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Research article

INTERACTIVE STUDIES OF ZINC WITH CADMIUM & ARSENIC ON SEED GERMINATION AND ANTIOXIDANT PROPERTIES OF PHASEOLUS AUREUS ROXB

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ABSTRACT: Seed developmental is essential stage in the plant life cycle that is highly protective against external stresses. Treatment of *Phaseolus aureus Roxb* with different concentration of cadmium and arsenic decreased the percentage of germination, germination index and vigour index. This effect was dose dependent and exhibits more inhibitory effects at higher dose of heavy metal. The supplementation of zinc (25 ppm) reduced the inhibitory effect of cadmium and arsenic by increasing the germination index and vigour index at low metal concentration (25 & 50 ppm). The phenolic content, catalase and FRAP activity reduced with increasing cadmium and arsenic concentrations compared to control seedling. Arsenic display relatively strong toxicity than cadmium. Reduced phenolic content, catalase and FRAP activity were increased significantly with the treatment of zinc at low heavy metal concentration (25 & 50 ppm). Enhancement in free proline and metal chelating activity as a response to heavy metal stress is reported. The amelioration of seedling growth by zinc supplementation under metal toxicity (at 25 and 50 ppm) was associated with further enhanced level of free proline and improved metal chelating activity. It was found that Zn was able to partially restore the seedling growth and reduced oxidative stress during seed development at low heavy metal concentration (25 & 50 ppm), whereas at higher concentration of heavy metal, zinc could not able to restored growth and biochemical parameters. In this study, we analyzed the growth promoting and antioxidative effect of zinc on arsenic and cadmium induced inhibitory effect in presence of low level of heavy metal.
***Key Words*:** Heavy metal, Seed germination, Cadmium, Arsenic

INTRODUCTION

The interactive effect of essential and heavy metals on plant growth and metabolism has become a subject of great interest in recent years. The nutritional quality and biomass production lead to reduced due to heavy metal stresses under various agricultural production systems [1]. Rapid industrialization contaminate soils in cultivated fields with toxic heavy metals and accumulate in the food chain is one of the major environmental and health problems of our modern society. Major anthropogenic sources of cadmium (Cd) are Cd-containing phosphate fertilizers, sewage sludge and industrial emissions. Most anthropogenic releases of arsenic (As) are to land or soil, primary in the form of pesticides or soil waste [2]. Therefore, arsenic & cadmium poisoning events of the human beings and livestock occur frequently**.** Heavy metal like arsenic and cadmium can be easily absorbed by plant roots and transported to shoots and can cause various toxic effects on the plants such as inhibition of seed germination, plant growth, reduced crop yield, and also alter normal metabolic pathway including respiration, photosynthesis and also affects nutrient uptake and homeostasis [3,4]. Blocking essential functional group or displacement of essential metal ion is the main mechanism of heavy metal toxicity [5]. Excessive uptake and accumulation of arsenic and cadmium are also known to disturb cellular redox environment causing oxidative stress including alterations in enzymes of the antioxidant defence system. It has been proposed that heavy metal can lead to oxidative stress by displacement of essential metals of proteins and enzymes, results in generation of ROIs [6]. Zinc plays a significant role in various enzymatic and physiological activities and performs many catalytic functions in plant system besides transformation of carbohydrates, chlorophyll and protein synthesis [7].

Zinc has known functions as micronutrients in plants, which is required as structural and catalytic components of protein and enzymes for normal growth and development of plant [8,9]. A deficiency of zinc is the most widespread micronutrient in crop plants [10]. There is need to study the combined effects of beneficial and toxic heavy metals on plants because most of them are present in an environment at the same time or on the same environment at different times. Germination & early seedling growth have been crucial step which are influence by heavy metal stress as compared to other stages of plant development [11].

