Arsenic and chromium hyperaccumulation by an ecotype of *Pteris vittata* – prospective for phytoextraction from contaminated water and soil

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This study was carried out to identify *Pteris vittata* plant in India, which is reported elsewhere as an arsenic hyperaccumulator. This is the first report of characterization of arsenic (As) accumulation in an Indian ecotype, which also shows chromium (Cr) hyperaccumulation in addition to As. Intact plantlets were grown in 20% Hoagland solution amended with up to 200 mg As or Cr l^{-1} medium. Plants absorbed and accumulated a significant amount of As and Cr in their biomass with high bioenrichment factor. As and Cr tolerance by spores and gametophytes under *in vitro* was also assessed. As and Cr accumulation in the gametophyte biomass was more when spores were directly germinated on As- and Cr-supplemented media. As and Cr accumulation in the gametophyte biomass was less when spores were germinated first on media devoid of As and Cr but subsequently grown on As- and Cr-amended media.

Keywords: Arsenic, chromium, hyperaccumulation, *Pteris vittata*, remediation.

ARSENIC (As) and chromium (Cr) metals are potential pollutants due to their toxic and carcinogenic[1] effects. Their compounds are widely used as pesticides, herbicides and wood preservatives[2,3]; tanning of skin and hide, chrome plating, dyes, pigments and wood preservation[4,5]. Environmental contagion and exposure to As and Cr is a grave concern – many sites around the world are contaminated by these metals. Extensive industrial use has resulted in their accumulation in soils and further, contamination of aquifers has become a serious environmental issue in some parts of the world including India[6,7]. Thus remediation of As- and Cr-contaminated soil has become an important environmental issue.

Traditional methods like soil washing, encapsulation, vitrification, precipitation, ion exchange, flocculation, carbon adsorption, etc. for heavy metal remediation are very expensive, laborious and often disrupt the environment. Most of the developing countries like India may not be able to afford such huge expenditure required for mitigating the heavy metal pollution by these modern technologies[8,9]. Therefore, R&D strategies are planned to develop eco-friendly and sustainable alternatives that are not costly and are viable on small as well as commercial scales. Phytoremediation is an emerging clean-up technology which uses plants to remove metals from contaminated sites[10]. This approach includes overall biological, chemical and physical processes that enable uptake, sequestration, degradation and metabolization of contaminants by plants[11,12]. It offers an attractive, non-intrusive, effective, aesthetically pleasing, socially-accepted method and an economical alternative to other methodologies. Phytoextraction[12,13] is one of the different approaches of phytoremediation which could be used to absorb heavy metals from the environment and accumulate in plant biomass[12,14]. As the plants absorb, concentrate and precipitate toxic metals from contaminated soils and accumulate in the biomass, phytoextraction is best suited for remediation of diffusely polluted areas, where pollutants occur only at relatively low concentration and not in greater depths of the soil[15]. Discovery of hyperaccumulator plant species, which have the unusual ability of accumulating metals such as As, Cr, zinc, nickel and copper to very high concentrations[16,17], has further boosted this technology.

Among the known As hyperaccumulators, *Pteris vittata* (brake fern) is one of the most efficient and most studied plants. It was first reported[18] as an As hyperaccumulator plant with potential for phytoremediation of As-contaminated soil. It was reported to accumulate up to 22,630 mg As kg^{-1} of its above-ground biomass (fronds), indicating its high tolerance to As and efficient mechanisms of As detoxification[18]. Although it is reported that this plant species is endemic in the sub-tropical region, there are a few reports on its availability and elucidation of conformity for As accumulation. In addition to this, it has also been reported[19,20] that different genotypes of *P. vittata* exhibit variation in their capability for As toler-

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ance and hyperaccumulation. Keeping these aspects in mind, efforts have been made to spot *P. vittata* endemic to the Indian sub-continent and, ascertain its capability for As and Cr tolerance and accumulation. An ecotype of *P. vittata* was identified and As and Cr tolerance and hyperaccumulation potentiality have been determined.

