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Effect of Arsenic-induced Toxicity on Morphological Traits of *Trigonella foenum-graecum* L. and *Lathyrus sativus* L. During Germination and Early Seedling Growth

Dibyendu Talukdar
Department of Botany, R.P.M. College, University of Calcutta, Uttarpara, Hooghly, West Bengal, 712258, India

Abstract: Effect of five different concentrations (0, 10, 20, 30 and 40 mg/L) of arsenic was studied on 11 different parameters of two important leguminous crops, namely *Trigonella foenum-graecum* L. (fenugreek) and *Lathyrus sativus* L. (grass pea) during germination and early seedling growth stage. Mean value of germination percentage, germination index and relative germination rate decreased with concomitant increase in arsenic-induced injury level in increasing concentration of arsenic in both plants and the effect was significant at 30 and 40 mg/L treatments. Fresh and dry weight of shoots, roots and their length also reduced significantly in these two treatment levels. There was significant accumulation of arsenic in tissues, and the effect was more severe on root than shoots. Based on the parameters responding to arsenic-induced stress, Arsenic Response Index (ARI) was developed for the first time in these two crops. Among the two crops, *T. foenum-graecum* L. exhibited better tolerance to arsenic-induced toxicity than *L. sativus* L. Considering mean and ARI value, 30 mg/L of arsenic was selected as toxic to *L. sativus* L., while the limit was 40 mg/L for *T. foenum-graecum* L. at the stages of germination and early seedling growth.

Key words: Arsenic response index, germination, *Lathyrus sativus* L., seedling growth, stress, *Trigonella foenum-graecum* L.

INTRODUCTION

Primarily as a sequel to drawing up huge quantities of groundwater by shallow tube-wells for agricultural purposes, and also some other factors like industrialization and modern agricultural practices, many aquifers have now been contaminated with various toxic metal compounds including arsenicals. The digging of tube-wells for drinking water supply into aquifers with high arsenic (As) concentration in southeast Asia has been described as the greatest mass poisoning in human history (Smith *et al*., 2000), with an estimated 51 million people or more exposed to elevated As in their drinking water in Bangladesh and West Bengal alone (Pearce, 2003). In recent times, the impact of irrigation with high As groundwater on soil and crop has now drawn more attention due to transfer of As to the food chain via groundwater-plant-soil system (Meharg and Rahman, 2003; Das *et al*., 2004; Rahman *et al*., 2008). The bioaccumulation of As in different crop plants including cereals, beans, vegetables and fruits has huge negative impact for public health issues in both rural and urban population, (Nickson *et al*., 1998; Katz and Salem, 2005), and this is of great environmental concern because arsenic is known to be a carcinogen and a powerful co-mutagen (Patra *et al*., 2004; Fayiga and Ma, 2006).

Both *Trigonella foenum-graecum* L (fenugreek or commonly as 'methi') and *Lathyrus sativus* L. (grass pea or commonly known as 'khesari') are cultivated as annual, winter legumes in India. Both the species can be grown easily in low-input, marginal environment, and are promising sources of calories, seed proteins, B-vitamins and minerals. Major fenugreek producing countries are India, Pakistan, Afganistan, Bangladesh, Argentina, Egypt, France, Yemen, Spain, Turkey, Morocco and China, and India is the largest producer of fenugreek in the world (Zohary and Hopf, 2000). In India, fenugreek or 'methi' is extensively used as spice, leafy vegetable and in medicine as a carminative, analgesic, anti-inflammatory as well as tonic for gastric troubles, diabetes, leucorrhea and as an important source of steroidal substance, diosgenin. The seeds of *Trigonella foenum-graecum* L. contain the most potent medicinal effects of the plant, and the role of phyto-estrogens and diosgenins to fight breast cancer and reduction of serum cholesterol has been widely recognized (Amin *et al*., 2009). On the other hand, grass pea has been grown for both food and forage in different parts of the world including Australia, the Mediterranean countries, North Africa, South America and predominantly in Indian subcontinent including several states of eastern, north-eastern and south-western part of India (Jackson and Yunus, 1984; Pandey *et al*., 1996; Campbell, 1997; Talukdar, 2009b). A renewed interest in

grass pea cultivation in different parts of Europe and its reintroduction in China is justified by the urgent need to recover marginal lands and to provide an efficient alternative for sustainable agriculture in low-input conditions (Granati *et al*., 2003; Yang and Zhang, 2005; Vaz Patto *et al*., 2006). However, vast areas of arsenic contaminated regions in different parts of the world including Bangladesh and state of West Bengal in India have been used for cultivation of these two pulse crops without any preliminary assessment of its toxic effect. The possibility of bioaccumulation of As in both *Trigonella* and *Lathyrus* therefore, may pose a serious threat to both human and cattle health.