The present study attempted on Mungbean (*Phaseolus aureus Roxb*) to investigate the impact of zinc on some of the cadmium and arsenic-induced negative effects on seed germination, plant growth and behaviour of some enzyme capacities involved in the redox regulation in the germinating seeds. The present work shall help to explain the growth promoting and antioxidative response of zinc on germinating seeds subjected to cadmium and arsenic treatment.

MATERIALS AND METHODS

Collection of Seed material

The certified seeds of *Phaseolus aureus Roxb* were purchased from Anand Agricultural University & seeds with uniform size; colour and weight were chosen for the present experiment. Seeds were surface sterilized by dipping in 0.1% $HgCl_2$ solution for 1.0 min to avoid fungal/bacterial contamination and washed properly five times with distilled water. The test solution of (1, 25, 50, 100, 250 ppm) of cadmium, zinc, arsenic were prepared with distilled water using cadmium chloride, zinc chloride and sodium arsenate respectively.

Germination assay

Collected seeds were washed and disinfected with diluted sodium hypochlorite (0.2%) and rinsed with distilled water twice. Germination test was carried out in 9 cm (diameter) sterilized petriplates having Whatman No. 1 filter paper. Seed were soaked in (i) distilled water (control), (ii) cadmium treated (1, 25, 50, 100, 250 ppm) (iii) arsenic treated (1, 25, 50, 100, 250 ppm) (iv) zinc treated (1, 25, 50, 100, 250 ppm) (v) cadmium treated as on (ii) supplemented with zinc (25ppm) (vi) arsenic treated as on (iii) supplemented with zinc (25ppm). For each treatment, 25 seeds were placed on double layered filter papers wetted with 0.7 ml ddH_2O or test solution. For every treatment, 5 replicates (Petri dishes), each with 25 seeds was maintained. Petri dishes were sealed and seeds were allowed to germinate under in dark at 26 ± 2°C. Test solution or distilled water was added in respective petriplates periodically. Seeds were considered germinated, if shoot extends to half of seed length and the radical extends to the seed length. Germinated seeds were recorded after 5 days and percentage of germination, germination index and vigour index were then calculated according to Singh & Singh [12]. The root and shoot length of seedlings in various concentrations of test solution were measured at the end of 7 day for each germinating seed.

BIOCHEMICAL ANALYSIS

Seed coat was removed and germinating seeds (1.0 g) were homogenized with cold sodium phosphate butter solution (0.1 M, pH 7.4) containing 0.1 mM EDTA, 0.5% (v/v) Triton X-100 and 0.5% (w/v) PVPP and centrifuged at 8,000 g for 15 min at 4 °C. The supernatant was used for biochemical analysis. The protein concentration was determined according to Lowry *et al.* [13] method using bovine serum albumin (BSA) as standards.

Estimation of total phenolics content

Total phenolics content in each extract was determined using a series of gallic acid as standard solutions (0.05-0.35mg/ml) as described by Slinkard and Singleton [14] but with some modifications. 0.1 ml of sample or standard was mixed with 2.0 ml of a 2% (w/v) Na_2CO_3 and vortexes vigorously. 0.1ml of 50% Folin-ciocalteu's phenol reagent was added after 3 min and each mixture was vortexes again. Reaction mixture was incubated for 30 min at room temperature and the absorbance was measured at 750 nm. The total amount of phenolic compounds was calculated as mg/g (gallic acid equivalents) from calibration curve of gallic acid standard.

Free proline

Proline was extracted with 3% sulphosalicylic acid, and determined according to Bates *et al*. [15]. It was determined in the supernatant by measuring the absorbance of the proline ninhydrin product formed at 520 nm. The amount of proline was calculated from the standard curve plotted with known concentrations of proline.

Determination of (Fe^{+2}) metal chelating ability

Ferrous ion (Fe^{+2}) chelating ability of the extract was measured using the method described by Dinis *et al*. [16]. EDTA was used as metal chelating standard. The % inhibition of ferrozine-Fe^{+2} complex formation were given by the following formula: Ferrous ion chelating activity (%) = $[1-(A_1-A_2)/A_0] \times 100\%$. Where, A_0 was the absorbance of the control (the mixture without extract), A_1 was the absorbance of the mixture in the presence of the extract and A_2 was the absorbance without Ferrozine.

