EVALUATION OF COMPARATIVE SCAVENGING EFFICIENCY OF *IN VITRO* GROWN *OCIMUM TENUIFLORUM* (TULSI) FOR COPPER AND LEAD USING ATOMIC ABSORPTION SPECTROSCOPY

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ABSTRACT: The comparative concentrations of Copper and Lead scavenged by *in vitro* grown *Ocimum tenuiflorum* were estimated with respect to its *in vivo* grown counterparts using Atomic Absorption Spectroscopy.*In vitro* plants were grown on 2.5 mg/l 2, 4-D in MS basal medium. Both these elements were estimated from the bioassays of both *in vitro* and field grown plants. *In vitro* grown *Ocimum tenuiflorum* showed lesser amount of both these elements as compared to field grown plants. Due to lesser amount of the elements in *in vitro* plant bodies, we claim that these plantlets in future could be able to extract more amount of heavy metals, when grown in industrially contaminated soils on a wider scale, proving them better phytoremediates.

Key words: Phytoremediation, Scavenging efficiency,*In vitro* plants, Atomic Absorption Spectroscopy, Heavy metals, Phyto growth regulators.

INTRODUCTION

Phytoremediation is now taken as the most emerging field of environmental biotechnology. Most of the soil contaminantions can be treated using different techniques but the heavy metal pollution specially of cultivated lands is a serious threat to the agricultural practices. The roots of the plants have natural ability to absorb heavy metals present in the soil, behaving as natural phytoremediates. Metals uptake using plants is an *in-situ* solution of the soil pollution, which is a low cost, environmental friendly, and is operated by solar energy (McCutcheon and Schnoor, 2003).

Some plants species act as hyper accumulators of the metals, depending upon their scavenging capacity and ability to deposit these metals in the different cellular compartments. These metals pass through the root cell membrane to the symplast, then metals could be passed to the vacuoles, (where their degradation occurs by enzymes) by membrane metal transporters, and could be deposited there with the help of special metal-binding proteins called metallothioneins.

Heavy metals are supposed to replace other essential metals in pigments of the cellular structure, destroying their natural balance (*Manios et al., 2003*). These metals 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-D., et al;* 1994). This technique is also useful for cultured plant organs to know the metal accumulation properties of each separate plant part e:g the removal of Sr^{2+} using shoots of *Solanum laciniatum* (*Kartosentono et al., 2001*), and Cd^{2+} hyper-accumulation by roots of *Thlaspi caerulescens* (Nedelkoska and Doran., 2000).

Ocimum tenuiflorum is a shrub reaching a height of 0.5 to 1.5m. It is branched, fragrant and erect, having hairs all over. The natural habitat varies from sea level to an altitude of 2000m. It is found growing naturally in moist soils (Heinrich, 2003). a member of the Lamiaceae (mint) family, is a native to tropical Asia. *Ocimum sanctum* is also known as 'TULSI', which means 'The incomparable one'. It is also called as 'HOLY BASIL' as it is one of the most sacred plants in Hindu Mythology. *Ocimum tenuiflorum* Plant is a prennial shrub reaching a height of 0.5 to 1.5m.. The natural habitat varies from sea level to an altitude of 2000m. It is found growing naturally in moist soils (Heinrich., 2003). *Ocimum tenuiflorum* gives good response during its *in vitro* growth, According to Lukmanul Hakim *et al.,* (2007),*O tenuiflorum* callus cultures were successfully initiated on MS medium supplemented with 2, 4-D (1 mg/l) combined with different concentrations (0.1-0.5 mg/l) of kinetin as plant growth regulators. Girija., *et al* in 2006 observed that maximum number of multiple shoots was obtained on MS medium supplemented with BAP (1.0mg/l) and Kinetin (2.0 mg/l) combination for both shoot tip and nodal

Banu and Bari., (2007) also established the protocol for multiplication and regeneration of ***O.tenuiflorum.*** **According to them,** highest percentage of shoot regeneration was obtained in 0.2 mg L^{-1} BAP.

Saha, *et al* (2010) worked on micropropagation of *Ocimum kilimandscharicum* and found that maximum growth was obtained on MS medium supplemented with different concentrations of BA, Kinetin and 2iP.

Atomic Adsorption 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 biological material .Therefore main objective of the present study is to evaluate and compare the cobalt and cadmium uptake by *in vivo* and *in vitro* grown *O. tenuiflorum* with the help of atomic absorption spectroscopy (AAS).

