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Arsenic accumulation in root and shoot *vis-a-vis* its effects on growth and level of phytochelatins in seedlings of *Cicer arietinum* L.

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Abstract: *Arsenic (As) contamination of water and soil has become a subject of prime interest due to its direct effect on human health through drinking water and food. In present study, two varieties (CSG-8962 and C-235) of chickpea, Cicer arietinum L., which is a major supplementary food in many parts of India and a valuable source of protein, has been selected to estimate the level of arsenate in root and shoot of five day old seedlings vis-à-vis effect of arsenate on seedling growth and induction of thiols including glutathione (GSH) and phytochelatins (PCs) and their homologues. Both varieties accumulated arsenate to similar levels and most of the metalloid was confined to roots, only about 2.5% was translocated to shoot. Plant growth was also not affected significantly in both the varieties. Arsenate exposure significantly induced the levels of thiols including PCs and homophytochelatins (hPCs). The induction of thiols was much higher in roots than shoots and was greater in var. C-235 between the two tested ones. Thus, both varieties tolerated and detoxified arsenic through chelation with GSH, PCs and hPCs, primarily in roots, however var. C-235 performed better.*

Key words: *Arsenic, Chickpea, Homoglutathione, Glutathione, Phytochelatins, Homophytochelatins*
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Introduction

Arsenic contamination is increasing day by day in many regions of the world and has become a global problem. The increased level of arsenic has mainly resulted from mining, industrial, agricultural and geochemical processes. Fly-ash, a by-product of thermal power plants, also contains arsenic and contributes to the arsenic pollution in the fly-ash affected areas. In industrialized nations, contamination is highly localized and pressure to use contaminated land and water by human is minimal (Kramer, 2005; Flora *et al*., 2007). But in developing countries, such as India and Bangladesh, which are among the most populated areas of the globe, there is pressure to cultivate on contaminated lands to feed the population (Meharg, 2004; Pandey, 2006) and it is difficult to clean such a huge area. Thus problem is dramatically severe due to food chain contamination and the only solution is to localize the metalloid in non-edible parts of the crops and seedlings (Sahu *et al*., 2007). It needs a thorough screening of varieties capable to grow on contaminated sites with good growth, yield and the potential to localize the metalloid in roots.

Plants synthesize low molecular weight peptides called phytochelatins (PCs), which are the polymers of GSH that make complexes with metals and metalloids and sequester them to vacuoles and thus detoxify their toxicity (Grill *et al*., 2006). Besides PCs, legumes are also known to synthesize homophytochelatins (hPCs), which contain homoglutathione (hGSH) instead of GSH, having the N-terminal β-alanine (Grill *et al*., 2006). Arsenic is reported to significantly induce the synthesis of PCs as well as hPCs (Gupta *et al*., 2004; Tripathi *et al*., 2007) and the complexes of arsenic with PCs as well as GSH have been demonstrated in various plants (Raab *et al*., 2004, 2005).

Thus it is worthwhile to study the accumulation and root-to-shoot partitioning of the metalloid, effect on growth and detoxification mechanisms in the edible plants. Chickpea (*Cicer arietinum* L.) is a supplementary food crop fulfilling major vegetarian protein requirement. The present study deals with the comparison of two varieties of chickpea, CSG-8962 and C-235, with respect to arsenate accumulation in roots and shoots *vis-a-vis* its effect on seedling growth and detoxification through thiols.

Materials and Methods

Two varieties of *C. arietinum* L., CSG-8962 and C-235, were selected for the present study. The seeds of test varieties were procured from Central Pulse Research Institute, Kanpur, U.P. The seeds were soaked overnight in double distilled water (ddw) and then kept in dark for 24 hr for germination. The germinated seeds were grown hydroponically for 4 days under a white fluorescent light (ca. 200 $\mu E\ m^{-2}\ s^{-1}$) at 25°C in glass flasks containing 150 ml 50% Hoagland medium (Haogland and Arnon, 1950) at pH 6.0. Seedlings were exposed to 20 μM arsenate, prepared by dissolving the salt sodium arsenate (Na_2HAsO_4) in 50% Hoagland solution, for 2 days. The seedlings kept in 50% Hoagland not containing arsenate served as control. After 2 days, seedlings were harvested, washed with ddw, blotted and separated in root and shoot. Length of root and shoot was measured with the help of metric scale and biomass was measured on fresh weight basis. For estimation of arsenate, samples were prepared and analyzed following Bleeker *et al.* (2003). Dried plant material (100 mg) was powdered and total arsenate was extracted by digesting in 2 ml 37% (v/v) HCl: 65% (v/v) HNO_3 (1:4