Materials and methods

Sporophyte and gametophyte culture

The ecotype of *P. vittata* was collected from Kerala, India, maintained in a glass house at the National Environmental Engineering Research Institute (NEERI), Nagpur and used in the experiments. Plants were acclimatized for 2 weeks in hydroponics containing 20% Hoagland nutrition solution[21] in 30–35°C temperature and 90% humidity. Acclimatized plants were treated with As (V) and Cr (VI) in 500 ml of 20% Hoagland nutrient solution supplemented with 0, 50, 100, 150 and 200 mg As l^{-1} medium ($Na_2HAsO_4 \cdot 7H_2O$) or $Cr(K_2Cr_2O_7)$. During the treatment, the volume of the Hoagland medium was maintained with double distilled water. After 20 days, the plants were removed, thoroughly washed with tap water followed by deionized water and roots, stems and leaves were separated.

Spores of *P. vittata* were decontaminated by soaking in 'Tween 80' for 10 min followed by 70% ethanol for 1 min and 0.1% $HgCl_2$ for 9 min and washed four times with autoclaved double distilled water. Decontaminated spores were aseptically germinated on agar-gelled Murashige and Skoog (MS) growth medium[22] amended with different concentrations of As or Cr and MS medium without metal supplement was used as control. For treatment experiments, spores were spread on MS medium amended with 15, 20, 25 and 50 mg As l^{-1} medium and 25, 50, 75 and 100 mg Cr l^{-1} medium. The gametophytes developed from their spores on As- or Cr-supplemented media or without As and Cr were subcultured to fresh MS media with the same or higher concentrations of As or Cr. Gametophytic biomass produced on As- and Cr-amended media were removed from the medium, washed with tap water followed by deionized water and used for As or Cr assay.

Effect of As and Cr on plant growth and hyperaccumulation

Four- to six-month-old sporophytes having three to six fronds were used for As and Cr accumulation experiments using Hoagland nutrient solution. Hoagland nutrient solution was amended with As or Cr as described here and one sporophytic plant was grown in each concentration of As and Cr. One plant was used per container and each treatment was repeated thrice. The effect of As and Cr on plant (sporophytes) growth was evaluated by measuring plant elongation, dry-mass accumulation and size of roots and shoots. Each plant was measured from the tip of its longest root to the stolon/crown, and from the base of the stolon to the main apex of the shoot to determine root length and shoot length respectively. Increase in size, texture and colour of the As- and Cr-treated *in vitro* gametophyte cultures were observed periodically at an interval of 15 days to assess their growth response.

Estimation of As and Cr in gametophyte and sporophyte biomass

Gametophytic and sporophytic biomass from control and treated experiments were oven dried at 60°C for 72 h, dry weight of each sample was recorded to determine biomass accumulation and used for As and Cr estimation in the biomass. Known amount of each sample was digested with concentrated HNO_3 (for estimation of As) or HNO_3 : $HClO_4$ :: 1 : 1 (for estimation of Cr) using a microwave oven (ETHOS 900) at 300 W for 15 min. The digested extract was filtered and diluted with double distilled water. As and Cr were estimated using ICP-AES and AAS respectively, against blank using As and Cr standards (MERCK, Germany).

Data analysis

All experiments were carried out in three replicates. Data presented here are expressed as the means ± SD of three independent experiments. These simple descriptive statistical measures served as the basis for interpretation of the data.

Result and discussion

The capability of *P. vittata* to accumulate As and Cr in tissue biomass was determined in Hoagland growth medium amended with different concentrations of As (V) and Cr (VI). As and Cr concentrations in leaf, stem and root biomass of *P. vittata* are presented in Tables 1 and 2, respectively. All the plants were healthy after 20 days of treatment with up to 200 mg As and Cr l^{-1} medium and did not show any phytotoxicity symptoms in comparison with the control, except slight necrosis at the edge and tip of the fronds. This indicates that this genotype of *P. vittata* is also tolerant to As, and further it is also tolerant to high concentration of Cr.

The highest total As accumulation in the sporophytic plant biomass of *P. vittata* was in 150 mg As l^{-1} medium (bioenrichment factor 138) (ratio of arsenic concentrations in plant biomass to As concentrations in growth medium).

