Heavy Metal Scavenging Evaluation of *in Vitro* Grown *Brassica Campestris* Var. Sarsoon from the Tanneries Contaminated Soil Using Atomic Absorption Spectroscopy

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Abstract: The study was conducted to evaluate and compare the scavenging efficiency of *in-vivo* and *in-vitro* grown *Brassica campestris* var. Sarsoon, for the uptake of Lead and Chromium(two major components of inorganic contaminants in the tanneries contaminated soils) using Atomic Absorption Spectroscopy. *In vitro* plants were grown on MS basal medium containing 2,4-D (2.5 mg/l).Both *in-vivo* and hardened *in-vitro* grown plants were shifted to contaminated soil near Kasur Tanneries. After 6o days of growth in this contaminated soil, the plants bioassays were subjected to Atomic Absorption Spectroscopy for the estimation of lead and Chromium uptake. The *in-vivo* grown *Brassica* plants absorbed much lesser amount of both the metals(2.69 &1.62ppm) as compared to *in-vitro* grown plants which showed higher ranges(4.61 &2.69 ppm) of Pb and Cr respectively. The results supported our idea that, in future, the *in-vitro* grown hyper accumulator plants specially weeds can be used as an effective and better tool of phytoremediation (compared to field grown ones) for the removal of heavy metals through their rhizosphere scavenging action, from the contaminated lands on a wider scale.

Keywords: Phytoremediation, *In- vitro* grown plants, Lead , Chromium, Inorganic, contaminants, Scavanging evaluation.

Introduction

Tanning industry always gives rise to serious environmental problems in countries lacking implementation of environmental regulations. Currently, the contamination of the environment by by–products of rural and mining industries is the most threatening problem. District Kasur in Punjab Province of Pakistan represents such an area, which is being spoiled by tanning industry due to deposition of its lethal exudates in the local soils, damaging their biological life on a wider scale.

Hundreds of thousands of acres of country's lands area are disturbed and polluted by these contaminants.Some of these lands are in remote locations making cleanup very difficult. Others have minimal funds for cleanup or are so large that cleanup becomes economically impractical.There is a need for low energy green technologies that can be applied at these sites.

Phytoremediation is considered as the most emerging field of environmental biotechnology. Most of the soil contaminants can be removed by many other physical methods but the heavy metal pollution of vast cultivated land is a serious threat to the agricultural biology because of their prolonged stay in the soil.The plants roots have

natural ability to absorb the heavy metals of the soil thus behaving as phytoremediates. Metals uptake using plants provides an environmental friendly solution of the soil pollution, which is a low cost, *in-situ* process, energized by solar energy (McCutcheon and Schnoor, 2003).

Many plants species behave as hyper accumulators of the metals, depending upon their scavenging efficiency, and ability to accumulate these metals in the different cellular compartments of their cells.The metals pass through the root cell membrane to the symplast, inside the cell, then metals could be passed to the vacuoles, (where they are degraded enzymatically) by membrane metal transporters, and are deposited there with the help of metallothioneins i:e metal-binding proteins.

Heavy metals replace other essential metals in pigments inside the cellular structure, destroying the natural balance of these molecules (Manios *et al*., 2003). They may cause oxidative stress too, especially transition metals like $Fe^{2+/3+}$ and $Cu^{+/2+}$ (Rivetta *et al.*, 1997).

Plant tissue culture provides a selected environment for the evaluation of many limiting factors. It is in extensive use nowadays, to obtain variants with variable tolerance to different biotic stresses (Ben-Hayyim, 1987; Santos-Díaz and Ochoa-Alejo, 1994). This technique is also found to be useful for cultured plant organs to know the metal accumulation properties by each separate plant part e:g the removal of Sr^{2+} using shoots of *Solanum laciniatum* (Kartosentono *et al*., 2001), and Cd hyper-accumulation by roots of *Thlaspi caerulescens* (Nedelkoska and Doran, 2000).