Development of safe legume crops for cultivation in arsenic contaminated soil is an important strategy to counter the detrimental effect of As accumulation in proteins and mineral rich legumes. Minimizing the uptake and translocation of As to edible parts would form the basis for improving crops (Tripathi *et al*., 2007) for which understanding of different morpho-physiological traits is extremely important from very early stage of growth and development (Bayuelo-Jiménez *et al*., 2002). Seed germination is the most critical first sage in seedling establishment, determining successful crop production (Almansouri *et al*., 2001). Many abiotic stress facors, like salinity, drought, are known to inhibit seed germination in different magnitudes leading to loss of crop establishments in legume crops (Bayuelo-Jiménez *et al*., 2002; Li, 2008). In *Trigonella foenum-graecum* L. and *Lathyrus sativus* L. rate of seed germination was affected only at high concentration of NaCl (Asaadi, 2009; Mahdavi and Sanavy, 2007). Among the legumes, effect of arsenic was studied in species of *Vigna*, *Pisum sativum* L., *Cicer arietinum* L., *Trifolium pratensis* L. and *Glycine max* Merril (Meharg and Hartley-Whitaker, 2002). Both *Trigonalla* and *Lathyrus* exhibited moderate level of tolerance to different abiotic stress responses (Vaz Patto *et al*., 2006; Sinha *et al*., 2007). Therefore, there remains the scope of improvement of these two crops against different soil stress factors including arsenic if appropriate parents and breeding techniques are adopted from very early stage of growth. Moreover, different genetic and cytogenetic tester stocks including trisomics, tetrasomics, polyploids and translocations have recently been developed in *L. sativus* L. (Talukdar, 2008, 2009a, c, 2010a, b). These stocks can be used to explore the genetic basis of metal stress tolerance in legume crops more effectively if comprehensive knowledge on different traits responsive to stress can be achieved. However, perusal of literature cites no information about the effect of arsenic on seed germination traits of these two crops. To improve the reliability and selection efficiency for arsenic tolerance, knowledge on arsenic-induced response of genotypes at the germination stage is particularly important for successful stand establishment and growth. The objectives of the present study, was therefore, to 1) investigate the effect of arsenic in different concentrations on seed germination traits of *Trigonella foenum-graecum* L. and *Lathyrus sativus* L. and 2) to develop a Arsenic Response Index (ARI) for these two crops at germination and early seedling growth stage.

MATERIALS AND METHODS

Plant materials: Dry, healthy and uniform-sized seeds of *Trigonella foenum-graecum* L. collected from farmers' fields and *Lathyrus sativus* L. improved variety BioL-212, collected from Pulses and Oil seeds Research Station, Berhampur, West Bengal, India were used in this experiment. The crops are well adapted in soil and climatic condition (soil pH 7.0, clay-alluvial, temperature 30/18ºC (day/night), relative air humidity 72±5% and 10-12 h photoperiod) of Gangetic West Bengal during winter season. The experiment was conducted during October-November of 2008 and 2009 at a research farm in Kalyani (22º59'N/88º29'E), West Bengal, India.