Table 1. As accumulation in different parts of *Pteris vittata* sporophyte plants after treatment in Hoagland's nutrient solution for 20 days containing 0–200 mg As l^{-1} medium

As treatment (in mg l^{-1})	As concentration (mg kg^{-1} dry biomass)				Bioenrichment factor	
	Leaf	Stem	Root	Total	Total	Aerial biomass
0	114 ± 44	650 ± 140	821 ± 761	1,585 ± 369	0	0
50	2,307 ± 882	1,299 ± 701	1,173 ± 1,012	4,779 ± 622	96	72
100	7,473 ± 7,169	1,784 ± 215	4,386 ± 2,953	13,643 ± 2,848	136	93
150	8,331 ± 7,156	6,400 ± 3,906	5,976 ± 4,235	20,707 ± 1,255	138	98
200	3,474 ± 1,767	2,745 ± 1,835	10,170 ± 8,193	16,390 ± 4,093	82	31

Values are means (n = 3) ± SD.

Table 2. Cr accumulation in different parts of *P. vittata* plant after treatment in Hoagland's nutrient solution containing 0–200 mg l^{-1} chromium for 20 days

Cr treatment (in mg l^{-1})	Cr concentration (mg kg^{-1} dry biomass)				Bioenrichment factor	
	Leaf	Stem	Root	Total	Total	Aerial biomass
0	16 ± 14	0 ± 0	7 ± 6	23 ± 8	0	0
50	611 ± 313	1556 ± 740	9,200 ± 7,993	11,367 ± 4,710	227	43
100	1,551 ± 120	3911 ± 2,280	17,427 ± 7,853	22,889 ± 8,567	229	55
150	2,609 ± 1,528	20,675 ± 9,498	12,019 ± 9,507	35,303 ± 9,036	235	155
200	2,099 ± 1,480	2,095 ± 759	8168 ± 2,415	12,362 ± 3,505	62	21

Values are means (n = 3) ± SD.

At this concentration, maximum As was accumulated in leaves (8331 mg) followed by stems (6400 mg) and least in roots (5976 mg) kg^{-1} dry weight of biomass (Figure 1 *a*). However, in treatment under 200 mg As l^{-1} medium, As accumulation was highest in the root biomass, i.e. 10,170 mg kg^{-1} dry biomass, which was much higher in comparison to As accumulated in leaf and stem. This denotes less As translocation to aerial biomass when treated with 200 mg As l^{-1} medium which was the highest As concentration in this study, presumably due to high toxicity. As accumulation in the biomass varied directly with increase in arsenic concentration in the nutrient solution, except in 200 mg As l^{-1} medium. This shows that 150 mg As l^{-1} medium is the threshold limit for As accumulation in biomass. As accumulation was more in frond in comparison to other parts, but significant amount of As also accumulated in stem and root (Table 1). Bioenrichment factor is an important parameter to assess the translocation and accumulation of metal in the plant biomass[23]. Bioenrichment factor for As accumulation increased with increase in As concentration up to 150 mg As l^{-1} medium but above this concentration, bioenrichment factor decreased (Figure 1 *b*). From the growth performance of plants, it was found that 50 mg As l^{-1} medium was optimum for As accumulation in the plant biomass without toxicity. This genotype of *P. vittata* also accumulated high amount Cr in the leaves, stems and roots when treated with different concentrations of hexavalent Cr($K_2Cr_2O_7$). The highest Cr concentrations in leaf, stem and root biomass of treated plants were 2609, 20,675 and 17,427 mg kg^{-1} dry biomass respectively (Figure 2 *a*). Cr accumulation in the plant biomass increased with increase in Cr concentration in the nutrient solution. Maximum bioenrichment factor for Cr accumulation was up to 235 (Figure 2 *b*) under treatment with 150 mg Cr l^{-1} medium. No toxicity symptoms were observed in the *P. vittata* sporophytes up to 100 mg Cr l^{-1} medium. Increase in Cr concentration above 150 mg l^{-1} resulted in acute toxicity of the plant, fronds started bleaching and dried. From the growth performance of plants it was found that optimum Cr concentration in the treatment medium for maximum accumulation in the plant biomass was up to 150 mg l^{-1}. This indicates that this plant is more tolerant to Cr in comparison to As. The results show that leaves of *P. vittata* accumulate more than 1000 mg Cr kg^{-1} dry biomass. To our knowledge, this is the highest value in comparison to other reported Cr hyperaccumulators[24].