MATERIALS AND METHODS

This piece of work was divided into two steps:

1. The *in-vivo* and *in -vitro* growth of *O. tenuiflorum.*
2. The estimation of Copper and Lead l,uptake by *in-vivo* as well as *in vitro* grown *O. tenuiflorum* using Atomic Absorption Spectroscopy

1. The *in-vivo* and *in -vitro* growth of *O. tenuiflorum*.

For *in-vivo* growth the certified seeds of *O. tenuiflorum* were sown in the regular soil of LCW University and plants were grown for 60 days.

For the *in-vivo* growth, the explants were taken from the wild *O. tenuiflorum*, cultured and then subcultured in the PGRs optimium media for 60 days. For the *in-vitro* growth, following protocols were followed.

a. Phyto growth Regulators (PGRs): MS (Murashige and Skoog, 1962) basal medium was used. Different PGRs were used separately and in combinations in MS basal medium as follows ,

i. 2, 4-D
ii. BAP
iii. NAA
iv. TDZ
v. BAP+2, 4-D
vi. 2, 4-D +BAP+NAA

b. Sucrose level: Sucrose was added to medium at 3% concentration (30g/l).

c. Culture environment temperature: The optimum temperature required for culture environment was maintained at 25±2°C.

d. Photoperiod: The cultures were incubated at 16 hours photoperiod (under cool light fluorescent tubes(with light intensity of 2000-3000 lux).

e. pH of the medium: The pH of the medium was adjusted between 5.6-5.7.

f. Plan of experiment and data recording: Three sets of each experiment were set 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 Levesque (2007).

2. Estimation of Copper and Cobalt uptake by *in-vivo* and *in-vitro O. tenuiflorum* using Atomic Absorption Spectroscopy: Atomic absorption spectroscopy is the "determination of concentration of elements by the ability of atoms to absorb radiant energy of specific wavelengths". Through the use of calibration curves, prepared from suitable standards, a high level of accuracy and precision (±1 to 3 %) is achieved for flame Atomic Absorption Spectroscopy (FAAS). This allows for compositional as well as traces impurity analysis. It is a sensitive technique that can determine the concentration of most elements at the part - per - million (ppm) levels. It 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 Cobalt and Cadmium uptake by *O. tenuiflorum (*Phytoremediation) 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.
b. Calibration range: The calibration curve was obtained by running the standards of 0, 2, 4, 8 ppm. A straight line was obtained between concentration and absorbance. All the points of the standard were tried to lie in the straight line, because the accuracy of the sample results is dependent on the exact absorbance of these standards.

Statistical Analysis: All the data were statistically analyzed through Analysis of Variance Technique following Steel *etal*., (1997) using SPSS software (Levesque,2007).The means were compared through Duncan's Multiple Range Test(Duncan,1955).

RESULTS AND DISCUSSION

1) *In vitro* growth of *Ocimum tenuiflorum*: Effect of different explants on callogenesis: Node explants gave maximum percentage of callogenesis i.e. 92% in the same medium composition i.e. 2.5 mg/l 2, 4-D in MS medium (Murashige and Skoog., 1962). The callus obtained from nodes was compact and dark green Leaf explants showed 45% callogenesis in 2.5 mg/l 2, 4-D in MS medium and the callus obtained was compact and yellowish green in color. Sixty five % callogenesis was seen in stem explants in 2.5 mg/l 2, 4-D in MS medium.Callus texture was compact with whitish green colour, whereas callogenesis was not observed at all, with root explants (Table 1).

Effect of different concentrations of 2, 4-D on callogenesis: The effect of different concentrations of 2, 4-D in MS medium on callogenesis using nodes as explants (the best callogenic explant) is given in table 2. Thirty cultures were inoculated with node explants in various 2, 4-D concentrations but best callogenesis (96.6%) was seen in cultures containing 2.5 mg/l 2, 4-D and a massive dark green, compact callus was observed.

Effect of different concentrations of BAP on callogenesis: The effect of different concentrations of BAP in MS medium on callogenesis using nodes as explants is shown in table 3. All of the 30 test tubes showed response but best response (80.5%) was shown by cultures having concentration of BAP 5 mg/l.