Ferric reducing antioxidant power (FRAP)

In the FRAP assay, blue colored Fe^{II}-tripyridyltriazine compound is formed from the colorless oxidized Fe^{III} form by the action of electron donating antioxidant. The change in absorbance was measured at 593 nm [17]. The standard curve was prepared by iron (II) sulfate solution and results were expressed as IC_{50} value of each seedling.

Catalase activity

Catalase activity was assayed spectrophotometrically by monitoring the decrease in absorbance of H_2O_2 at 240 nm according to the method of Noctor and Foyer [18]. Unite activity was taken as the amount of enzyme, that decomposes 1.0 M of H_2O_2 in one minute.

Metal accumulation in germinated Seeds

The cadmium and arsenic content were estimated employing a Perkin-Elmer (Analyst Model 300) atomic absorption spectrophotometer equipped with an air-acetylene burner. The heavy metal content was expressed as mg g^{-1} of seedling sample.

RESULTS

The results of the present study revealed that cadmium and arsenic adversely influenced the germination process and antioxidant properties of seed during early development. The present results also showed the positive effect of zinc supplementation (25 ppm) over the inhibitory effect of cadmium and arsenic on seed germination and antioxidant properties at lower concentration (25 and 50 ppm) but does not exhibits beneficial effect at higher concentration (100, 250 ppm) of cadmium and arsenic. Effect of studied heavy metal cadmium, arsenic and essential metal zinc on the growth of *Phaseolus aureus Roxb* seedling is shown in Table 1. Germination index and vigour Index of seed treated with cadmium, arsenic or in combination with zinc are listed in Table 2. Treatment of *Phaseolus aureus Roxb* with different concentration (25, 50, 100, 250 ppm) of cadmium chloride and arsenic decreased the germination index and Vigour Index. Zinc causes strong inhibition of seedling growth at high concentration (250 ppm). Application of zinc (25 ppm) in the form of zinc chloride was effective to reduce the inhibitory effect of cadmium and arsenic by increasing the percentage of germination, GI and VI at 25 & 50 ppm compared to respective control, but higher concentration (100 and 250 ppm) did not show a beneficial effect. Arsenic was evidently more effective in inhibiting germination than cadmium.

Table-1 : Effect of various concentrations of Cd, As & its interaction with zinc supplementation (25ppm) on seed germination. In bracket shows percentage of germination compared to control.

Treatments (ppm)	Control	Zn	Cd	Cd + Zn	As	As + Zn
0	23.4 ± 1.8 (93.6)	-	-	-	-	-
1	-	24.1 ± 1.2 (96.4)	22.0 ± 1.4 (88.0)	24.5 ± 0.7 (98.0)	20.6 ± 1.7 (82.4)	23.5 ± 1.4 (94.0)
25	-	23.5 ± 1.1 (94.0)	17.4 ± 2.1 (69.6)*	21.5 ± 1.4 (86.0)†	13.4 ± 1.2 (53.6)*	18.5 ± 0.9 (74.0)*†
50	-	22.0 ± 1.8 (88.0)	14.3 ± 1.6 (57.2)*	19.5 ± 1.3 (78.0) †	10.5 ± 1.5 (42.0)*	14.5 ± 1.1 (58.0) *†
100	-	20.8 ± 1.5 (83.2)	11.5 ± 1.7 (46.0)*	15.5 ± 2.2 (62.0)*	6.2 ± 0.9 (24.8)*	7.0 ± 1.5 (28.0)*
250	-	12.8 ± 2.8 (51.2)*	8.5 ± 2.4 (34.0)*	9.5 ± 1.5 (38.0)*	-	-

Values are means of three replicate ± SD. * denotes significant difference from control at $p < 0.05$; † indicate significant difference between Cd or As treated with Cd or As + Zn treated seed at $p < 0.05$ level

Table 2 : Germination index (GI) and Seedling Vigour Index (SVI) at various concentrations of Cd or As and along with zinc supplementation (25ppm). In bracket shows Seedling Vigour Index (SVI).