Germination efficiency of *P. vittata* spores and As and Cr tolerance of gametophytes (haploid prothallium developed after spore germination) were assessed on agar-gel MS growth media amended with As (V) and Cr (VI). Spore germination was 100% in the growth medium amended with 15, 20, 25 mg As l^{-1} medium and 25, 50, 75 mg Cr l^{-1} medium, whereas spore germination was 24% in 50 mg As l^{-1} and 100 mg Cr l^{-1} growth media, in comparison to the control. The reduction in spore germination efficiency indicated that the As concentration > 25 mg l^{-1} and Cr concentration > 75 mg l^{-1} were toxic and beyond tolerance of these haploid germplasms of the plant. Nevertheless, the tested concentrations of As and

Cr are reasonably high as far as the haploid nature of the spores and gametophytes is concerned. Further, the As and Cr tolerance of the haploid structures conform to the genetic potentiality of this genotype.

The gametophytes produced *in vitro* in As-free MS culture medium and brought up further on As-amended media supplemented with 50, 100 and 200 mg As l^{-1} medium accumulated 6141, 13,109 and 36,659 mg As kg^{-1} dry weight respectively, in a span of 96 weeks (Table 3). Whereas spores germinated in As-amended media supplemented with 15, 20, 25 and 50 mg As l^{-1} medium to produce gametophyte accumulated 3433, 5989, 8442 and 17,786 mg As kg^{-1} dry weight correspondingly within four weeks only (Table 4). Chromium accumulation in *P. vittata* gametophyte was also evaluated. Gametophyte produced *in vitro* in Cr-free MS culture medium and brought up further on Cr-amended media supplemented with 50, 100, 150 and 200 mg As l^{-1} media accumulated 1864, 5300, 8577 and 15,257 mg Cr kg^{-1} dry weight respectively, after 96 weeks (Table 5). Whereas spores germinated *in vitro* in MS media supplemented with 25, 50, 75 and 100 mg Cr l^{-1} medium to produce

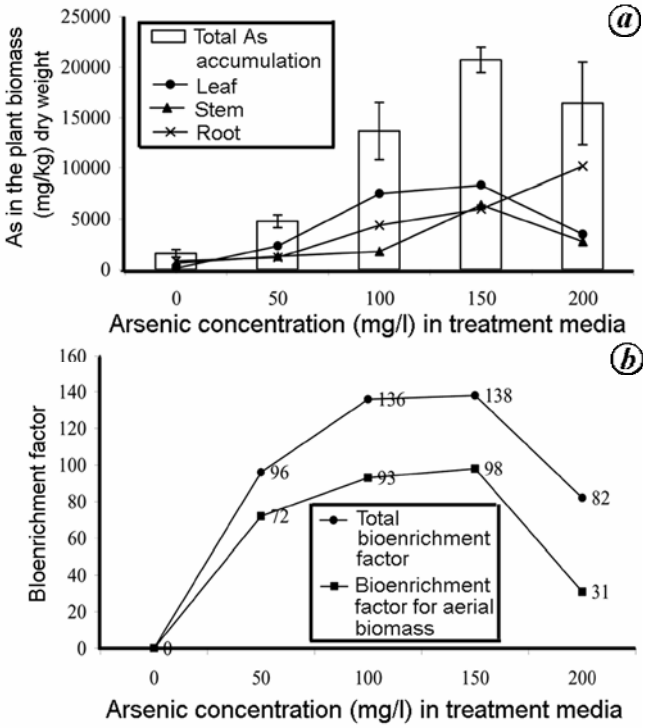

Figure 1. ***a***, As accumulation in *Pteris vittata* plant biomass grown in As-supplemented Hoagland's liquid medium. ***b***, Bioenrichment efficiency of As accumulation in *P. vittata* plant biomass treated with arsenic in hydroponics culture.