Effect of different concentrations of BAP and 2, 4-D on callogenesis: Effect of different concentrations of BAP + 2, 4-D in MS medium on callogenesis using nodes as explants is shown in table 4. Thirty cultures were inoculated containing different combinations of 2, 4-D + BAP. Callogenesis was observed in all the combinations but best callogenic results (66.6) were observed in 2.0 mg/l BAP+ 1.5 mg/l 2, 4-D.

Effect of different concentrations of 2, 4-D, BAP and NAA on callogenesis: Table 5 shows the effect of various concentrations of 2, 4-D + BAP + NAA in MS medium on callogenesis using nodes as explants. All culrures showed good results but best callogenesis (93.3%) was observed in medium containing 1.0 mg/l 2, 4-D + 0.5 mg/l BAP + 0.5 mg/l NAA.Saha, *et al*(2010) worked on micropropagation of *Ocimum killimandscharicum* and found that maximum growth was obtained on MS medium with different concentrations of BA, Kinetin and 2iP.

Effect of different concentrations of BAP and Kinetin on callogenesis: Effect of different concentrations of BAP + Kinetin in MS medium on callogenesis using

nodes as explants is given in table 6. Rate of callogenesis (93.3%) was found to be best in medium supplemented with 5.0 mg/l BAP + 5.0 mg/l kinetin. Girija, *et al* observed that maximum number of multiple shoots was obtained on MS medium supplemented with BAP (1.0mg/l) and Kinetin (2.0mg/l) combination for both shoot tip and nodal explants.

Effect of different concentrations of TDZ on callogenesis and regeneration: Table 7 shows the effect of different concentrations of TDZ on callogenesis and regeneration using nodes as explants. 30 cultures containing medium with TDZ were inoculated. Rate of callogenesis was higher than regeneration. The best concentration of TDZ was found to be 18 µM for callogenesis as well as regeneration .The callus was compact, granular and light green in color along with regenerated plantlets.

Effect of liquid medium and solidifying agents on callogenesis and regeneration:

Effect of solidifying agents on *in vitro* growth of *Ocimum tenuiflorum* is given in table 9. Three types of media were used:

- Liquid medium
- Agar solidified medium
- Phyta gel solidified medium

Thirty cultures were inoculated but only 4 to 9 cultures showed minor response in liquid medium containing 2.0 mg/l and 2.5 mg/l 2, 4-D (Table 8).

Rate of callogenesis and regeneration was found to be best in phyta gel solidified medium, good in agar solidified medium and poor in liquid medium. Liquid medium gave 30% callogenesis but no regeneration whereas agar solidified medium showed 60% callogenesis and 50% regeneration. Maximum callogenesis and regeneration was obtained in phytagel solidified medium i.e.98% callogenesis and 90% regeneration.

Effect of different temperature ranges on callogenesis and regeneration: Table 10 shows the effect of different temperature ranges on callogenesis and regeneration of *Ocimum tenuiflorum.* It was observed that the most suitable temperature range was 25±2 for *in vitro* growth. The callogenesis rate was 95 % at this temperature while regeneration was 45%. Shilpa., *et al(2010)* grew *in vitro* plants of *Ocimum sanctum* from root explants at 25°C in 16 hrs photoperiod.

Effect of different pH levels on callogenesis and regeneration: Table 11 shows the effect of different pH levels on *in vitro* growth of *Ocimum tenuiflorum* .pH 5.7 were found to be the best for the *in vitro* growth. At this pH level, callogenesis rate was 91% and regeneration rate was 29%.Gogoi and Kumaira(2011) got regeneration of *Ocimum sanctum* at a pH of 5.7 on a solidified medium.

During the present piece of work, the best explant selected for *in vitro* growth of *Ocimum tenuiflorum* was the nodal portion of mature plant taken from the field. It was inoculated in the MS medium in order to form callus mass. According to Lukmanul Hakim *et al* (2007), callus cultures were successfully initiated on MS medium supplemented with 2, 4-D (1 mg/l) combined with different concentrations (0.1-0.5 mg/l) of kinetin as plant growth regulators. During present study, the best medium composition for the *in vitro* growth of *Ocimum tenuiflorum* was 2, 4-D (2.5 mg/l).

Girija *et al*., (2006) observed that maximum number of multiple shoots was obtained on MS medium supplemented with BAP (1.0mg/l) and Kinetin (2.0 mg/l) combination for both shoot tip and nodal explants but during the present piece of work, it was concluded that a combination of 5.0mg/l BAP and 5.0 mg/l Kinetin was suitable for callogenesis.

