrate Fractions

The seedlings/plant tissue (1 g) was fixed in boiling 90 % (v/v) ethanol in a ratio 1:10. The fixed plant material was homogenised with a glass pestle and mortar in 90 % (v/v) ethanol, and centrifuged at 800 g. The alcohol soluble fractions and the subsequent washings of the residue were mixed and made to volume. The alcohol insoluble fraction was collected in pre-weighed petri-dishes, and was dried first at room temperature and then in an electric oven at 70 °C. The amount of alcohol insoluble fraction was determined and was used for the determination of starch. Sugars were estimated in the alcohol soluble fractions (Nelson 1944).

2.4.1 Sugar

The concentration of reducing and total sugar was determined by the method of Nelson (1944). A suitable aliquot was drawn from the alcohol soluble fraction in 50 mL test tubes along with 5 mL of water for the estimation of sugars. Alcohol was evaporated and sugars were brought to the aqueous phase by heating the solution in a boiling water bath. The pigments in the solution were precipitated by treating with 0.5 mL saturated solution of lead acetate and filtered off. The filtrate was then treated with 1 mL of dibasic sodium biphosphate (saturated) and filtered. The filtrate was made to volume (10 mL) and sugars were estimated in a suitable aliquot drawn from this solution. For estimation of reducing sugars, in a 50 mL test tube an aliquot of 5 mL was taken and 1 mL of copper reagent was added to it. The tubes were covered with glass bulbs and were kept in boiling water bath for 20 min. After cooling water was allowed to drain from bulbs and 1 mL of arsenomolybdate reagent was added. The volume was made to 25 mL.

The optical density of the solution was measured at 490 nm. Similarly total sugars (reducing and non-reducing) were estimated in a suitable aliquot after hydrolysis of the non-reducing sugars with 1 mL solution of 1 % (v/v) invertase. A few drops of toluene were also added to prevent microbial growth. The reaction mixture was kept overnight at 5 °C in a refrigerator and total sugars were estimated in the same way as reducing sugars (Nelson 1944). The concentration of non-reducing sugars was calculated as the difference in the concentration of total and reducing sugars. The results were expressed as mg sugar/100 mg fresh weight.

2.4.2 Starch

Starch was estimated by the method of Montgomery (1957). Oven dried alcohol insoluble fraction (equivalent to 20 mg) was solubilised in 2 mL distilled water in centrifuge tubes in a boiling water bath for 15 min. The material was macerated with a glass rod after adding a pinch of acid washed sand for 3 min and again for 2 min after adding 1.5 mL of concentrated perchloric acid. The mixture was centrifuged at room temperature for 10 min. The process was repeated twice and the supernatant fraction was made to volume 20 mL. A suitable aliquot (2 mL) was drawn in a test tube, to which 0.2 mL 80 % (w/v) phenol was added followed by rapid addition of 5 mL hot concentrated sulphuric acid. The mixture was allowed to cool at room temperature and its optical density was measured at 490 nm. The values were referred to the standard calibration curve prepared from soluble starch. The results were expressed as mg starch/100 mg fresh weight.

2.4.3 Phenols

The concentration of total phenols was estimated in the alcohol extract by the method of Swain and Hillis (1959). A suitable aliquot from alcohol soluble material after removing pigments was taken and Folin-ciocalteu reagent was added exactly after 3 min. Super saturated Na_2CO_3 solution was added to stop the reaction. This reaction mixture was set aside for 1 h to develop colour. The optical density of the solution was measured at 660 nm. The concentration of phenol was determined by referring the readings to a standard calibration curve prepared from A.R. grade phenol. The results were expressed as mg phenol/100 mg fresh weight.

2.5 Soluble Proteins and Amylase Activity

The soluble proteins in the leaf extracts were estimated by the method of Lowry et al. (1951). Protein was precipitated with 20 % (w/v) chilled TCA and the mixture was kept for at least 1 h at 4 °C in a refrigerator. The contents were centrifuged and residue was washed with distilled water to remove TCA. The water washed residue was treated with 80 % (v/v) acetone to remove pigments. The clean residue was dissolved in 0.1 N NaOH at 80 °C in a water bath for 10 min. After centrifugation a suitable aliquot was drawn and reacted with 0.5 mL Folin ciocalteu reagent. The optical density of the mixture was measured at 640 nm. The readings were referred to a standard calibration curve prepared from crystalline Bovine serum albumin and the concentration of proteins was expressed as mg protein/100 mg fresh weight of tissue. The activity of amylase was estimated by the method of Katsuni and Fukuhara (1969). Results were expressed as mg starch hydrolyzed/100 mg fresh weight.