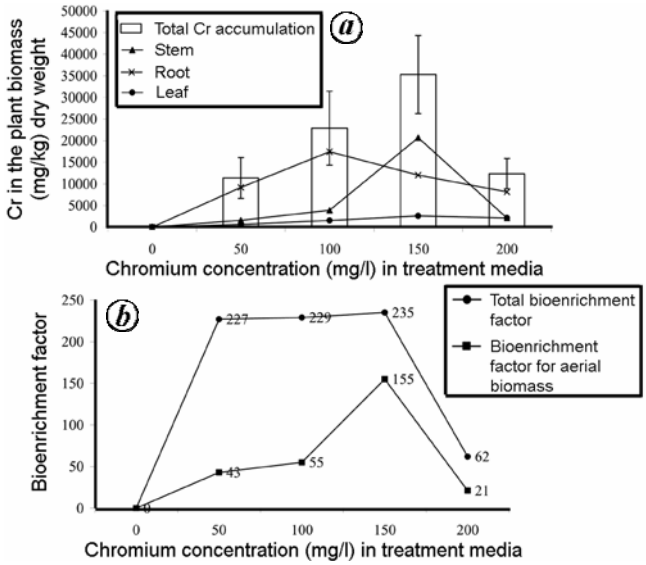

Figure 2. ***a***, Cr accumulation in *P. vittata* plant biomass grown in Cr-supplemented Hoagland's liquid medium. ***b***, Bioenrichment efficiency of Cr accumulation in *P. vittata* plant biomass treated in hydroponics culture supplemented with Cr.

Table 3. As accumulation in gametophyte biomass after 96 weeks; grown by *in vitro* spore germination in As-free growth medium followed by subculture on arsenic-supplemented medium (0–200 mg l^{-1})

As treatment (in mg l^{-1})	As concentration (mg/kg dry biomass)	Bioenrichment factor
0	259 ± 75	0
50	6,141 ± 232	123
100	13,109 ± 618	131
200	36,659 ± 1198	183

Values are means (n = 3) ± SD.

Table 4. As accumulation in the gametophyte biomass after 4 weeks; grown by *in vitro* spore germination in MS culture medium supplemented with 0–50 mg l^{-1} arsenic

As treatment (in mg l^{-1})	As concentration (mg kg^{-1} dry biomass)	Bioenrichment factor
0	355 ± 10	0
15	3,433 ± 405	229
20	5,989 ± 338	299
25	8,442 ± 286	338
50	17,786 ± 274	356

Values are means (n = 3) ± SD.

Table 5. Cr accumulation in the gametophyte biomass after 96 weeks; grown by *in vitro* spore germination in Cr-free MS media followed by subculture to Cr-supplemented (0–200 mg l^{-1}) media

Cr treatment (in mg l^{-1})	Cr concentration (mg kg^{-1} dry biomass)	Bioenrichment factor
0	9 ± 0.25	0
50	1,864 ± 120	37
100	5,300 ± 206	53
150	8,577 ± 536	57
200	15,257 ± 172	76

Values are means (n = 3) ± SD.

gametophytes accumulated 1845, 3431, 4116 and 5246 mg Cr kg^{-1} dry biomass correspondingly within four weeks only (Table 6). The results showed that spore germination under As and Cr stress, and the gametophytes developed from these spores accumulated higher amount of As and Cr in their biomass on further growth in As- and Cr-amended media. While spore germination in As- and Cr-free medium, and gametophytes developed from these spores accumulated less amount of As and Cr in the gametophyte biomass on further growth under As- and Cr-amended media. The bioenrichment factors for both As and Cr accumulation in the gametophyte biomass were increased with increase in As and Cr concentration within 96 weeks of culture in the media (Tables 3 and 5). At the same time, after only 4 weeks of culture, bioenrichment factor for As accumulation in the gametophyte biomass increased as As concentration in the medium increased (Table 4), but in the same duration, bioenrichment of Cr accumulation in the biomass decreased with increase in Cr concentration in the medium (Table 6). The results also indicate that the gametophytes developed from spores which were germinated in As-amended media have higher As translocation capacity (Figure 3 *a* and *b*) as relatively more amount of As was accumulated in the gametophyte biomass in 4 weeks in comparison to 96 weeks. On the other hand, in the case of Cr, translocation to the gametophyte biomass which were developed from spores germinated in MS Cr-free medium was more, in comparison to the gametophyte biomass grown by spore germination in Cr-supplemented medium (Figure 4 *a* and *b*). The hyperaccumulators are characterized to have bioenrichment factor > 1 (ref. 23). The findings that this ecotype of *P. vittata* also has high bioenrichment for As and Cr indicate that this genotype is a hyperaccumulator for As and Cr . This plant can be potentially used for phytoextraction of As and Cr from contaminated soils and water in phytoremediation system. Further studies are in progress to determine the molecular determinants (protein(s) and gene(s)) conferring As and Cr tolerance and accumulation in this plant. Activity assay of the arsenate reductase enzyme, identification of the gene and its phylogenetic relationship with other reported organisms using the available database are under progress. Analyses of total proteome of control and treated plants are under progress to identify the over- and under-expressed proteins with reference to As stress. It is envisaged to study similar aspects with reference to Cr stress. Further studies are required to assess resilience of the sporophytes to As and Cr stress and effect of field parameters on accumulation of these metals which are prerequisites for designing a phytoremediation system for remediation of contaminated soil and water.