Banu and Bari (2007) also established the protocol for multiplication and regeneration of ***Ocimum sanctum.*** **According to them,** highest percentage of shoot regeneration was obtained in 0.2 mg L^{-1} BAP. Whereas during the present study, 18µM TDZ was found to be best PGR for the regeneration of ***Ocimum tenuiflorum*** **from the callus tissues.**

2) Estimation of Copper (Cu) and Lead (Pb) in *in vivo* and *in vitro Ocimum tenuiflorum* by Atomic Absorption Spectroscopy:

a. Concentration of Copper (Cu) and Lead (Pb) in the leaves of *in vivo* grown *Ocimum tenuiflorum* by estimated by Atomic Absorption Spectroscopy : Concentration of Copper (Cu) and Lead (Pb) in *in vivo* grown *Ocimum tenuiflorum* was determined with the help of Atomic Absorption Spectroscopy and leaves were used as biomass material. The concentration of Copper (Cu) was found to be 4.83 ppm while that of Lead (Pb) was 0.44 ppm as indicated in Table 12. Three concordant readings were taken to confirm the results.

b. Concentration of Copper (Cu) and Lead (Pb) in the leaves of *in vitro* grown *Ocimum tenuiflorum estimated* by Atomic Absorption Spectroscopy: The concentration of Copper (Cu) and Lead (Pb) in *in vitro* grown *Ocimum tenuiflorum* was evaluated with the help of Atomic Absorption Spectroscopy which was found to be 0.29 ppm for Copper (Cu) and 0.031 ppm for Lead after taking three concordant readings as shown in Table 13.

c. Comparison of Copper (Cu) and Lead (Pb) concentrations during *in vivo* and *in vitro* growth of *Ocimum tenuiflorum* :

Comparison of Copper (Cu) and Lead (Pb) concentrations during *in vivo* and *in vitro* growth of *Ocimum tenuiflorum* by Atomic Absorption Spectroscopy is shown in table 14.

Concentration of Copper in the leaves of *in vivo* grown plants was found to be 4.83 ppm whereas in the

leaves of *in vitro* grown plants, its concentration was found to be 0.29 ppm. Concentration of Lead in *in vivo* grown plants of *Ocimum tenuiflorum* was found to be 0.44 ppm whereas its concentration in the leaves of *in vitro* grown *Ocimum sanctum* plants was 0.031 ppm. Lanning and Schrenk., (1977) also reported a rapid and simple atomic absorption spectroscopic method for determining copper in plants. The methods have been applied to alfalfa, whole grain corn, whole grain sorghum, sorghum leaves, sunflower leaves and rice leaves.

The present study indicated that the concentration of Copper (Cu) and Lead (Pb) in *in vivo* grown *Ocimum tenuiflorum* was higher than that of *in vitro* grown *Ocimum tenuiflorum* plants which led to the conclusion that filed grown (*in vivo*) plants of *Ocimum tenuiflorum* absorbed more Copper (Cu) and Lead (Pb) from the soil as compared to *in vitro* grown *Ocimum tenuiflorum* (which were grown in controlled cultural conditions).

Table 1: Response of different explants of *Ocimum tenuiflorum* used for callogenesis on MS medium supplemented with 2.5mg/l 2, 4-D.

Type of explants used	Number of cultures	Callogenesis (% mean)	Texture and color of callus
Leaf	30	45 ± 0.02^{c}	Compact, dark green
Node	30	92 ± 0.12^{a}	Compact,yellowish green
Stem	30	65 ± 0.09^{b}	Compact, light green
Root	30	0	–

The means with different letters in each column are significantly different according To Duncan's Multiple Range Test (<0.05 p value) ± Standard error of the mean.

Table 2: Effect of different concentrations of 2, 4-D in MS medium on callogenesis using nodes as explants.

2, 4-D used mg/l	Number of cultures	Callogenesis (%mean)	Texture of callus	Color of callus
1.0	30	26.6 ± 0.02^{c}	Compact granular	Yellowish green
1.5	30	33.3 ± 0.05^{c}	Compact granular	Yellowish green
2.0	30	50 ± 0.04^{b}	Compact granular	Yellowish green
2.5	30	96.6 ± 0.01^{a}	Compact granular	Yellowish green
3.0	30	63.3 ± 0.02^{b}	Compact granular	Yellowish green

The means with different letters in each column are significantly different according to Duncan's Multiple Range Test (<0.05 p value) ± Standard error of the mean.