Atomic Absorption Spectroscopy is an alternative, simple and rapid technique for quantitative isolation of the group of eight elements (Al, Ca, Cd, Cu, Fe, Mg, Pb and Zn) from vegetable material (Wieteska *et al*.,1998). The proposed method allows to obviate the organic matrix destruction stage, shortens the analyte dissolution time, reduce cost, and minimize hazards of loss and contamination. Therefore main objective of the present study is to evaluate and compare the Lead and chromium uptake by *in vivo* and *in vitro* grown *B.compestris* var Sarson with the help of Atomic Absorption Spectroscopy (AAS).

The present research was aimed to study the accumulation of heavy metals (Pb & Cr) in the plant body of *B.Campestris*. It can help identify the comparative efficiency potential of plants to remediate the metals from the contaminated soil in which they were cultivated.This study also examined the growth performance and physiological responses of these plants under contamination stress.

So by employing this method, one may be able to find the effect of heavy metal contamination to the plant body as well as to give practical implementation of phytoremediative use of tissue cultured plants and their future prospects on a wider scale in future.

Materials and Methods

This piece of work was divided into two steps:

1. The *in-vivo* and *in -vitro* growth of *B.campestris* var Sarson and the hardening of the *in-vitro B.compestris* and its transfer, along wit *in-vivo* grown *B.campestris*, to the Pb and Cr polluted soils of Kasur tanneries for a period of 60 days.

2. Estimation of Lead and Chromium uptake by these plant(grown in contaminated soil) using Atomic Absorption Spectroscopy.

1. The *in-vivo* and *in -vitro* growth of *B.campestris*.

For *in-vivo* growth the certified seeds of *B.campestris* were sown in the normal soil of Lcw university and were grown for 60 days. For the *in -vivo* growth, the explants were taken from the wild *B.campestris*, were cultured and then subcultured in the PGRs optimium media for 60 days.
For the *in-vitro* growth, following protocol was followed.

a. Medium and Phyto Growth Regulators (PGRs):

MS (Mrashaige and Skoog, 1962) basal medium was used. Different PGRs were used separately and in combinations in MS basal medium as follows to select the best one.
i. 2, 4-D,2.5mg/l
ii. BAP, 0.5mg/l
iii. NAA,1.0mg/l
iv. 2, 4-D,2.5mg/l+BAP,0.5mg/l

b. Physical Factors:

Sucrose was added to medium at 3% concentration (30g/l).The optimum temperature required for culture environment was maintained at 25±2°C. The cultures were incubated at 16 hours light period (under cool light fluorescent tubes,with light intensity of 2000-3000 lux) and the pH of the medium was adjusted between 5.6-5.7.

c. Hardening and shifting of the plants to the Kasur Tanneries contamoinated soil

In vitro grown plants were shifted to the sterilized soil of different grades and and gradually to the normal regular soil in order to harden the plants.These plants and other set of field grown plants were shifted to the contaminated soil near Tanneries located in vicinity of Kasur city .

d. Plan of experiment and data recording: Three sets of each experiment were designed with three replica of each experiment**.** The cultured explants were observed after inoculation and the contamination percentage, percentage of callus formation and number of frequency of micro-propagated plants per explants after given culture period was worked out.Mean deviation was calculated after using SPSS software(Levesque,2007) following Steel *et al* (1997).

2. Estimation of Lead and Chromium uptake by *in-vivo* and *in-vitro B.campestris* using Atomic Absorption Spectroscopy (AAS) after their shifting to Pb and Cr contaminated soils:

Atomic Absorption Spectroscopy (AAS) provides accurate quantitative analysis for metals in water, sediments, soils or rocks.Samples are analyzed in solution form, so solid samples must be leached or dissolved prior to analysis.The second step of the study was to estimate the Lead and Chromium uptake by *B.campestris(* Phyto remediation) using AAS. All chemicals and reagents used in the study were of analytical grade and were used without further purification. Solutions were prepared in double distilled water.

a. Preparation of Biomass: Elements in plants parts cannot be detected directly by atomic absorption spectroscopy, so solutions for plants were prepared by wet digestion method and then samples were analyzed to determine the concentration of metal ions. After collecting leaves of plant they were washed with double distilled water to remove dust from plant. These leaves were then dried in an oven. The dried plants were then digested. The same procedure was done with *in vitro* grown plants except that regenerated plants were not sterilized.

b. Methods for digestion: The dried plant leaves were weighed separately and 5.0g of then was taken in a round bottom flask. The dried material was ashed in crucible muffle furnace at 500C for 1 hour . The residue was then wet digested by Hcl/HNo_3 5ml (1:3) and heated till dryness. After drying 5ml of HNO_3 was added in the same beaker and heated for 5-10 minutes. The volume was adjusted up to 50 ml with double distilled water and then was filtered . The sample solutions were ready to be aspirated in AAS. These sample solutions of *in vivo* and *in vitro* grown leaves were kept at 4°C with UV protection in amber bottles.