Seed germination and measurement of growth: Seeds of both crops were incubated at 4ºC for a few days and then surface-sterilized with 5% sodium hypochlorite. The experiment was carried out under 70% relative humidity at 25ºC with a 12 h photoperiod following a guide line of ISTA (2008) to test the germination in five different concentrations of arsenic (0, 10, 20, 30 and 40 mg/L), prepared as solution of sodium arsenate, in three replicates with completely randomized block design. A sample of 20 dry and healthy seeds of each genotype per treatment per replicate was sown in 9 cm diameter petriplates with a tight-fitting lid on two filter papers, and 9 mL of distilled water was used to soak filter papers for the first treatment. This was used as control set. Seeds were allowed to germinate in above described condition on filter paper (Whatman No. 2) in Petri dishes soaked in a solution of the respective arsenic concentration. The seed germination was evaluated after every 12 h. Seeds were considered to be germinated with the emergence of both plumules and radicles. The germinating seeds were counted at daily intervals. Germination percentage, germination index, relative germination rate and relative arsenic-injury rate were determined in each concentration (mean of three replicates) by the following formulas of Li (2008) with some modifications for the present materials:

$$\text{Germination percentage} = \frac{\text{Total no. of seeds germinated} \times 100}{\text{Total no. of seeds taken for germination}}$$

Germination index (GI)

$$= \frac{\text{Total no. of germinated seeds}}{\text{Total no. of germination days required}}$$

Relative germination rate

$$= \frac{\text{Germination percentage in arsenic concentration}}{\text{Germination percentage in control}}$$

Relative arsenic-injury rate

= (Germination percentage in control - Germination percentage in arsenic treatment) / Germination percentage in control

After 15 days of sowing, final germination percentage was calculated. Length and fresh weight (g) of shoot and roots were then measured. After that, the seedlings were oven dried at 65ºC for 72 h, and dry weight (g) was estimated. Tolerance to arsenic was determined by calculating arsenic response index, ARI = (value from salt treatment) /(value from control) × 100% for each trait. The experiment was repeated in two consecutive seasons, but no significant difference was found across seasons. Thus, data from one season is presented here.

Estimation of arsenic from plant samples: Plant samples were collected and brought to the arsenic laboratory with proper labeling, to monitor the arsenic accumulation in different parts of the plant. The samples were appropriately labeled, dried in an air-oven at 105ºC for 24 h. Then a portion (1 g) of the ground samples were digested on a sand bath with tri-acid mixture (HNO_3: H_2SO_4: $HClO_4$: 10 : 1 : 4 , by volume) to obtain a clear digest. The arsenic content of the digested sample was measured by the use of AAS (Perkin Elmer AA-400, Norwalk, CT, USA) following manufacturer's instruction.

Statistical analysis: Statistical analyses were performed using software STATISTICA 6.0 (StatSoft, Inc. Tulsa, U.S.A) by one-way ANOVA and t-test. A probability of $p<0.05$ was considered significant.

RESULTS

Arsenic-induced stress response at germination stage: At germination stage, the two crops behaved in similar fashion in control, 10 and 20 mg/L As treatments, but significant variation ($p<0.05$) in mean value of germination percentage, germination index and rate of germination was estimated between two genotypes at higher concentration of 30 mg As/L and 40 mg As/L (Table 1). Concentration at which means of three seed germination traits reduced to nearly 50% level of control value was considered as critical. At 0 mg/L (used as control) germination was 100%, and at 10 mg/L mean value for the trait was 95 and 85% for *T. foenum-graecum* and *L. sativus*, respectively. Thereafter, it began to decrease in different magnitudes at higher levels of arsenic concentration, and 50% reduction of control level was recorded in *Trigonella* at 40 mg/L treatments and in *Lathyrus* at 30 mg/L As concentration (Table 1).

Germination Index (GI) was not significantly different between control and 10 mg/L As concentration in the two varieties. However, at 20 mg/L, the index value decreased by 1.2-fold in *Trigonella* and by 1.3-fold in *Lathyrus*, and 50% reduction of control value was recorded at 30 mg/L in *Lathyrus* and at 40 mg/L in *Trigonella* (Table 1). Arsenic Response Index (ARI) for germination index was 100% at control (0 mg/L) treatment, but it then reduced following the trend of mean values of these two traits in both the varieties (Fig. 1). Lowest ARI (44%) was recorded in *L. sativus* at 40 mg/L As treatment.

Relative rate of germination was found reduced with increasing concentration of arsenic in both varieties. At 0, 10 mg As/L and 20 mg/L, the germination rate was comparable, but at higher concentration this rate began to

Table 1: Effect of five arsenic treatments on germination percentage, germination index relative germination rate and injury rate in *Trigonella foenum-graecum* L. and *Lathyrus sativus* L.