Table 3: Effect of different concentrations of BAP in MS medium on callogenesis using nodes as explants.

BAP used (mg/l)	Number of cultures	Callogenesis (%mean)	Texture of callus	Color of callus
1.0	30	16.6 ± 0.02^{d}	Compact granular	Light green
2.0	30	36.6 ± 0.01^{c}	Compact granular	Light green
3.0	30	53.3 ± 0.06^{b}	Compact granular	Light green
4.0	30	70 ± 0.07^{ab}	Compact granular	Light green
5.0	30	80.5 ± 0.02^{a}	Compact granular	Light green

The means with different letters in each column are significantly different according to Duncan's Multiple Range Test (<0.05 p value) ± Standard error of the mean.

Table 4: Effect of different concentrations of BAP + 2, 4-D in MS medium on callogenesis using nodes as explants.

BAP + 2, 4 D used (mg/l)	Number of cultures	Callogenesis (%mean)	Texture of callus	Color of callus
1.0 + 2.5	30	30 ± 0.012^{cd}	Compact	Yellowish green
1.5 + 2.5	30	40 ± 0.05^{bc}	Granular	Light green
1.5 + 2.0	30	50 ± 0.04^{b}	Compact	Brownish green
1.0 + 1.5	30	66.6 ± 0.51^{a}	Granular	Bright green
1.0 + 1.5	30	60 ± 0.12^{a}	Granular	Yellowish green

The means with different letters in each column are significantly different according to Duncan's Multiple Range Test (<0.05 p value) ± Standard error of the mean.

Table 5: Effect of different concentrations of 2, 4-D + BAP + NAA in MS medium on callogenesis using nodes as explants.

2, 4-D + BAP + NAA used (mg/l)	Number of cultures	Callogenesis (%mean)	Texture of callus	Color of callus
0.5 + 1.5 + 1.5	30	36.6 ± 0.32^{d}	Compact granular	Light green
1.0+ 0.5 + 0.5	30	93.3 ± 0.14^{a}	Compact granular	Light green
1.5 + 2.0 + 2.0	30	40 ± 0.025^{cd}	Compact granular	Light green
2.0+ 2.0 + 2.5	30	56.6 ± 0.014^{b}	Compact granular	Light green
2.5 + 2.5 + 2.0	30	63.3 ± 0.01^{b}	Compact granular	Light green

The means with different letters in each column are significantly different according to Duncan's Multiple Range Test (<0.05 p value) ± Standard error of the mean.

Table 6: Effect of different concentrations of BAP + Kinetin in MS medium on callogenesis using nodes as explants.

BAP + Kin used (mg/l)	Number of cultures	Callogenesis (%mean)	Texture of callus	Color of callus
1.0+ 1.0	30	33.3 ± 0.01^{d}	Granular friable	Light green
2.0+ 2.0	30	50 ± 0.02^{bc}	Granular friable	Whitish green
3.0+ 3.0	30	60 ± 0.12^{bc}	Granular friable	Whitish green
4.0+ 4.0	30	83.3 ± 0.50^{b}	Granular friable	Light green
5.0+ 5.0	30	90 ± 0.014^{a}	Granular friable	Whitish green

The means with different letters in each column are significantly different according to Duncan's Multiple Range Test (<0.05 p value) ± Standard error of the mean.

Table 7: Effect of different concentrations of TDZ in MS medium on callogenesis andregeneration using nodes as explants.

TDZ used (µM)	Number of cultures	Callogenesis (%mean)	Regeneration (%mean)	Texture and color of callus
10	30	33.3 ± 0.12^{cde}	5 ± 0.16^{a}	Compact, granular, Light green
12	30	46.6 ± 0.025^{bc}	13.3 ± 0.12^{b}	Compact, granular, Light green
14	30	56.6 ± 0.01^{bc}	15 ± 0.025^{c}	Compact, granular, Light green
16	30	63.3 ± 0.07^{ab}	26.6 ± 0.02^{d}	Compact, granular, Light green
18	30	70 ± 0.08^{a}	39 ± 0.13^{d}	Compact, granular, Light green

The means with different letters in each column are significantly different according to Duncan's Multiple Range Test (<0.05 p value) ± Standard error of the mean.

Table 8: Effect of liquid medium supplemented with 2, 4-D in MS medium on callogenesis using nodes as explants.