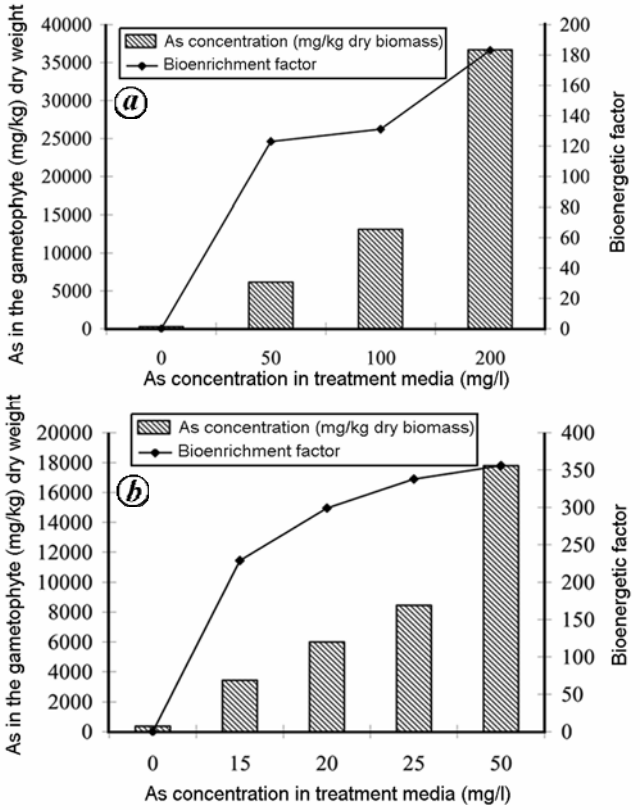

Figure 3. ***a***, As accumulation in *P. vittata* gametophyte after 96 weeks; grown by *in vitro* spore germination in As-free MS culture media followed by subculture on As-supplemented media. ***b***, As accumulation in *P. vittata* gametophyte after 4 weeks; grown by *in vitro* spore germination in As-supplemented MS culture media.

Table 6. Cr accumulation in the gametophyte after 4 weeks; grown by *in vitro* spore germination in MS growth medium supplemented with 0–100 mg l^{-1} chromium

Cr treatment (in mg l^{-1})	Cr concentration (mg kg^{-1} dry biomass)	Bioenrichment factor
0	6 ± 1	0
25	1845 ± 30	74
50	3431 ± 79	69
75	4116 ± 47	55
100	5246 ± 357	52

Values are means (n = 3) ± SD.

Handling of the plant biomass with high metal concentration such as As or Cr concentration is a topical issue. However, this issue is similar to the problem faced by other physico-chemical methods adopted for water treatment for abatement of As and Cr pollution. Further treatment of sludge, rejects, consumed membranes or volatile gases generated by the treatment are materials of great environmental concern. However, the phytoremediation process has some merits in comparison to the other processes. Once metals are accumulated in the plants biomass they can be harvested, suitably processed and disposed with available secure methods. In cases where the contaminants are degraded to harmless compounds, disposal may not be required. However, due to the elementary nature of the metal ions they are non-destructive and non-degradable; however, they could be