	Treatment (mg/L)				
Traits+	0 (control)	10	20	30	40
Germination percentage					
T. foenum-graecun L.	100±0.00	95±0.04	80±0.02	65*±0.05	45*±0.07
L. sativus L.	100±0.00	85±0.02	80±0.06	50*±0.05	35*±0.01
Germination index					
T. foenum-graecum L	5.0±0.01	4.61±0.05	4.29±0.04	3.75*±0.02	2.50*±0.1
L. sativus L.	5.0±0.02	4.52±0.03	4.00±0.07	2.60*±0.08	2.22*±0.1
Relative germination rate					
T. foenum-graecum L.	1.00±0.00	0.95±0.04	0.80±0.02	0.65*±0.05	0.45*±0.07
L. sativus L.	1.00±0.00	0.85±0.02	0.80±0.06	0.50*±0.05	0.35*±0.01
Injury rate					
T. foenum-graecum L.	0.00±0.0	0.05±0.03	0.20±0.02	0.35±0.05	0.55±0.07
L. sativus L.	0.00±0.0	0.15±0.02	0.20±0.06	0.50±0.04	0.65±0.00
One-way ANOVA					
Genotypes(F-value)	ns	ns	ns	*	*

+: values are means of three replicates with ± standard error; *: significant at 5% level; ns: not significant

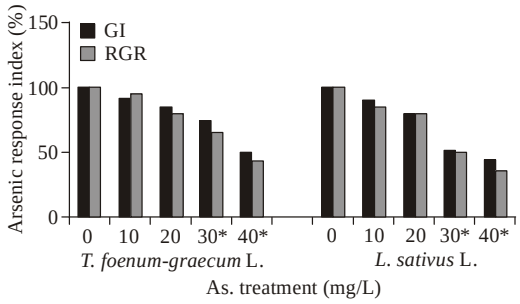
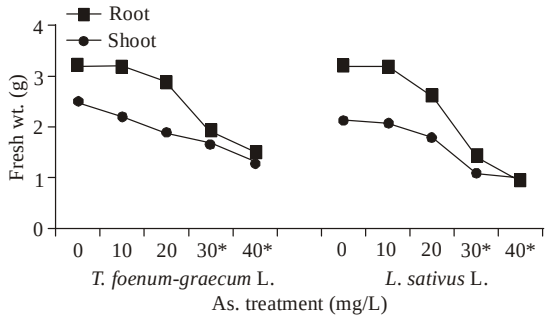


Fig. 1: Arsenic response index (ARI) for germination index (GI) and relative germination rate (RGR)



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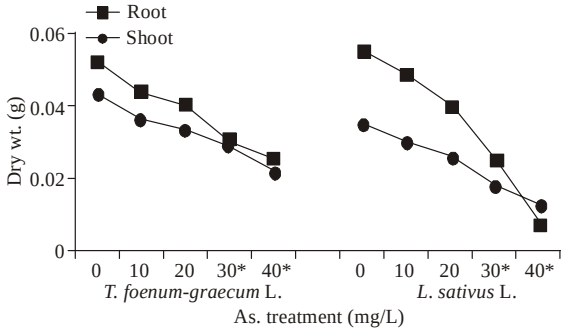
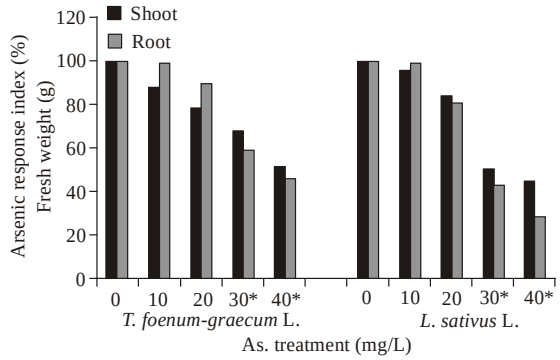


(b)

Fig. 2: Change in mean value of fresh and dry weight of roots and shoots, respectively, note the effect on root at 30 and 40 mg/L As treatments

decrease. As compared with control, the reduction was significant for both the varieties at 30 mg/L (1.5-fold in *Trigonella* and 2.0-fold in *Lathyrus*) as well as 40 mg/L (2.2-fold in *Trigonella* and 2.9-fold in *Lathyrus*) treatment (Table 1). This parameter also indicated the value of ARI for rate of germination (Fig. 1).