2.6 DNA and RNA Content

Estimation of DNA and RNA content was performed according to the method of Nieman and Poulsen (1963). The plant tissue (0.5 g) was fixed in chilled 95 % (v/v) ethanol. The frozen samples were homogenized in same above 10 mL chilled 95 % ethanol. It was then centrifuged

and the supernatant was discarded. The sediment was extracted by centrifuging with 10 mL of each of the reagents in the order: 95 % ethanol, 50 % ethanol, 0.2 N perchloric acid, 95 % ethanol, boiling absolute alcohol and ether (3:1) and finally in pure ether.

The residue was suspended with 5 mL NaOH (0.3 N). It was then acidified to pH 1 using concentrated PCA and then centrifuged. The supernatant so obtained equals the RNA fraction. Residue was re-suspended with 2 mL H₂O followed by addition of 2 mL 1 N PCA. Residue was again suspended with 3 mL of 0.5 N PCA heated at 70 °C for 15 min. It was then centrifuged at 2 °C. Supernatant equals the DNA fraction. Final volume of DNA fraction was made to 5 mL with 0.5 N PCA. RNA was measured at 259.5 nm and DNA fraction at 261 nm. The readings were referred to standard calibration curves and final values were expressed in percentages.

2.7 Statistical Analysis

Statistical analysis of data was done by one way ANOVA using software program Sigmastats 3.5. It was followed by comparison of mean values using Holm Sidak method at $p \leq 0.05$.

3 Results

Effect of arsenate (Na₂HAsO₄·7H₂O) applied singly or with 40 ppm phosphate (K₂HPO₄) was studied in black gram seedlings using the parameters of growth, pigment contents, carbohydrate metabolism, nucleic acid and soluble protein contents (Table 1).

3.1 Growth

Arsenic applied in the form of sodium arsenate (Na₂HAsO₄·7H₂O) at 100 and 200 µM proved to be quite detrimental for the growth of black gram seedlings. Both shoot and root lengths reduced significantly under increasing concentrations of arsenic. From 14.6 cm in the control, shoot length dropped to 10.7 cm at 100 µM As and to 7.6 cm at 200 µM As. Similarly, length of root also showed a linear decrease on increasing arsenic doses. However, reduction in the length of root was more severe than shoot length (Fig. 1a). At highest arsenic dose, i.e., 200 µM, the drop in shoot length was 47.94 % compared to control, whereas the drop in length of roots were 56.48 % compared to control (i.e., 0 µM As). Biomass of seedlings also reduced at higher arsenic levels (Fig. 1b). Maximum decrease of 40.07 % in fresh weight of seedling was observed at 200 µM As over the control. However, dry weight of seedlings significantly dropped (69.88 %) to 0.0247 g at 200 µM As with respect to 0.082 g in control.

Phosphate application showed positive alterations in all studied parameters of growth. When phosphate together with 100 µM As was supplied to the seedlings, its shoot length was 14.267 cm against 10.767 cm at only 100 µM As treatment. This value (14.267 cm) was quite near the length of shoot obtained in control. In 200 µM As + P also, seedlings showed better growth than in 200 µM As alone. The increase in shoot length from 7.6 cm at 200 µM As to 11.9 cm at 200 µM As + P was quite appreciable. Root length also increased and improved under phosphate application. Root length of seedlings at 100 µM As + P was even more than control. Similarly, from 5.6 cm at 200 µM As, the root length rose to 8.933 cm when phosphate was supplied together with same As dose. Fresh weight and dry weight of seedlings also showed similar trends on phosphate treatments. Like for root length, the value obtained for fresh weight of seedlings at 100 µM As + P was higher than the control. From 0.16 g at

Table 1 Effects of arsenate applied either singly or in combination with phosphate on the growth parameters, photosynthetic pigments, carbohydrate metabolism, nucleic acids and soluble protein content of 15 days old black gram seedlings (values are expressed as 'per seedling')