Treatments (ppm)	Control	Zn	Cd	Cd + Zn	As	As + Zn
0	4.82 (249.1)	-	-	-	-	-
1	-	4.82 (272.1)	4.8 (168.9)	4.9 (217.6)	4.12 (128.5)	4.7 (176.7)
25	-	4.7 (240.6)	3.48 (69.6)	4.3 (139.3)	2.68 (38.6)	3.7 (84.4)
50	-	4.4 (186.6)	2.86 (49.2)	3.9 (102.2)	2.1 (21.4)	2.9 (31.9)
100	-	4.16 (141.4)	2.3 (32.7)	3.1 (62.2)	1.24 (4.5)	1.4 (3.36)
250	-	2.56 (33.8)	1.7 (10.5)	1.9 (15.9)	0	0

Table 3: Effect of various concentrations of heavy metal (cadmium, arsenic) & its interaction with zinc supplementation (25ppm) on metal chelating activity of seedling. Metal chelating activity expressed in IC_{50} value.

Treatments (ppm)	Control	Zn	Cd	Cd + Zn	As	As + Zn
0	125.3 ± 8.9	-	-	-	-	-
1	-	198.6 ± 10.8*	185.4 ± 12.6*	207.3 ± 19.5*	171.4 ± 14.3*	198.4 ± 14.5*
25	-	325.2 ± 25.5*	225.2 ± 15.5*	425.8 ± 22.3*†	212.2 ± 21.8*	387.8 ± 21.9*†
50	-	174.8 ± 15.0*	134.6 ± 12.7	194.8 ± 13.7*†	120.4 ± 13.9	171.6 ± 12.7 *†
100	-	130.5 ± 20.8	100.3 ± 10.4 *	138.6 ± 14.4	115.1 ± 12.4	113.4 ± 18.6
250	-	100.5 ± 15.7	105.3 ± 12.5	110.6 ± 11.5	105.6 ± 10.3	88.7 ± 14.6

Values are means of three replicate ± SD. * denotes significant difference from control at $p < 0.05$; † indicate significant difference between cadmium or arsenic treated alone with Zn application treated seed at $p < 0.05$ level.

Total phenolic content of seedling was consistently reduced with increasing concentration of Cd and As presented in Fig-1. The total phenolic content does not altered at 1.0 ppm of cadmium and arsenic. The combined treatment of zinc with cadmium or arsenic significantly improved phenolic content at 25 and 50 ppm but could not recovered at higher concentration (100 and 250 ppm) compared to respective control. Our results indicate that accumulation of prolin increase with increasing Cd and arsenic concentration in dose dependent manner compared to control seedling (Fig-2). In response to cadmium and arsenic stress, supplementation of zinc further enhanced the proline content significantly compared to respective control at 1 and 25 ppm. The non-significant change was observed in proline content at 50, 100 and 250 ppm of arsenic and cadmium in combination with zinc. Fig-3 represents the effect of As and Cd alone or in combination with zinc on catalase activity of seedling. Cd and As toxicity decreased the catalase activity in seedling with increasing the concentration of metal. Catalase activity not affected at 1.0 ppm of cadmium and arsenic compared to control. Supplementation of zinc along with Cd or As (at 1 & 25 ppm) significantly increased the catalase activity compared to respective control. The combined treatment of zinc with Cd or As at 50, 100 and 250 ppm could not restored catalase activity to control. The effect of studied heavy metal Cd, As and Zn individually and supplementary of Zn (25 ppm) with cadmium and arsenic on metal chelating activity of seedling growth is presented in Table 3.