Relative arsenic-injury rate calculated on the basis of germination percentage in control and treated population in each of the five treatments was 0 in control treatment, but the value increased with increasing concentration of As in both the crops (Table 1). In comparison to control, the injury rate increased marginally in 10 and 20 mg/L



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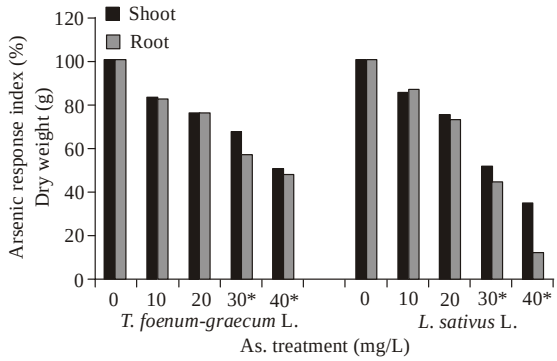


(b)

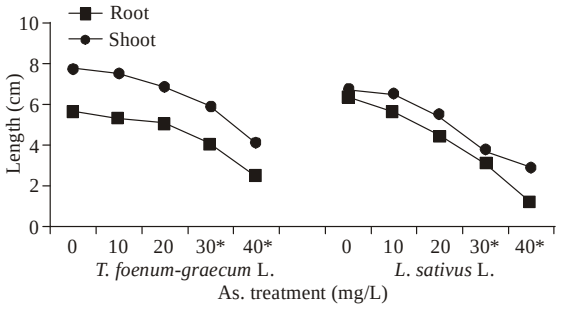
Fig. 3: ARI for fresh and dry weight of roots and shoots, respectively

treatment, but the rate became significant from 30 mg/L As level. The two crops differed significantly by relative injury rate at 30 and 40 mg/L treatments, and highest injury was manifested by *Lathyrus* seeds at 40 mg/L As treatment (Table 1). Arsenic-induced injury being 0 in control treatment ARI value of this parameter was not calculated.

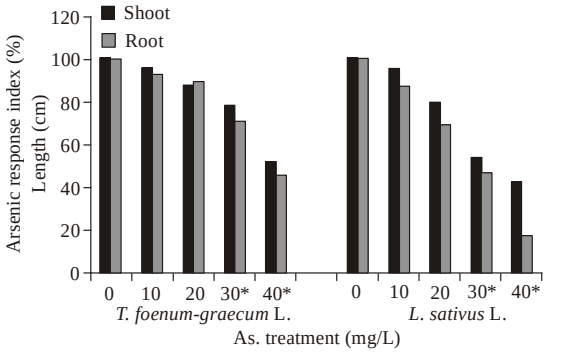
Arsenic-induced stress response at early seedling growth stage: Seven parameters were tested on 15-day (days after sowing) old seedlings under As treatment. Mean value for fresh weight (g) of root (FWRG), dry weight (g) of root (DWRG), fresh weight (g) of shoot (FWSG), dry weight of shoot (DWSG), length (mm) of root (LR) and length (mm) of shoot (LS) decreased with increasing concentration of As. In comparison to control, the reduction in the means of fresh as well as dry weight of shoot and root was not significant at 10 mg/L treatment in both the crops. However, mean values of these four traits reduced significantly at 30 mg/L, and as compared to control, 50% reduction of the traits was estimated at 30 mg/L in *L. sativus* and at 40 mg/L for *Trigonella foenum-graecum* L. In both crops, fresh and dry weight of roots were much higher than those of shoot at initial stage of

treatment, but it reduced drastically from 30 mg As/L treatment and touched the value of shoot (Fig. 2a and b). The effect was more severe in *L. sativus* L. than in *T. foenum-graecum* L with significant differences ($p<0.05$) between crops were estimated at 30 and 40 mg/L As treatment. ARI calculated from mean value of both treated and control population was 100% for the traits at control treatment, and as compared to it, no significant change was observed at 10 and 20 mg/L As treatment (Fig. 3a and b). At 30 mg/L, ARI for FWRG and DWRG was 59% and 57% in *T. foenum-graecum* and was 43 and 45% in *L. sativus* L, respectively. ARI for FWSG and DWSG at 30 mg/L was calculated as 68 and 67% in *Trigonella* and 50 and 51% in *Lathyrus* variety BioL-212. At 40 mg/L, reduction in ARI value for these four traits was higher in *L. sativus* than *T. foenum-graecum* L (Fig. 3a and b).