Parameters	Control	100 μM As	200 μM As	100 μM As + P	200 μM As + P
Shoot length [cm]	14.6±0.306	10.767±0.788*	7.6±1.323*	14.267±0.406	11.9±0.153
Root length [cm]	12.867±0.593	9.1±0.493*	5.6±0.231*	13.367±0.348	8.933±0.521*
FW of seedling [g]	0.267±0.0219	0.223±0.0088	0.16±0.0173	0.293±0.047	0.203±0.0433
DW of seedling [g]	0.082±0.0042	0.036±0.0038*	0.0247±0.0029*	0.0773±0.0018	0.061±0.0021*
Chl a [mg/g fw]	0.258±0.00485	0.154±0.00182*	0.124±0.00147*	0.182±0.00501*	0.133±0.0041*
Chl b [mg/g fw]	0.194±0.00295	0.143±0.00332*	0.122±0.00165*	0.162±0.0044*	0.131±0.00199*
Total chl [mg/g fw]	0.450±0.00989	0.282±0.00522*	0.241±0.00429*	0.330±0.00543*	0.259±0.00449*
Car [mg/g fw]	0.100±0.00015	0.073±0.00037*	0.061±0.00007*	0.078±0.00022*	0.065±0.00037*
Protein [mg/ 100 mg tissue]	0.919±0.100	0.759±0.0074	0.62±0.0529*	0.777±0.0212	0.728±0.00543
% DNA	0.0202±0.00009	0.0164±0.0002*	0.013±0.0001*	0.0243±0.0003*	0.0204±0.00003
% RNA	0.339±0.0020	0.234±0.0035*	0.203±0.0028*	0.492±0.0036*	0.470±0.0022*
Total sugars [mg/ 100 mg fw]	0.329±0.00008	0.504±0.00014*	3.078±0.00026*	0.355±0.00014*	0.756±0.00008*
Reducing sugars [mg/ 100 mg fw]	0.267±0.00061	0.352±0.00026*	1.342±0.00018*	0.345±0.00007*	0.581±0.00014*
Non-reducing sugars [mg/ 100 mg fw]	0.062±0.0002	0.151±0.00008*	1.736±0.00055*	0.010±0.00012*	0.175±0.00009*
Starch [mg/ 100 mg fw]	0.436±0.0049	1.227±0.0007*	1.662±0.00274*	0.944±0.00128*	1.082±0.0004*
Phenol [mg/ 100 mg fw]	0.018±0.00008	0.022±0.00017*	0.033±0.00004*	0.017±0.00012*	0.025±0.00012*
Amylase [mg starch hydrolyzed /100 mg fw]	0.503±0.002	0.477±0.0054	0.425±0.0056*	0.496±0.0129	0.438±0.0129*

Means±SE, *n*=3, *As* = Na₂HAsO₄·7H₂O, *P*=40 ppm K₂HPO₄, *Chl* = Chlorophyll, *Car* = Carotenoid, * statistically significant difference (*P*≤<0.001), multiple comparisons vs Control group (Holm Sidak method) Overall significance level = 0.05

200 μM As, fresh weight of seedlings increased to 0.203 g at 200 μM As + P treatment. Dry weight of seedlings also increased upon phosphate application.

3.2 Photosynthetic Pigments

Arsenic exposure greatly damaged the photosynthetic machinery of black gram plants (Fig. 1c). There was 51.94 % reduction in the levels of chlorophyll_a at 200 μM As over control. From 0.258 mg/g fresh weight at control, chlorophyll_a dropped to 0.154 and 0.124 mg/g fresh weight at 100 and 200 μM , respectively. Chlorophyll_b and total chlorophyll followed similar trends. Both chlorophyll_b and total chlorophyll dropped significantly at 100 μM and 200 μM As doses. The drop in chlorophyll_b was 26.29 % and 37.11 % at 100 μM and 200 μM As doses, respectively, compared to control. Total chlorophyll declined to 37.33 % and 46.44 % at 100 μM and 200 μM As doses, respectively, over control.

Carotenoids, accessory photosynthetic organic pigments, found in the chloroplasts and chromoplasts of plants, are also powerful antioxidants. Analysis of carotenoid content in black gram seedlings was performed. At 100 μM and 200 μM As doses, carotenoid content significantly declined 27 % and 39 % over control, respectively.