v/v) at 140°C for 7 hr, after which the volume was adjusted to 10 ml with demineralized water. Arsenate concentrations were determined on a flame atomic absorption spectrophotometer (GBC Avanta Σ, Australia), which was coupled to a GBC Hydride Generation System (HG900) for arsenate estimation.

For analysis of PCs, hPCs and related thiols, plant material was extracted in an equal volume (1 ml g^{-1} tissue) of 10% (w/v) of 5-sulfosalicylic acid (SSA) at 4°C as described previously (Inouhe *et al*., 1994). The separation of PCs and hPCs in acidic extracts was carried out by post column derivatization with 75 µM 5, 5'-dithiobis (2-nitrobenzoic acid) in 50 mM phosphate buffer on a reverse-phase HPLC column (Hibar Lichrosorb RP-18, Cica-Merck, Darmstadt, Germany) connected to a pump (L-7110, Hitachi, Japan) following the method of Mendum *et al*. (1990) with some modifications as described previously (Gupta *et al*., 2005). The peak of various thiols was monitored at 412 nm using a UV-visible detector (L-7420, Hitachi).

Results and Discussion

The accumulation potential of both varieties was similar and found to be low (Table 1). Most of the metalloid accumulated was confined to the roots and only small proportion *i.e.* 2.5% was translocated to shoots in both varieties. The growth of the plants exposed to arsenate was determined in terms of effects on length and weight of roots and shoots. In both varieties, plant growth was not affected to significant levels by the metalloid (Table 2). Though control plants of var. CSG-8962 showed better growth than var. C-235, after exposure to arsenate, var. C-235 performed better than var. CSG-8962. The root length of var. CSG-8962 exposed to arsenate was similar to that of control while root and shoot weight and shoot length slightly decreased. In var. C-235 either no effect or a slight increase in root and shoot growth parameters was observed upon exposure to arsenate (Table 2).

Table - 1: Accumulation of arsenic in roots and shoots of *Cicer arietinum* L. var. CSG-8962 and C-235 after 2 day of exposure to 20 µM arsenate. Values are mean of triplicates ± SD

Cultivar	Plant tissue	As content (µg g^{-1} dw)
Var. CSG8962	Roots	47.34±2.29
	Shoots	1.17±0.047
C-235	Roots	45.32±3.51
	Shoots	1.08±0.033

The HPLC profile of thiols has been presented in Fig. 1, 2. The peaks of cysteine, GSH, hGSH, PC_2, hPC_2, PC_3 and hPC_3 have been identified and quantified in Table 3, 4. Control profiles of roots showed peaks of cysteine, GSH, hGSH and PC_2, whereas that of shoots showed only cysteine, GSH and PC_2. The level of cysteine, GSH and PC_2 was higher in shoots than in roots was higher in both varieties and was higher in var.CSG-8962 when compared between the two varieties in control plants. Arsenic exposure significantly induced the level of all the thiols with more being in roots than shoots of both the varieties. The maximum level of cysteine was observed in shoots of var.CSG-8962 exposed to 20 µM arsenate which was about 2-fold higher than in control. The level of GSH and hGSH were induced by about 20-fold in roots of var.235, whereas by about 14 and 9 fold, respectively in var. CSG-8962. Among PCs only PC_2 was found to be in detectable levels in control plants, however arsenate exposure induced significantly high amount of PCs and hPCs. In roots, the level of PC_3 was maximum followed by hPC_3>PC_2>hPC_2 in both varieties and the total amount of PCs (PC_2+hPC_2+PC_3+hPC_3) was higher (about 2 fold) in C-235 than CSG-8962. In shoots, PCs followed the order PC_2>hPC_2>PC_3 and the total amount of PCs (PC_2+hPC_2+PC_3) was about 4-fold higher in var. C-235 than var. CSG-8962.