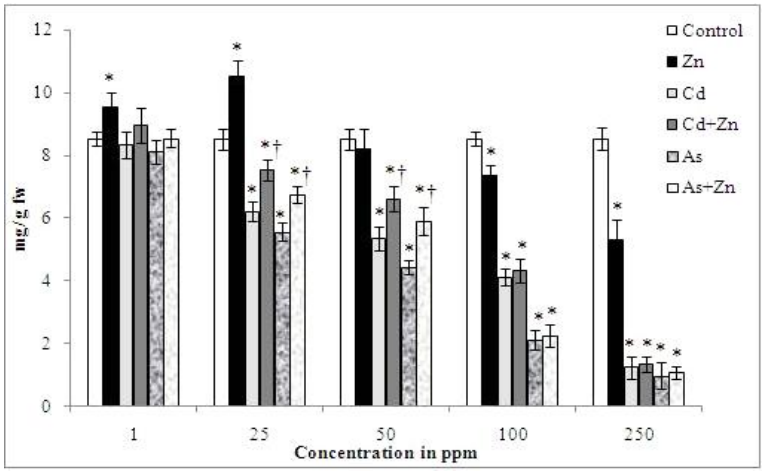

Figure-1: Effect of cadmium or arsenic along with zinc supplementation on phenolic content in germinating seeds. All values are three replicate ± SD. * and † indicate significant difference compared to control and metal treated respectively at *P<0.05.*

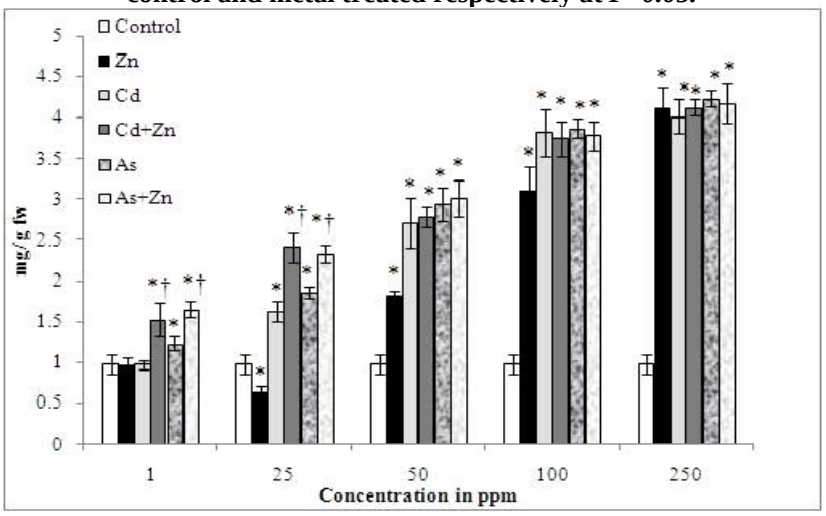

Figure-2: Effect of Cd or As along with Zn supplementation on proline level in germinating seeds. All values are three replicate ± SD. * denotes significant difference from control at *p < 0.05;* † indicate significant difference between Cd or As treated and along with Zn treated seed at *p < 0.05* level

The metal chelating activity of seedling was found to be increased at 1 & 25 ppm of Zn, Cd, and As compared to control. The inhibitory effect of Zn, Cd, and As was observed on metal chelating of seedling at concentration of 50 ppm onwards. The beneficial effect of Zn supplementation with Cd or As was observed at 25 & 50 ppm on metal chelating activity as indicated by significantly increased activity of metal chelating compared to respective control.

FRAP activity were negatively correlated with concentration of cadmium and arsenic presented in Fig-4. FRAP activity decreased significantly with increasing dosages. The FRAP activity increased (at 1 & 25 ppm) on zinc treatment but declined at 50, 100 and 250 ppm compared to respective control. After 7 days of the growth, the accumulation of Zn, Cd and As content is given in Fig-5. Cd is found to be more accumulated in the seed than As. Zinc accumulation increased was progressively in the seedling with increasing concentration. Zinc addition significantly reduced the Cd and As level in seedling growth. The result indicate that low concentration of zinc had micronutrient and antioxidative like effect on seed germination.