Phosphate application significantly improved the levels of photosynthetic pigments. Content of chlorophyll_a, chlorophyll_b and total chlorophyll enhanced significantly in the presence of phosphate. Chlorophyll_a increased from 0.154 mg/g at 100 μM As to 0.182 mg/g at 100 μM As + P treatment, while it increased from 0.124 mg/g at 200 μM As to 0.133 mg/g at 200 μM As + P treatment. Chlorophyll_b showed an increase from 0.143 mg/g at 100 μM As to 0.162 mg/g at 100 μM As + P dose. Total chlorophyll in the presence of phosphate increased from 0.282 mg/g at 100 μM As to 0.330 mg/g at 100 μM As + P treatment.

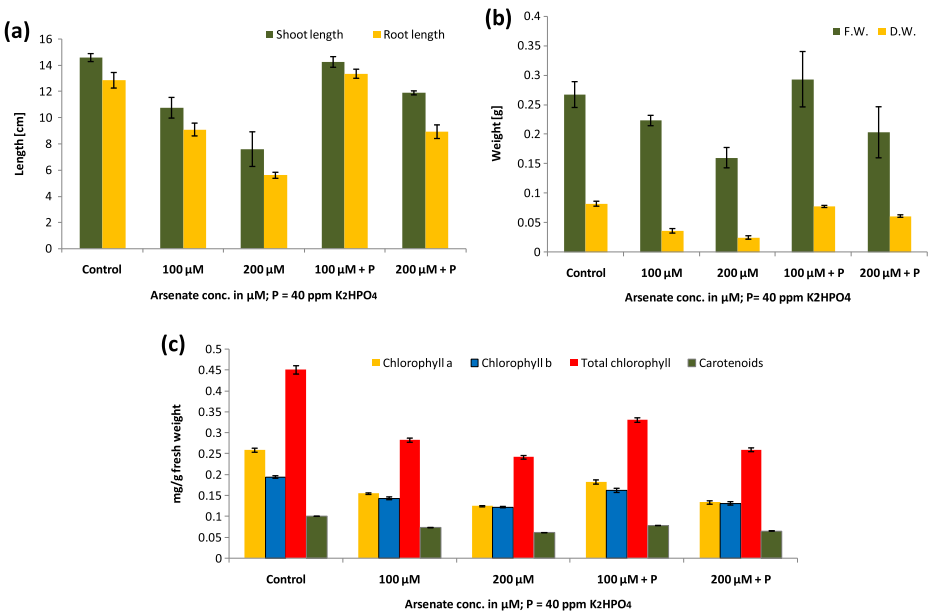


Fig. 1 Effect of arsenate with or without phosphate on (a) shoot and root length; (b) fresh weight (F.W.) and dry weight (D.W.) and; (c) photosynthetic pigments of black gram seedlings

However, carotenoid contents improved only marginally in presence of phosphate as compared to only As treatment.

3.3 Nucleic Acids and Soluble Protein Content

Heavy metals are known to damage DNA, either directly or indirectly through production of ROS. Here also, arsenic exposure significantly reduced the amount of DNA, RNA and soluble protein contents of the plant tissue. DNA content of black gram tissue decreased by 35.64 % at 200 μM As over control and the drop in RNA content was 40.12 % at 200 μM As over control (Fig. 2a). DNA % reduced from 0.0202 in control to 0.0164 at 100 μM As treatment, while RNA % declined from 0.339 to 0.234 at 100 μM As treatment. In case of protein, from 0.919 mg/100 mg fresh weight at control, protein levels reduced to 0.759 at 100 μM As treatment and to 0.62 at 200 μM As treatment, respectively (Fig. 2b).

Phosphate forms an integral part of nucleic acid and its application is bound to influence the levels of DNA and RNA. % DNA increased from 0.0164 at 100 μM As to 0.0243 at 100 μM As + P treatment, while it increased from 0.013 at 200 μM As to 0.0204 at 200 μM As + P treatment. Similarly, % RNA also showed increment from 0.234 and 0.203 at 100 and 200 μM As, respectively, to 0.493 and 0.470 at 100 μM As + P and 200 μM As + P, respectively. Soluble protein content also increased substantially on phosphate application.

3.4 Carbohydrate Metabolism

Under carbohydrate metabolism, parameters studied were total sugars comprising both reducing and non-reducing sugars, starch and phenol content of seedlings together with activity of amylase enzyme.