2, 4-D used in liquid medium (mg/l)	Number of cultures	Callogenesis (%mean)	Texture of callus	Color of callus
1.0	30	0	–	–
1.5	30	0	–	–
2.0	30	13.3 ± 0.12^{b}	Soft, granular	Light brown
2.5	30	30 ± 0.024^{a}	Soft, granular	Light brown

SThe means with different letters in each column are significantly different according to Duncan's Multiple Range Test (<0.05 p value) ± Standard error of the mean.

Table 9: Effect of solidifying agents on *in vitro* growth of *Ocimum tenuiflorum.*

State of Medium (MS basal medium)	Number of cultures	*In vitro* growth rate (%) mean	
		Callogenesis	Regeneration
Liquid medium (without Solidifying agent)	30	30 ± 0.35^{c}	0
Agar solidified Medium	30	60 ± 0.25^{b}	50 ± 0.12^{b}
Phyta gel solidified medium	30	98 ± 0.12^{a}	90 ± 0.05^{a}

The means with different letters in each column are significantly different according to Duncan's Multiple Range Test (<0.05 p value) ± Standard error of the mean.

Table 10: Effect of temperature on *in vitro* growth of *Ocimum tenuiflorum.*

Serial number	Temperature ranges (ºC)	Number of cultures	*In vitro* growth rate (mean)%	
			Callogenesis	Regeneration
1.	21±2	30	30 ± 0.12^{d}	15 ± 0.025^{cd}
2.	23±2	30	39 ± 0.13^{c}	20 ± 0.04^{c}
3.	25±2	30	95 ± 0.012^{a}	45 ± 0.03^{a}
4.	27±2	30	46 ± 0.25^{c}	27 ± 0.21^{b}
5.	29±2	30	60 ± 0.14^{b}	36 ± 0.25^{a}

The means with different letters in each column are significantly different according to Duncan's Multiple Range Test (<0.05 p value) ± Standard error of the mean.

Table 11: Effect of pH on *in vitro* growth of *Ocimum tenuiflorum*:

Serial number	pH ranges	Number of test tubes inoculated	*In vitro* growth rate (%mean)	
			Callogenesis	Regeneration
1.	5.1	30	27 ± 0.02^{e}	18 ± 0.01^{b}
2.	5.3	30	41 ± 0.14^{cd}	5 ± 0.16^{a}
3.	5.5	30	59 ± 0.24^{b}	31 ± 0.25^{c}
4.	5.7	30	91 ± 0.045^{a}	29 ± 0.25^{c}
5.	5.9	30	38 ± 0.045^{d}	21 ± 0.45^{c}

The means with different letters in each column are significantly different according to Duncans' Multiple Range Test (<0.05 p value) ± Standard error of the mean.

Table 12: Concentration of Copper (Cu) and Lead (Pb) in the leaves of *in vivo* grown *Ocimum tenuiflorum* determined by Atomic Absorption Spectroscopy .

Element	Biomass used	Concentration of element (ppm)	Mean concentration of element (ppm)
Copper (Cu)	Leaf from field grown plants	4.83	4.83
		4.83	
		4.83	
Lead (Pb)	Leaf from field grown plants	0.44	0.44
		0.44	
		0.44	

Table 13: Concentration of Copper (Cu) and Lead (Pb) in *in vitro* grown *Ocimum Tenuiflorum* determined by Atomic Absorption Spectroscopy.

Element estimated	Biomass used	Concentration of element (ppm)	Mean concentration of element (ppm)
Copper (Cu)	*In vitro*regenerated leaves	0.29	0.29
		0.29	
		0.29	
Lead (Pb)	*In vitro* regenerated leaves	0.031	0.031
		0.031	
		0.031	

Table 14: Comparison of Copper (Cu) and Lead (Pb) concentration during *in vivo* and *in vitro* growth of *Ocimum tenuiflorum* by atomic absorption spectroscopy

Element estimated	Condition of growth	Concentration of element (ppm) mean
Copper (Cu)	*In vivo*	4.83
	In vitro	0.29
Lead (Pb)	*In vivo*	0.44
	In vitro	0.031

We conclude from these results that tissue cultured plantlets can be better natural hyperaccumulates and scavengers due to lesser amount of elements in their bodies because they were grown in the controlled growth environment as compared to field grown plants which have now lesser absorbing capacity due to more amount of already absorbed elements in their tissues. This piece of work can be helpful for future research on *in vitro* grown *Ocimum tenuiflorum* as possible natural Copper (Cu) and Lead (Pb) scavenger and stress tolerant plant for polluted and contaminated soils of our country.