Arsenate is taken up by plants through phosphate transporters (Abedin *et al*., 2002) and is also transported through phosphate channels (Dhankher *et al*., 2006). The less accumulation of arsenate in the present study by two varieties of chickpea may be due to high affinity of transporters for phosphate than arsenate. In many plants arsenic tolerance is achieved through suppression of high-affinity phosphate/arsenate transporter (Macnair *et al*., 1992). Further, phosphate and arsenate exert antagonistic effect on each other during plant uptake and transport (Meharg and Macnair, 1992). Phosphate is reported to reduce the influx of arsenate in the arsenic tolerant *Holcus lanatus* and hyperaccumulator, *Pteris vittata* (Hartley-Whitaker *et al*., 2001; Tu and Ma, 2003). In the present study also, the nutrient medium contained high amount of phosphate (500 µM), which might have suppressed the uptake. The observed less translocation of arsenate in this study may be attributed to competition of phosphate with arsenate for root to shoot translocation. Their chelation and sequestration in the roots would have also been responsible for the less transportation to the shoots.

Less effect on growth parameters is attributed to less accumulation and efficient detoxification of the metalloid. In var.

Table - 2: Effect on root and shoot growth of *Cicer arietinum* L. var. CSG-8962 and C-235 after 2 day of exposure to 20 µM arsenate. Values are mean of triplicates ± SD

Cultivar	Treatment	Growth parameters			
		Root length (cm)	Root weight (g)	Shoot length (cm)	Shoot weight (g)
Var. CSG8962	Control	13.52±0.86	0.29±0.02	18.86±1.44	0.28±0.04
	20 µM As	13.51±0.50	0.24±0.05	16.00±0.29	0.25±0.03
C-235	Control	12.13±1.04	0.18±0.09	14.60±1.51	0.17±0.03
	20 µM As	12.82±1.05	0.20±0.03	14.63±1.02	0.19±0.02