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Results and Discussion

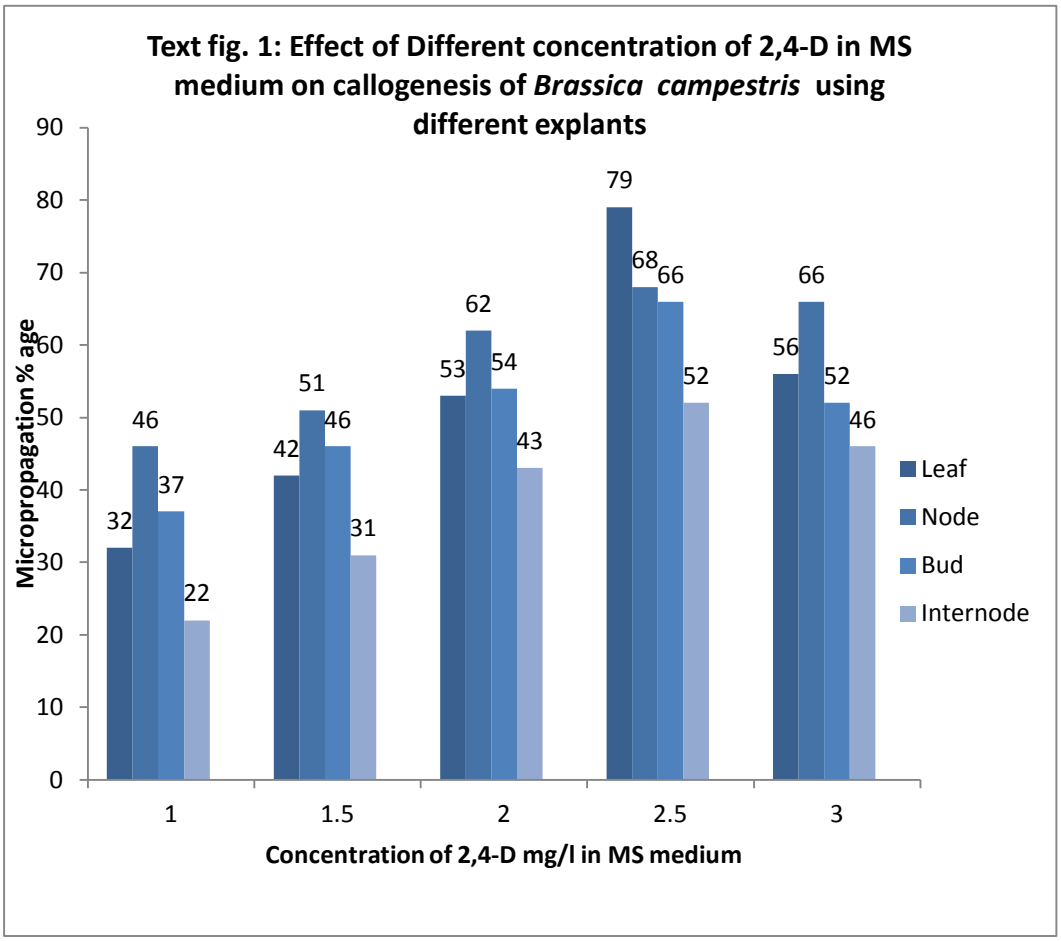
It was found that among all the PGRs and their combinations 2, 4-D was the best PGR for the *invitro* growth of *Brassica campestris.* The effect of different concentration of the 2, 4-D (mg/l) on *in vitro* growth of *Brassica campestris* in MS medium using different explants is given in table 1 and text figure 1. Twenty-five cultures were inoculated for each explant and the best response was observed in leaf explants and gave maximum percentage of *invitro* growth i.e. 79% in the medium containing 2, 4-D (2.5 mg/l), whereas the minimum percentage i.e. 46% was found in 2, 4-D (1.0mg/l).Nodal explants gave highest percentage of growth i.e. 68% in 2, 4-D (2.5 mg/l), while in medium containing 2, 4-D (1.0mg/l) showed a lowest percentage of growth i.e. 33%. The bud explants showed the maximum percentage of growth i.e. 66% in 2, 4-D (2.5mg/l), whereas the minimum percentage of growth i.e. 37% was found in 2, 4-D (1.0mg/l). Internode explants gave the maximum percentage of growth i.e. 52% in the medium containing 2, 4-D (2.5mg/l), while the minimum growth i.e. 22% was found in 2, 4-D (1.0mg/l).