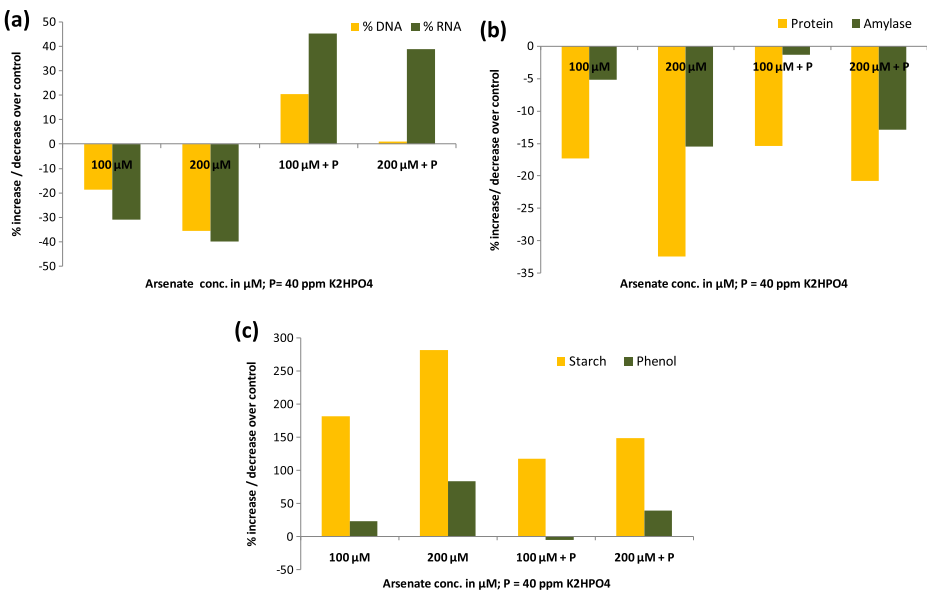


Fig. 2 Graphs showing % increase or decrease in (a) DNA & RNA contents; (b) protein content and amylase activity and; (c) starch and phenol contents over control in black gram seedlings on application of arsenate with or without phosphate

Arsenic exposure led to the accumulation of sugars in plant tissue (Fig. 3). Sugar content (i.e., reducing, non-reducing and total sugars) increased at both 100 and 200 μM As doses. There was a significant increase in the level of total sugars, however, the increase in non-reducing sugar was more than reducing sugar. From 0.329 mg/100 mg fresh weight at control, content of total sugars increased to 3.078 mg/100 mg fresh weight at 200 μM As application. At the same levels, reducing sugars increased from 0.269 to 1.342 mg/100 mg fresh weight, while enhancement in the levels of non-reducing sugar was from 0.062 at control to 1.736 mg/100 mg fresh weight at 200 μM As.

Together with sugar, starch content also increased with increase in the doses of arsenic. From 0.436 mg/100 mg fresh weight at control, starch content rose to 1.227 at 100 μM As and upto 1.662 at 200 μM As. Phenol content in black gram seedlings also showed similar trend. Phenol content rose to 83.33 % at 200 μM As over control (Fig. 2c). Amylase enzyme, which is responsible for hydrolysis of starch, showed reduction in its activity on arsenic application. Amylase activity declined 15.5 % at 200 μM As with respect to control (Fig. 2b).

On phosphate application, sugar contents in plants significantly dropped to levels nearly similar to the control. A similar trend followed with starch and phenol content. Both of these parameters dropped quite consistently. Starch content showed reduction from 1.227 mg/100 mg fresh weight at 100 μM As to 0.944 at 100 μM As + P treatment (Fig. 2c). Similarly it reduced from 1.662 mg/100 mg fresh weight at 200 μM As to 1.082 mg/100 mg fresh weight on phosphate treatment. Phenol content reduced from 0.033 mg/100 mg fresh weight at 200 μM As to 0.025 mg/100 mg fresh weight at 200 μM As + P treatment (Fig. 2c). Activity of amylase showed improvement in the presence of phosphate. Its activity increased in As + P treatments.