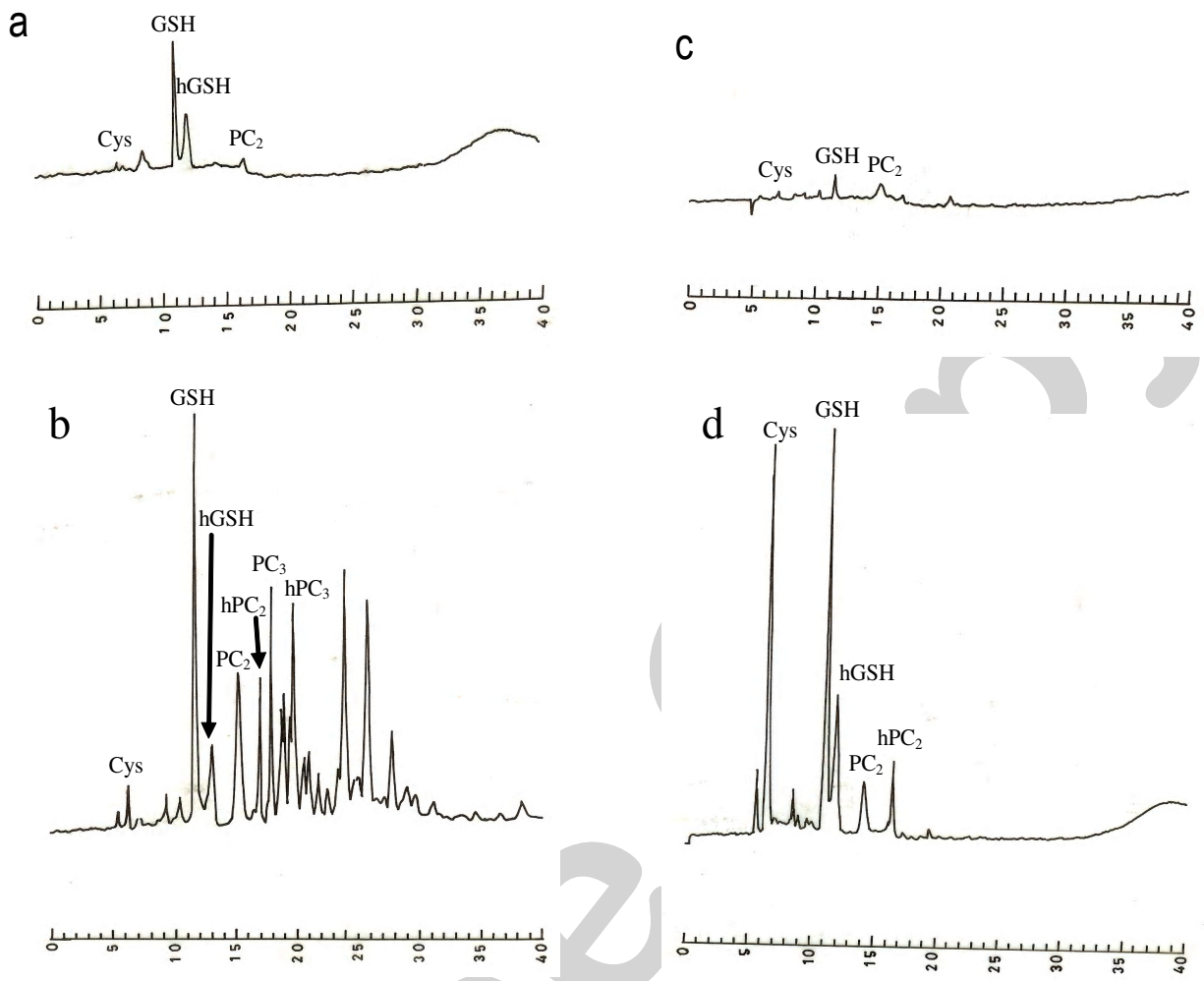

Fig. 1 : HPLC-profiles of PCs, hPCs and related thiols in crude extracts of *Cicer arietinum* L. var. CSG-8962 exposed to 20 µM arsenate for 2 day in (a) control root, (b) 20 µM arsenate root, (c) control shoot and (d) 20 µM arsenate shoot. The root and shoot tissues were separately frozen at -80°C in the presence of 100 mM sodium ascorbate and immediately extracted with 10% SSA. The extract was analyzed by post-column HPLC method (Mendum *et al*., 1990)

Table - 3: Induction of PCs and hPCs and effect on level of thiols in roots of *Cicer arietinum* L. var. CSG-8962 and C-235 after 2 day of exposure to 20 µM arsenate. Values are mean of triplicates ± SD. Cysteine is expressed in nmol g^{-1} fresh weight, GSH and hGSH in µmol g^{-1} fresh weight and while PCs and hPCs in nmols of GSH equivalents g^{-1} fresh weight

Parameters	CSG-8962		C-235	
	Control	20 µM As	Control	20 µM As
Cysteine	1.6±0.2	2.1±0.2	1.1±0.03	5.7±0.3
GSH	3.6±0.25	51.9±3.55	4.0±0.61	79.6±3.6
hGSH	1.2±0.02	10.2±0.19	0.8±0.035	15.7±1.75
PC_2	0.08±0.001	25.8±1.65	0.072±0.002	46.9±2.81
hPC_2	ND	12.5±0.88	ND	32.4±1.92
PC_3	ND	39.4±2.7	ND	78.5±5.5
hPC_3	ND	40.8±4.9	ND	72.7±4.32

ND = Non detectable

Table - 4: Induction of PCs and hPCs and effect on level of thiols in shoots of *Cicer arietinum* L. var. CSG-8962 and C-235 after 2 day of exposure to 20 µM arsenate. Values are mean of triplicates±SD. Cysteine is expressed in nmol g^{-1} fresh weight, GSH and hGSH in µmol g^{-1} fresh weight and while PCs and hPCs in nmols of GSH equivalents g^{-1} fresh weight