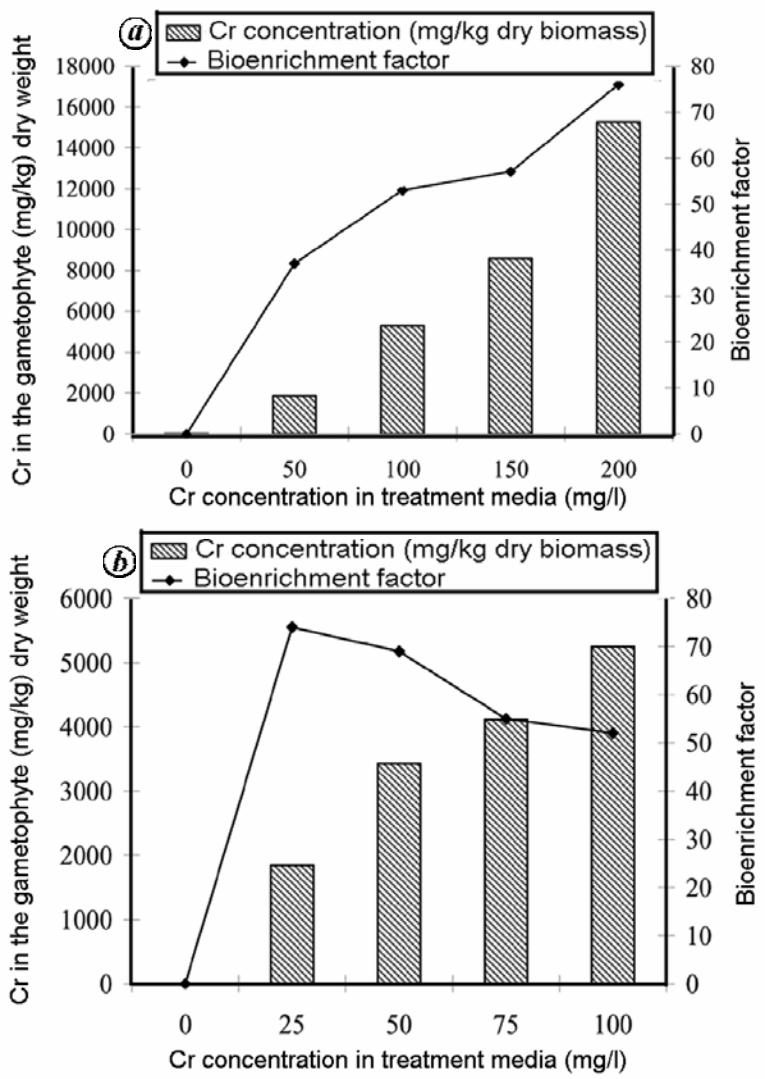

Figure 4. ***a***, Cr accumulation in *P. vittata* gametophyte after 96 weeks; grown by *in vitro* spore germination in Cr-free growth media followed by subculture on Cr supplemented media. ***b***, Cr accumulation in *P. vittata* gametophyte after four weeks; grown by *in vitro* spore germination in Cr-supplemented growth media.

converted to harmless form through biocycles. The most commonly mentioned process for dealing with metal-enriched plant material is controlled incineration, which results in ash with a high metal content. An economically feasible method for metal recovery from the incinerated ash needs to be developed. Conventional disposal methods such as land filling could also be possible in some instances. Other methods of plant tissue treatment currently under investigation include drying under sun, heat and air, composting, pressing, compacting and leaching[14].

Conclusion

This ecotype of *P. vittata* accumulates up to 20,707 mg As kg^{-1} dry weight and up to 35,303 mg Cr kg^{-1} dry weight with bioenrichment factor up to 138 and 235, respectively. *In vitro* raised gametophytes, by spore germination on As- and Cr-supplemented growth media, show higher As and Cr accumulation in the gametophyte in comparison to gametophytes developed in As- and Cr-free growth media. Efficiency of spore germination was less at higher concentration of As (50 mg l^{-1} medium) and Cr (100 mg l^{-1} medium). These studies elucidate that this *P. vittata* genotype collected from India has the inherent ability to tolerate As (V) and ability for accumulation in the sporophytic and gametophytic biomass. *P. vittata* has been reported As tolerant and hyperaccumulator, but they are found elsewhere. This study enumerates that the genotype of this Indian ecotype is also As tolerant and a potential hyperaccumulator. In addition to As tolerance, it was found that this ecotype is also tolerant to high concentration of Cr (VI) and able to accumulate Cr in its sporophytic and gametophytic

biomass which is a new finding adding to its ability for hyperaccumulation. These attributes of this genotype make it a potential plant for use as an alternative system for remediation of As- and Cr-contaminated soil and water.

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