4 Discussion

Arsenic being a non-essential element for plants is regarded highly phytotoxic. Arsenic restricts growth of plant (Stoeva et al. 2003). Its exposure not only results in physiological

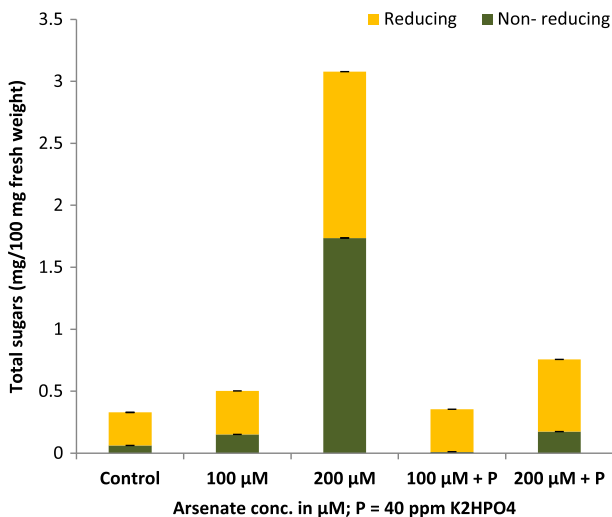


Fig. 3 Effect of arsenate with or without phosphate on total sugars, reducing and non-reducing sugars of black gram seedlings

damages (Marin et al. 1992; Wells and Gilmour 1977) but also reduces crop productivity (Stepanok 1998). In the present study, arsenic application resulted in the decrease of growth of black gram seedlings. It was observed that both biomass and height decreased markedly with increasing arsenic in treatment solution. Reports have shown that plants treated with arsenic, showed symptoms like seed germination inhibition, reduction in plant growth and yield etc. (Frans et al. 1988; Tsutsumi 1980; Carbonell-Barrachina et al. 1995; Tang and Miller 1991; Marin et al. 1992) The drop in length of root was greater when compared to shoots on arsenic application on black gram seedlings. Plant roots are the first to come in contact with arsenic and are likely to be damaged most. Arsenic accumulates mainly in the roots (Marin et al. 1992, 1993) and to a lesser degree in aboveground organs, decreases the biomass production, plant height and leaf area, which are the typical symptoms of phytotoxicity. There are various reports of toxic effects of arsenic on growth of roots of many plants (Sneller et al. 1999; Van Den Broeck et al. 1998; Hartley-Whitaker et al. 2001). Fresh and dry weight both were decreased drastically in black gram seedlings. Similar results were observed in bean plants when exposed to arsenic. Arsenate application reduced biomass and height of bean seedlings together with leaf area and other physiological parameters (Stoeva et al. 2005). Here in this study, reduction of dry weight was more than reduction of fresh weight in black gram seedlings. Also, it was important to note that overall reduction in root was greater than that of shoots, reflecting greater sensitivity of growing roots to As treatment. Retardation of root elongation is considered one of the commonly visualized symptoms of metal toxicity (Wang et al. 2003) These findings are also in agreement with report of marked reduction of root length in As-treated rice varieties (Dasgupta et al. 2004).

Arsenic toxicity resulted in reduction of photosynthetic pigments in black gram seedlings. Similarly, Miteva et al. (2005) observed the reduction in pigments content in tomato when exposed to arsenic. Choudhary et al. (2010a) also reported a decrease in pigment levels in rice seedlings when exposed to higher doses of arsenic. Krupa et al. (1996) postulated that determination of the chlorophyll and carotenoids contents can serve as marker of heavy metal pollution in plants. Metal stressed plants usually substitute their Mg-ion in the chlorophyll molecule with any available heavy metal in low-light conditions which leads to extreme disturbances in photosynthetic activity of plants by breakdown of photosynthesis. In previous works, it has been shown that increase in arsenic concentration in rice leaf, not only injured the membrane of chloroplast, but also disorganized its structure (Miteva and Merakchiyska 2002). Rahman et al. (2007) observed that photosynthesis in rice plants is badly affected by arsenic toxicity which accounted for reduction in both its growth and yield. Similar decline in pigment contents were observed in other plant species under arsenic toxicity, e.g., red clover plants (Mascher et al. 2002) and bean plants (Stoeva et al. 2005). The photosynthetic pigments are believed to be the targets of toxic As effect (Miteva and Merakchiyska 2002).