Mean and ARI for length of 15 d old root (LR) and shoot (LS) also varied significantly ($p<0.05$) between 0 mg/L and concentration higher than 20mM (Fig. 4a and b). In general, ARI for LR in five treatments varied 0%-%, and for LS it ranged between 0% and % in *Trigonella* and 0%-% for LR and 0%-% For LS in *Lathyrus*.



(a)



(b)

Fig. 4: Change in mean value and ARI for length of shoot and root, respectively

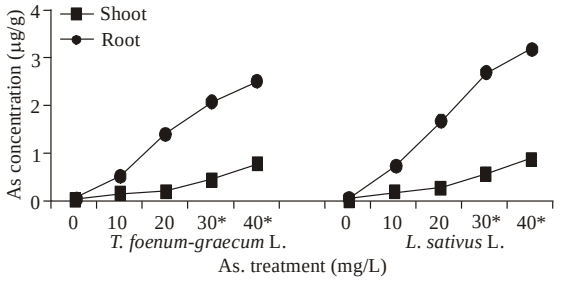


Fig. 5: Arsenic accumulation in shoot and root indicating higher accumulation in roots than shoots

Accumulation of arsenic in 15-day old root was considerably higher than shoot (Fig. 5) in both the crops. It increased with increasing doses of As, and in comparison to its accumulation in shoots, it increased 4.7-fold in *Trigonella* and 5.1-fold in *Lathyrus* roots at 30 mg As/L treatment. The differences in accumulation of arsenic between shoots and roots were further widened at 40 mg As/L treatment, and highest accumulation was estimated in *Lathyrus sativus* L. roots at this level.

DISCUSSION

Response of two crops to five different regimes of arsenic (As) treatments was tested by mean values of germination percentage, germination index and its rate, relative injury rate during germination and fresh and dry weight of root and shoot and their length at early seedling stage. Barring relative injury rate and arsenic concentration in tissues, Arsenic Response Index (ARI) value was calculated on the other nine parameters. Results in Table 1 and Fig. 1-5 indicated decrease in germination percentage, germination index and germination rate under As treatment of 20, 30 and 40 mg/L, and as compared to control the values were significant at 30 and 40 mg/L. Two crops also differed at these two treatments. Mean value and corresponding ARI value suggested that *Trigonella foenum-graecum* L. seeds could germinate well up to 30 mg/L, while significant decrease in germination percentage in seeds of *Lathyrus sativus* L. was observed in this treatment. This led to 50% reduction of germination percentage at 30 mg/L in *Lathyrus* as compared to *Trigonella* (40 mg/L).

Both mean and ARI values of Germination Index (GI) decreased with increasing concentration of As in both the varieties. However, the values were higher in *Trigonella* than *Lathyrus* in all the treatment regimes, indicating shorter time required for *Trigonella* seeds to germinate than *Lathyrus* seeds under As-induced stress. Higher increase in corresponding number of germination days attributed to lower GI value in *Lathyrus sativus* seeds than *Trigonella*.

Reduction in relative rate of germination as compared to control was exhibited with increasing concentration of As, and the effect was significant at 30 and 40 mg/L treatments for both the varieties. However, 50% reduction of control value for the trait was exhibited by *Trigonella* at 40 mg/L and by *Lathyrus* seeds at 30 mg/L, suggesting better tolerance of former in As-induced toxicity during germination stage.