Parameters	CSG-8962		C-235	
	Control	20 µM As	Control	20 µM As
Cysteine	14.3±1.65	28.2±1.52	14.6±0.95	23.10±2.2
GSH	23.5±1.85	47.2±2.43	15.5±2.05	42.6±1.5
hGSH	ND	12.3±1.51	ND	20.3±1.72
PC_2	2.34±0.13	1.2±0.2	2.01±0.11	4.9±0.45
hPC_2	ND	0.93±0.05	ND	3.2±0.15
PC_3	ND	0.72±0.01	ND	2.7±0.21

ND = Non detectable

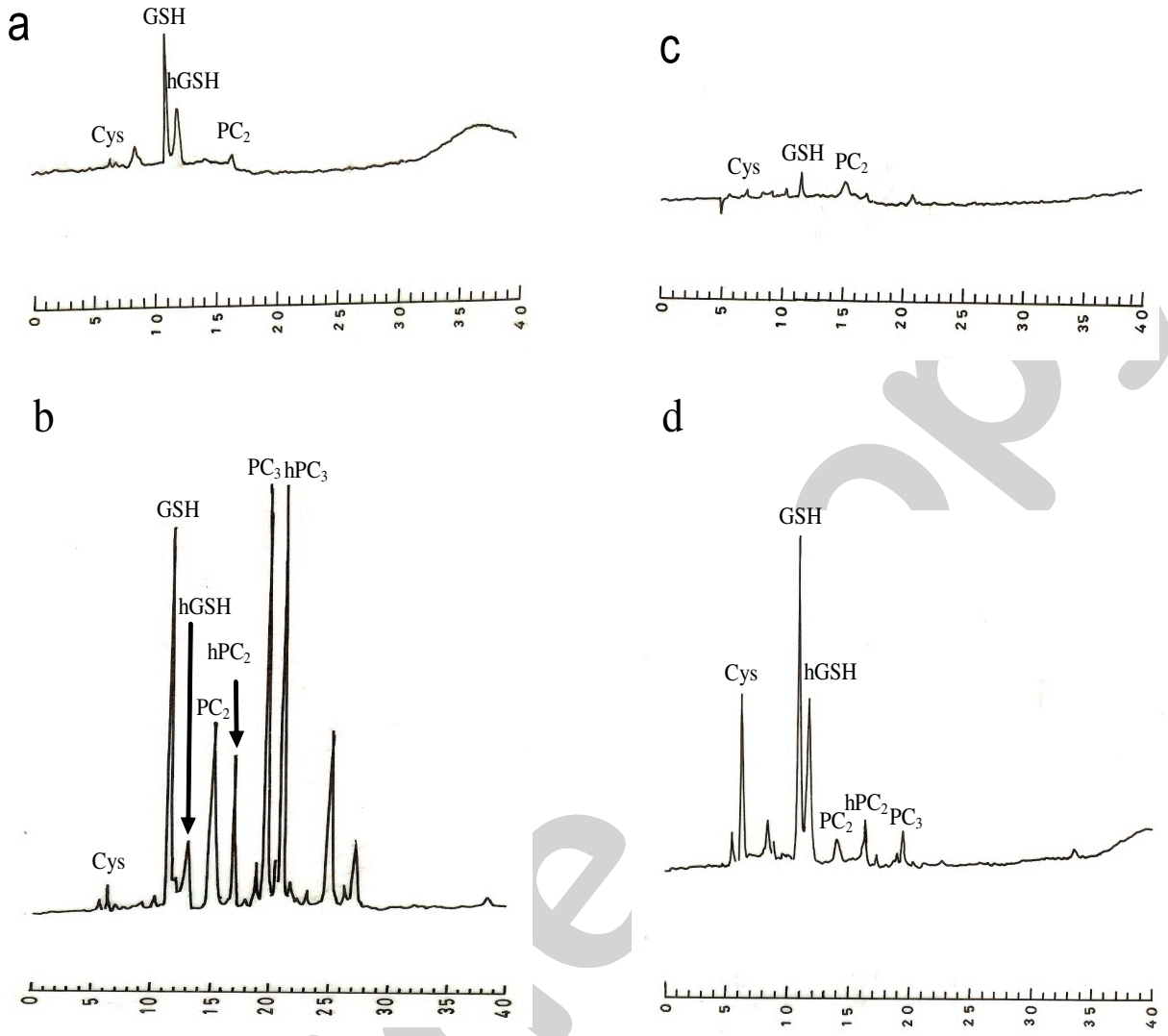

Fig. 2 : HPLC-profiles of PCs, hPCs and related thiols in crude extracts of *Cicer arietinum* L. var. C-235 exposed to 20 µM arsenate for 2 day in (a) control root, (b) 20 µM arsenate root, (c) control shoot and (d) 20 µM arsenate shoot. The root and shoot tissues were separately frozen at -80°C in the presence of 100 mM sodium ascorbate and immediately extracted with 10% SSA. The extract was analyzed by post-column HPLC method (Mendum *et al*., 1990)

C-235 there was slight increase in growth of plants that were exposed to arsenate. Such an increase in plant growth under arsenate stress has been previously reported by Mascher *et al*. (2002). Though in this study, the level of metalloid in the seeds was not investigated, in our previous study (Gupta *et al*., 2006), we observed that seeds of *C. arietinum* var. CSG-8962 and C-235 accumulated very low amount of toxic metals. It is thus supposed that chickpea has internal detoxification and storage capacity to accumulate metals in roots and leaves allowing less translocation to seeds and hence it is likely that seeds may accumulate very less amount of arsenic also. Investigation of thiols seems correlated with the detoxification potential thus less toxicity in terms of growth parameters.

The significant induction in level of cysteine upon arsenate exposure is ascribed to induction of its biosynthetic pathway. Heavy metals are reported to induce the sulfur reduction pathway including the enzymes like ATP sulfurylase, APS reductase, serine acetyl transferase (SAT) and cysteine synthase (CS) (Rausch and Wachter, 2005). GSH and PCs are the main arsenic chelating ligands, which were induced to high levels in the present study. Availability of GSH is crucial for metal detoxification as it can bind metal directly or through synthesis of PCs (Raab *et al*., 2004, 2005). In addition, GSH is used as reductant for direct or arsenate reductase catalyzed reduction of arsenate to arsenite, which is a primary requisite for the complex formation (Rosen, 1999; Dhankher *et al*., 2002; Bleeker *et al*.,