Table.1: Effect of different concentration of 2,4-D (mg/l) on *in vitro* growth of *Brassica campestris* using different explants.

Sr. no.	Expalnts Used	2,4-D(mg/l) used	Number of cultures inoculated	Callogenesis and micropropagation mean (%)	LSD value
I.	Leaf	1.0	25	33±0.51^{cd}	1.46
		1.5	25	42±0.58^{c}	
		2.0	25	53±0.57^{b}	
		2.5	**25**	**79±0.34^{a}**	
		3.0	25	56±0.22^{b}	
ii.	Node	1.0	25	46±0.11^{cd}	1.23
		1.5	25	51±0.39^{c}	
		2.0	25	62±0.41^{b}	
		2.5	**25**	**68±0.21^{a}**	
		3.0	25	66±11^{b}	
iii.	Bud	1.0	25	37±57^{cd}	1.67
		1.5	25	46±0.58^{c}	
		2.0	25	54±0.31^{b}	
		2.5	**25**	**66±0.47^{a}**	
		3.0	25	52±0.37^{b}	
iv.	Internodes	1.0	25	22±0.99^{cd}	1.83
		1.5	25	31±0.52^{c}	
		2.0	25	43±0.41^{b}	
		2.5	**25**	**52±0.32^{a}**	
		3.0	25	46±1.32^{b}	

± =Standard error of the mean

The mean with different letter in each column are significantly different according to Duncan's multiple range test (0.005p value)



As far as, physical factors were concerned, it was noted that maximum and minimum *in vitro* growth, i.e 79% and 20% were seen at 25±2°C and 18±2°C respectively (Table 2). It was also observed that *Brassica campestris* in liquid medium gave only 5% *in vitro* growth. Whereas, solidified medium (using Difco-Bacto agar as solidifying agent) gave 79% *in -vitro* growth for the plant (Table 3). The effect of different photoperiods was also observed and it was concluded that 16hrs photoperiod (3000 lux) showed 80% *in vitro* growth and zero hour photoperiod (complete dark) gave minimum, i.e.10% *in vitro* growth rate of the plant (Table 3). Most suitable pH value for *in vitro* growth was found to be 5.7 with 79% i. e maximum percentage. Where as for pH 5.9 minimum, i.e. 25% *in vitro* growth was noted for *Brassica campestris.*

Table 2: Effect of temperatures & pH on *in vitro* growth of leaf explants of *Brassica campestris* in MS medium using 2, 4-D,2.5mg/l.

S. No	Physical factors	Ranges	Maximum %*In vitro* growth (% mean)
1.	Temeprature (°C)	25±2	**79 ±1.93**
2.	pH	5.7	**79±2.08a**

Table 3: Effect of Agar Solidified medium & Photoperiod on *in vitro* growth of leaf explants of *Brassica campestris* in MS medium using 2,4-D,2.5mg/l

S. No	Physical factors	Maximum% *In vitro* growth (% mean)
1.	16 hrs Photoperiod (3000 lux)	**79±2.60**
3.	Agar Solidified Medium	**79±1.78**