REFERENCES

Banu, L.A. and Bari Miah, M.A. Protocol establishment for multiplication and regeneration of ***Ocimum sanctum*** Linn. An important medicinal plant with high religious value in Bangladesh, *Journal of Plant Sciences*, 2 (5): 530-537(2007).

Ben-Hayyim, G. Relationship between salt tolerance and resistance to polyethylene glycol-induced water stress in cultured *Citrus* cells, *Plant Physiology* , 85(20): 430-433(1987).

Duncan, D .B. Multiple Range and Multiple F Tests. *Biometrics*, 11(1): 11-42 (1955).

Girija, S, S. Kavitha, and S. Deepavathi. Direct multiple shoot regeneration from shoot tip and nodal explants of ***Ocimum sanctum*** L. (Tulsi): A medicinal herb. *Plant Cell Biotechnology & Molecular Biology*, 7(1-2): 23-28(2006).

Gogoi, K and S.Kumiara. Callus mediated plantlet regeneration of *O.tenuiflorum* using axillary buds as explants.*Intl J Pl Sc*,2(1):001-005(2011).

Heinrich, M. Ethnobotany in México and the search of anti-inflammatory natural Products*, Personal Care Ingredients, Formulation, Manufacture,* 4: 51-55(2003).

Kartosentono. S, N. Ana, I. Gunawan and Z. Noor Cholies. Phytoremediation of Sr^{2+} and its influence on the growth, Ca^{2+} and solasodine content of shoot culture of *Solanum laciniatum*. *Biotechnology Letters*, 23 (2): 153-155(2001).

Lanning, F. C and W.G.Schrenk. Comparison of Atomic Absorption Flame Spectroscopy and the Official AOAC Method for determining Copper in plant tissues. *Transactions of the Kansas Academy of Science,*80 (1/2): 41-46(1977)

Levesque, R. SPSS Programming and Data Management: A Guide for SPSS and SAS Users, Fourth Edition (2007), SPSS Inc., Chicago Ill.

Lukmanul Hakkim, F. C, Gowri Shankar, and S. Girija, Chemical composition and antioxidant property of holy basil (***Ocimum sanctum*** L.) leaves, stems, and inflorescence and their *in **vitro*** callus cultures. *Journal of Agricultural & Food Chemistry*, 55(22): 9109-9117(2007).

McCutcheon, C. S and J. L. Schnoor, Phytoremediation, Transformation and Control of Contaminants. John Wiley-*Interscience,* New Jersey, 987 : 39435-39431(2003).

Manios, T, E.I Stentiford and P.A. Millner. The effect of heavy metals accumulation on the chlorophyll concentration of *Thypha latifolia* plants, growing in a substrate containing sewage sludge compost and watered with metaliferus water. *Ecological Engineering*, 20(1):65-74(2003).

Murashige, T and F.Skoog. A revised medium for rapid growth and Bioassays with tobacco tissue cultures, Physiol. Plant, 15: 473-49(1962).

Nedelkoska, T. V. and P.M. Doran. Hyperaccumulation of Cadmium by hairy roots of *Thlaspi caerulescens, Biotechnology and Bioengineering*, 67 (5): 607-615(2000).

Rivetta, A, N. Negrini and M. Cocucci. Involvement of Ca^{2+}-calmodulin in Cd^{2+} toxicity during the early phases of radish (*Raphanus sativus* L.) seed germination, *Plant, Cell and Environment*, 20(5):600-608(1997).

Saha, S, T. Dey and P. Ghosh.Microprpagation of *O.kilimandscharicum* Guerke. *Acta Biologica Cracovienansia* ,52(2):50-58(2010).

Santos-D,D.S. María and O-A. Neftalí. PEG-tolerant cell clones of chili pepper: growth, osmotic potentials and solute accumulation, *Plant Cell, Tissue and Organ Culture*, 37 (1): 1-8(1994).

Shilpa,K, C. Selvakumar, A.Senthil and B.S.Lakshami.*In vitro* root cultures of *Ocimum sanctum* and elevation of its free radicle scavanging activity.*plant Cell Tissue organ Culture*,101:105-109(2010).

Steel, R.G. D, J. H. Torrie and D. A. Dickey. Principles and Procedures of Statistics. A biometrical approach. 3rd Ed., McGraw Hill Book Co, New York, USA. (1997).