2006). The enhanced level of GSH in arsenate exposed plants might be due to induced transcription of genes of GSH biosynthesis such as γ-glutamylcysteine synthetase and glutathione synthetase (Xiang and Oliver, 1998). In higher plants, arsenic is reported to significantly induce the synthesis of phytochelatins (PCs) (Schat *et al*., 2002; Grill *et al*., 2006; Tripathi *et al.*, 2007) and are considered to be essential for both normal and constitutive tolerance to arsenic (Hartley-Whitaker *et al*., 2001; Schat *et al*., 2002; Li *et al*., 2004). Further, induced levels of PCs and GSH suggest the induction of whole PC biosynthetic pathway as hypothesized earlier for cadmium and arsenic by Inouhe (2005). Members of the fabales synthesize hPCs in addition to PCs (Grill *et al*., 1986; Piechalak *et al*., 2002). In the present study also, significant amount of hPCs have been synthesized. Synthesis of PCs and hPCs in chickpea, has been demonstrated in our earlier studies (Gupta *et al*., 2004, 2005). The complexes of arsenic with GSH and PCs has been demonstrated in various plants like *Silene vulgaris, Rauvolfia serpentina, Holcus lanatus, P. cretica, Helianthus annuus* and *Brassica juncea* (Sneller *et al*., 1999; Schmöger *et al*., 2000; Raab *et al*., 2004, 2005, Montes-Bayón *et al*., 2004). The complexes of metals and metalloids have been suggested to be sequestered into vacuole as a final step of detoxification. The sequestration of As^{III}-GS_3, complexes has been demonstrated from fungi (Ghosh *et al*., 1999) to higher plants (Bleeker *et al*., 2006).

From the present study it may concluded that the chickpea plant induced high levels of arsenic chelating thiols and showed less accumulation of arsenic in shoots. Thus these plants seem to have high tolerance towards arsenic toxicity and may be grown in arsenic contaminated areas without any major risk of significant accumulation of arsenic in aerial parts.

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