Nucleic acid and protein contents were found to decrease on arsenic application in black gram seedlings. This is in accordance with earlier reports, like in maize and bean plants reported by Stoeva et al. (2003, 2005), who have shown reduction in the levels of protein on arsenic application. Similarly, a reduction in amount of RNA and DNA contents was noticed in tomato leaves when they were treated with heavy metal cobalt (Gopal et al. 2003). As(V) is easily converted into As(III) in plant cell, which results in binding with proteins having sulfhydryl group. This results in interference in protein functions. Arsenic leads to generation of reactive oxygen species (ROS) that has the capacity to damage both proteins and nucleic acids (Panda et al. 2010). ROS are known to cause various lesions in DNA. In DNA, according to Imlay and Linn

(1986), both deoxyribose sugar and nitrogenous base are prone to oxidation, leading to damage in its structure. In general, heavy metals bring a considerable decrease in the rate of all metabolic processes of the cell, including nucleic acid synthesis, cell division and protein contents (Singh 2005). Likewise, heavy metal lead was supplied in germinating rice seedlings, and activity of lead acetate was studied with respect to RNA, DNA and protein contents. All these were reported to be greatly reduced both in endosperm and embryo with increasing concentration of lead and with concomitant increase in amino acid content in embryo (Singh 2005). Jain and Gadre (1997) also reported reduced total RNA and protein in maize tissues on application of sodium arsenate.

Proteins subjected to oxidative stress results in enhanced susceptibility to proteolysis (Davies 1987). Also, it is observed that sulphur containing amino acids and thiol groups, especially are very prone to oxidative damage.

Arsenic exposure led to the accumulation of sugars in plant tissue of black gram seedlings. Sugar contents (i.e., reducing, non-reducing and total sugars) were found to be increased at both 100 and 200 μM As doses. This is in accordance with the report concerning rice which showed an increasing trend in reducing, non-reducing and total sugars in the presence of arsenic (Jha and Dubey 2004). Sugar buildup under stressful environment leads to osmoregulation and helps in protection of biomolecules. Starch content in black gram was increased with increase in the doses of arsenic. Choudhary et al. (2010b) also reported a small increase in starch content in rice seedlings on arsenic exposure. Phenol content increased in plants with increase in the doses of arsenic. These results are in agreement with the observations of Gopal et al. (2003) who observed increase in phenol contents in tomato when treated with heavy metal cobalt. Amylase enzyme, which is responsible for hydrolysis of starch, showed reduction in its activity on arsenic application in seedlings of black gram. This is in agreement with the findings of Jha and Dubey (2004) and Choudhary et al. (2010b) where they have shown that arsenic exposure resulted in decline in the amylase activity in rice seedlings. Soluble sugar helps in basal metabolism of plant under stressed environment, by allowing it to increase adequate carbohydrate storage reserve (Hurry et al. 1995).

Phosphate application helped in ameliorating arsenic toxicity in black gram seedlings. Arsenic is similar to phosphate in many ways, being placed in the same group in the periodic table; it shares similar electron configuration and chemical properties with phosphate. Likewise, both compete for the same uptake carriers in the root plasma lemma of plant (Ullrich-Eberius et al. 1989; Meharg and Hartley-Whitaker 2002). In the present study, black gram seedlings showed positive alterations tested under the purview of arsenic treatment alone. Whether growth, photosynthetic pigments, content of protein and nucleic acids or carbohydrate metabolism is concerned, all improved in the presence of phosphate application in black gram seedlings. Thus, it can be interpreted that amelioration of arsenic toxicity by phosphate application was because of enhanced phosphorus content in the plant which helped plant combat arsenic stress. Plant growth is better in presence of phosphate with less oxidative stress as compared to As treatments. Also, more cytoplasmic phosphate prevents the cell from As(V) toxicity. Pigna et al. (2009) reported that when phosphate was given together with arsenic in irrigation water, it prevented As toxicity and growth inhibition in wheat grown in uncontaminated soil. Swarnakar and Mukherji (2005) stated that amelioration of As(V) toxicity was possible when mungbean seeds were pretreated with phosphate salts for 24 h. In another experiment with rice, phosphate application showed positive alterations in various parameters tested under the purview of arsenic treatment alone. Phosphate application has been shown to increase growth and metabolism of rice seedlings together with carbohydrate metabolism (Choudhary et al. 2010a; b).

5 Conclusion

The present study indicated that arsenic checks the growth and development of black gram seedlings. Thus, black gram proved to be quite sensitive towards arsenic toxicity. Phosphate application was analyzed as an ameliorating agent for arsenic toxicity and it definitely proves to be one of the important remedial methods.

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Conflict of Interests Authors declare that they have no conflict of interests.

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