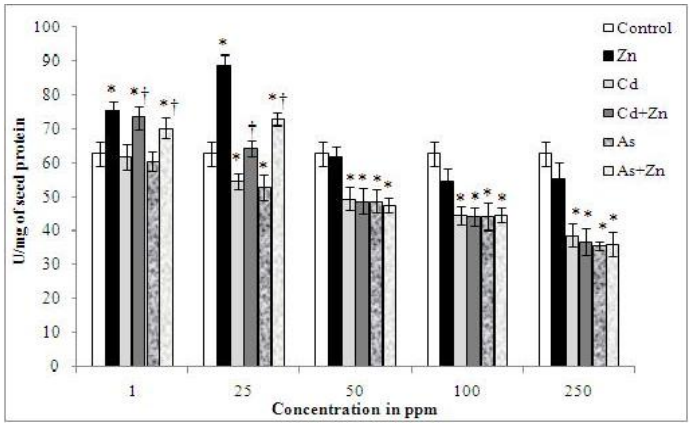

Figure-3: Catalase activities in mungbean seedling treated with cadmium or arsenic along with zinc supplementation. All values are three replicate ± SD. * and † indicate significant difference compared to control and metal treated respectively at *P<0.05*

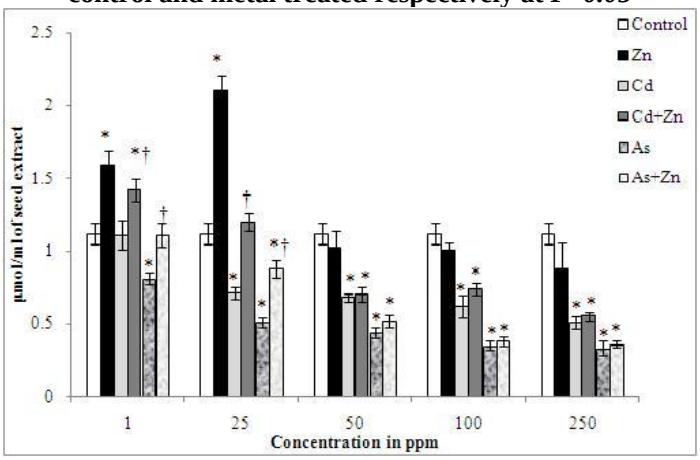

Figure-4: Effect on total antioxidant capacity (FRAP) in mungbean seedling treated with Cd or As along with Zn supplementation. Data are means of three replicate ± SD. * and † indicate significant difference compared to control and metal treated respectively at *P<0.05*

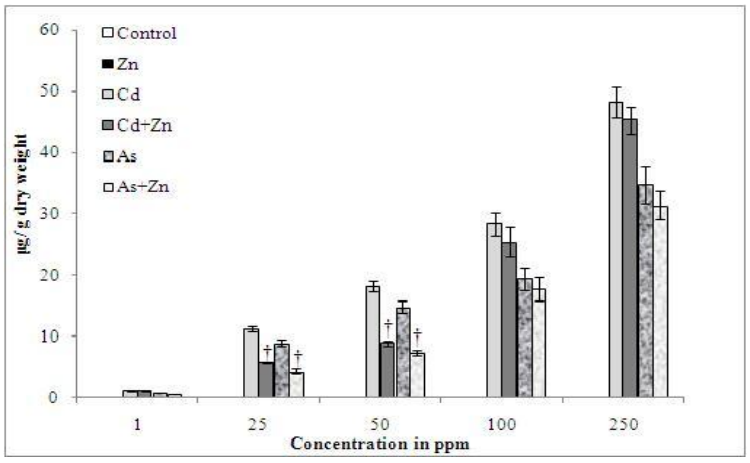

Figure-5: Effect of zinc application on bioaccumulation of cadmium and arsenic in mungbean seedling. Data are mean of 3 replicates with Standard Deviation. †indicate significantly (P<0.05) different from Cd or As treated seedling.