On the basis of observations made for physical factors and nature of media it was concluded that maximum *in vitro* growth i.e 79% was seen at 25±2°C and minimum *in vitro* growth of *Brassica campestris,* i. e 20% was observed at 18±2°C. The agar solidified MS medium supplemented with 2,4-D (2.5mg/l) gave 79% *in vitro* growth. Ebrahim, *et al.* (2000) reported influence of medium, solidification and pH value on *in-vitro* micropropagtion of shoot tip explants. The effect of different pH ranges on *in -vitro* growth of *B.campestris* were also studied during the present piece of work. It was observed that the most suitable pH range was 5.7 for *in vitro* growth (Table 2). At this pH, callus formation was 80% where as 5.9 pH gave minimum growth (25%). Ebrahim, *et al.* (2000) studied the effect of pH on the *in -vitro* growth of *Maranta leuconeura.* A medium with pH of 5.7 resulted in the maximum multiplication rate, shoot strength and leaves differentiation. *Maranta leuconeura* can be successfully micropropagated at pH

5.7 irrespective of nature of media, either it is liquid or solid .

The effect of different photoperiods on micropropagation of *Brassica campestris* was also recorded. It was observed that the most suitable photoperiod was 16 hours.. At this photoperiod 79% *in -vitro* growth was observed and the minimum photoperiod result was 10% at 0hours photoperiod. In 2004, Morini, *et.al.* (1991) studied the effect of different photoperiods on *in-vitro* growth of plum rootstock.Three photoperiods i:e16h (control), 12h and 8h were applied,with a PAR of 39 mol m^{-2} sec^{-1}. Tips collected from *in- vitro* established shoots were used. Growth medium was MS. Shoot proliferation after 45 days of growth was not statistically different between 12 h and 16 h of light, while the 8-h photoperiod gave a much lower rate of shoot formation.

The second step of this research work was to determine concentration of two mineral elements i.e; Lead (Pb) and Chromium (Cr) in *in vivo* and *in vitro* grown plant tissues of *B.campestris* after their shifting to the Kasur tanneries contaminated fields so that their comparison may be carried out.

In *in vivo* grown *Brassica campestris* gave Lead and Chromium uptake up to 1.21 and 1.62 ppm. Where as in *in vitro* grown *Brassica campestris* uptake for Lead (Pb) Chromium (Cr) uptake was found to be 2.69& 4.61ppm respectively.

The concentration of Chromium (Cr) and Lead (Pb) was determined by Yuwai *et al;* (1991) in *in vivo* grown plant tissues of *Brassicaeae*. According to them was Chromium (Cr) 3.54ppm and Lead (Pb) was 5.97ppm. One of the major factors influencing trace mineral uptake in plants is the composition of the soil.

This study also leads to the conclusion that *in vitro* grown plants can behave as natural scavengers if planted to the chemically polluted soils on large scale in future.

Table. 4: Concentration of Cr and Pb in *In-vivo* plant material of *B.campestris* determined by Atomic Absorption Spectrometry (AAS)

Plant used	**Plant tissue**	**Heavy metal**	**Concentration(ppm)**	**Mean%**
Field grown *Brassica campestris*	Leaf Explants	Pb	2.21 2.21 2.21	2.21±0.000
		Cr	1.62 1.62 1.62	1.62±0.000

Table. 5: Concentration of Cr and Pb in *In-vitro* plant material of *B.campestris* determined by Atomic Absorption Spectrometry (AAS)

Plant used	**Plant tissue**	**Heavy metal**	**Concentration(ppm)**	**Mean%**
In vitro grown *Brassica campestris*	Leaf Explants	Pb	4.61 4.61 4.61	4.61±0.000
		Cr	2.69 2.69 2.69	2.69±0.000

Table 4 and 5 show that Lead (Pb) and Chromium (Cr) both are in high quantity in *in vitro* grown plant tissues as compared to *in vivo* grown plant tissues which indicate that the composition of the media and soil plays an important role in mineral uptake of plants.This study also leads to the conclusion that *in vitro* grown plants can behave as natural scavengers if planted to the chemically polluted soils.In future, the purified in-*vitro* grown hyper accumulator plants can be used as the natural phytoremediates and heavy metal scavangers of the toxic elements e:g Pb and Cr, for the treatment of contaminated and polluted agricultural lands on commercial scale.

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