Cumulative effect of As treatment at different concentrations on germination percentage was manifested by As-induced injury rate. The relative As-induced injury rate increased with increasing concentration of As in both the varieties with severest effect was recorded at 40 mg/L treatment. Injury rate was 0 at control and marginal difference with control at 10 mg/L and at 20 mg/L suggested tolerance of both the genotypes at lower concentration of As. Significant increase in injury rate at 30 and 40 mg/L was mainly due to decrease in seed germination percentage of both the varieties. However, value was much higher in *Lathyrus* than *Trigonella*, revealing better tolerance of *Trigonella* than *Lathyrus* at higher level of As treatment.

Among the parameters responding to As-induced toxicity at early seedling stage, reduction in length as well as weight of both root and shoot was highly significant in both the crops at higher treatment regimes. Munns (2005) described dry weight measurement as one of the realistic criteria in stress perception as it manifested cumulative effect of stress on different parameters at particular growth stage. Mean and ARI values for these six traits reduced under As-induced stress. Significant differences between the genotypes were also observed for these traits, and *Trigonella* performed better than *Lathyrus*. It was important to note that reduction in fresh weight, dry weight and length of root was more than those of shoot, indicating higher sensitivity of growing roots to As treratment in both crops. Inhibition of root elongation is considered one of the frequently observed symptom of metal toxicity (Wang *et al*., 2003) This finding was in accordance with report of significant reduction of root length in As-treated different rice varieties (Dasgupta *et al*., 2004). Arsenate is the dominant form of arsenic in aerobic soils and is an analogue of phosphate, competing for the same uptake carriers in the root plasmalemma (Meharg and Macnair, 1992). Studies on arsenate toxicity have shown that plant species not resistant to arsenic suffer considerable stress upon exposure, with symptoms ranging from inhibition of root growth through to death (Macnair and Cumbes, 1987; Paliouris and Hutchinson, 1991; Barrachina *et al*., 1995), although mechanisms of arsenite uptake by plant roots are still not clearly understood.

Over-accumulation of arsenic in roots as compared with shoots in 15-d-old seedlings in the present material of *Trigonella* and *Lathyrus* might be due to the fact that the upward transport of As from roots was inhibited by its high toxicity to the membranes of radicle (Wauchope, 1983; Barrachina *et al*., 1995). Higher accumulations of arsenic in roots than stem and leaves has also been reported in many other leguminous crops like cowpea (Huq *et al*., 2009), lentil (Ahmed *et al*., 2006), alfalfa (Maclauchlan *et al*., 1988), *Prosopis* (Mokgalaka-Matlala *et al*., 2008) and in many non-leguminous crops like arum (Parvin *et al*., 2006), turnip (Carbonell-Barrachina *et al*., 1998), marigold and ornamental arum (Huq *et al*., 2005), rice (Huq and Naidu, 2005), wheat (Kapustka *et al*., 1995) and in lodgepole pines (Yamare, 1989).

Response of legumes to arsenic-induced toxicity was investigated in a limited number of crops, but no study was carried out during germination. In *Glycine max* Merril., Milivojević *et al*. (2006) reported decrease in phosphorus content with increasing As concentration, while in *Pisum sativum* L. Paivoke (2003) found negative correlation between growth parameters and arsenate treatment. Negative impact of As-induced toxicity on plant growth parameters and yield components was also reported in lentil (Ahmed *et al*., 2006).

CONCLUSION

In conclusion, it can be said that the phytotoxic effect of arsenic was manifested by significant reduction of different parameters at germination and seedling growth stage in both the plants. Considering the 50% reduction of control level in mean value of 10 parameters- seed germination percentage, germination index, relative germination rate and relative As injury rate, FWRG, DWRG, FWSG, DWSG, LR and LS and Arsenic Response Index (ARI) value of nine traits (except injury rate) as determining criteria for As tolerance, it was concluded that both the crops are tolerant to As treatment up to 20 mg/L, but became sensitive at higher concentration during early growth stage. However, *Trigonella foenum-graecum* L. showed better tolerance than *Lathyrus sativus* L. The level of 30 mg As/L was concluded as toxic to *L. sativus* L, while the limit was 40 mg As/L for *T. foenum-graecum* L. during germination and early seedling stage. It was worth mentioning that both the plants showed capability to accumulate significant amount of arsenic in their tissues and root accumulated higher level of arsenic than shoot. The information gathered may provide a better strategy in understanding of As-induced toxicity in legume crops in future breeding programs.

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