DISCUSSION

Heavy metals in soils are an increasing concern of environmental pollution. A negative correlation between heavy metal doses and the germination percentage was observed in seeds. The germination rates continuously decreased with increasing of As and Cd doses. It was found in this study that arsenic was most inhibitive effect on germination of *Phaseolus aureus Roxb* [19]. Research showed that Cd and As inhibited germination and seedling growth of several crops when concentration of metal was increased. There have been various reports on the effect of heavy metals Cd & As on germination dynamics, decrease in fresh weight of seedlings [20,3]. The germination percentage can be considered as a sensitive indicator for As and Cd cytotoxicity. Zn supplementation significantly increased percentage of seed germination, germination index, and vigour index. Zn is the most critical micronutrient affecting protein synthesis and required at the early stage of seed development [10]. Several studies are available showing that the concentrations of Zn in seeds are positively correlated with germination [21]. The negative effect of salinity on growth of mungbean could be mitigated by application of zinc [22]. Zn is required in the actively growing parts of plants and is highly mobile in germinating seeds. Mrozek and Funcelli [23] reported that germination capacity of seeds was inhibited by Cd or As and stimulated by Zn. The simulative effect of zinc on germination and seedling vigour, observed in the present study agrees with the findings of. Excess Zn inhibits seed germination, plant growth [23] as observed in the present experiment. The retardation in seedling growth could be attributed due to high accumulation of heavy metal [24,25].

Our investigations showed alterations in the activities of the catalase enzymes, FARP activity and phenolic content which play an important role in the defence mechanism of plants in relation to As and Cd exposure. Cd and As inhibited catalase activity has been shown by several workers [26]. Cd and As induce accumulation of H_2O_2 can result in the inactivation of catalase followed by decrease of its activity [27]. It might be due to inhibition of enzyme synthesis or change in enzyme conformation [28]. Our results agrees with the findings of Hamid *et al.* [29] who found that phenolic content of plants were decreasing with increasing levels of heavy metal. Phenolic compounds are shown to have strong antioxidant activity increased with treatment of zinc. The zinc treatment resulted in a considerable rise of catalase enzyme activity, FRAP activity and phenolic content in seedling exposed either to arsenic or cadmium, fact proving activation of detoxification mechanisms. Zn has protective effect against photoxidative damage catalyzed by ROS in chloroplasts [30].

Increased levels of proline in seedling under heavy metal might be due to protein degradation. Proline has been shown to alleviate metal-induced oxidative stress by scavenging harmful ROS [31]. Proline is known to accumulate under heavy metal exposure and considered to involve in stress resistance. Free proline has been found to chelate metal ion in plants by forming non-toxic metal-proline complex and protect enzymes and cellular structures [32]. Similarly, the uptake and toxicity of Cd and As can be moderated significantly in the presence of essential metal nutrients like Zn [33]**.** According to our results, zinc supplementation has significantly decreased the accumulation of cadmium and arsenic in seedling. Zn as a micronutrient could have a positive effects on final yield but if concentration of Zn exceeded from optimum level, it could have toxic effects on plant [34]. Cd and zinc have been found to be accumulated in plants by the same transporters [35] which reduced the accumulation in seedling with zinc treatment in present experiment.

CONCLUSION

The present study revealed that the growth inhibitory effect of cadmium and arsenic on *Phaseolus aureus Roxb* has been reduced significantly by the application of lower level of zinc (25 ppm). Application of zinc could not overcome the toxicity of cadmium and arsenic beyond levels of 50 ppm. Therefore, it suggested that for optimal use of zinc as a fertilizer, first an experiment performed to check zinc level in farm soil and irrigational water before starting to planting and using fertilizers.

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