Evaluation of Bacopa Monnieri as a Suitable Hyperaccumulant for Copper Contaminated Soil using in vitro Techniques

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Abstract—Various heavy metals like as, Cu, Zn, Pb, Hg etc. affect the environment by affecting soil properties, its fertility, biomass and crop yield and ultimately human health. Therefore, heavy metal pollution poses a great threat to the environment and mankind. In order to maintain good quality of soil and water and keep them free from contamination, continuous efforts have been made to develop technologies that are easy to use, sustainable and economically feasible. Phytoremediation as an emerging technology that employs the use of higher plants for the clean-up of contaminated environment has been reported using several plant species. Presently an attempt has been made to study the efficiency of in vitro regenerated multiple shoot cultures of Bacopa monnieri to accumulate copper from the plant medium. Copper sulphate (CuSO45H2O) was added to the shoot multiplication medium at varying concentrations of 0; 20; 40 and 80 mg/ltr media. The effect of copper on plant growth, chlorophyll content and production of pharmaceutically important bacosides from the regenerants was examined. It was observed that copper sulphate acted as an elicitor for plant growth at a concentration of 20 mg/l. Although, when the concentration was increased from 20 mg/l to 80 mg/l it resulted in suppression of plant growth. The efficiency of copper removal was highest in plants treated with 40 mg/l of copper sulphate and decreased upon increase in concentration to 80 mg/l. Bio-concentration factor (BCF) calculated for all the treatments was found to be maximum (3.03) at 40 mg/l followed by 20 mg/l (2.7) and 80 mg/l (1.91). Bacoside content measured by HPLC was found to be maximum in the cultures treated with copper at a concentration of 40 mg/l (12.405 mg/g DW) followed by 80 mg/l (8.091 mg/g DW), control (8.047 mg/g DW) and 20 mg/l (1.127 mg/g DW). The study suggests the practical application of B. monnieri plants which can act as hyperaccumulators for the removal of copper in industries where the metal is a major contaminant.

Key words: Brahmi, Multiple Shoot Culture, Phytoremediation, Bio-Concentration Factor, Bacoside Production, Chlorophyll Estimation

I. INTRODUCTION

Heavy metals are among the most common environmental pollutants, and their occurrence in water and biota indicate the presence of natural or anthropogenic pollutants. Accumulation and distribution of heavy metals in soil, water and environment is increasing at an alarming rate causing deposition and sedimentation in water reservoirs and affecting aquatic organisms as well [1,2,3]. Phytoremediation is considered to be a green revolution in the field of innovative clean up technologies and is evolving as a cost effective, environment friendly technology to clean up contaminated environments [4,5,6,7,8]. The success of phytoremediation process to clean up the metal contaminated soil and water depends upon the type of the plants being used for the purpose. Plants suitable for phytoremediation purpose should be fast growing, having high biomass and should be tolerant to pollution. Till now, over 400 hyperaccumulator plants including members of families Asteraceae, Brassicaceae, Caryophyllaceae, Cyperaceae, Cunoniaceae, Fabaceae, Flacourtiaeaceae, Euphorbiaceae, Lamiaceae, Poaceae and Violaceae have been identified. Various plants which have been studied for their phytoremediation potential include Helianthus, Brassica, Arabidopsis, Phyllanthus etc. Among water plants, water ferns Azolla, Lemna, Pteris, Elodea Echhornia, Ceratophyllum, Myriophyllum, Phragmites and Bacopa have been studied [9,10,11,12] as hyperaccumulating plants for the removal of heavy metals from the environment. The removal of metals from solution using plants offers an attractive alternative, because it is solar driven and can be carried out in situ, minimizing cost and human exposure [13,7]. Researchers from various countries [5,6,14,15,16,17] have done lot of research on phytoremediation but little information is available from our country [18,19,20] in general and Jammu and Kashmir in particular. The objectives of the present study were thus to investigate:

1) Elicitation effect of Copper on plant growth,
2) Accumulation of Copper by Bacopa monnieri,
3) Effect of Copper accumulation on bacoside content of in vitro cultured plants,
4) To calculate the Bio-concentration factor (BCF) so as to establish it as a model system, this, in future would help us to get rid of copper from contaminated soils and waters.

II. MATERIALS AND METHODS

A. Plant Material

Multiple shoot cultures of Bacopa monnieri were selected from established cultures maintained in the Tissue Culture Lab of SMV DU. The phytoremediation potential of B. monnieri for Copper and its effect on shoot culture growth and Bacoside content was analyzed. A total of four treatments were prepared each containing MS media + sucrose (3%) + BAP (1 mg/l as PGR) spiked with different concentrations of copper sulphate i.e. 0 mg/l (control), 20 mg/l, 40 mg/l and 80 mg/l. The pH of the media was adjusted at 5.8-6.0 prior to addition of Agar (7 g/l, w/v), and the media was autoclaved at 120 OC for 20 minutes. The explants (clump of multiple shoots each having an average of 10 shoots/cluster), were transferred on to culture media contained in conical flasks (4 explants flask).
B. Incubation Conditions:
Cultures were incubated under controlled photoperiod and temperature conditions in the culture room with a light intensity of 35 µmol/s provided by cool white fluorescent tubes (Philips) at 25 ± 20C (day) and 20 ± 10 C (night) throughout the experimental duration. After four weeks, shoot cultures were harvested and subjected to analysis for chlorophyll, bacoside and heavy metal contents.

C. Heavy Metal Analysis
Harvested plant samples were weighed for fresh weight, air dried until constant dry weight was reached. Dry weight of the samples was recorded and it was ground to a fine powder using silica pestle mortar, to pass through a 2 mm mesh size. 1.0 gm of the powdered plant material was put in a pre- weighed porcelain crucible and placed in a cool muffle furnace. The temperature of the muffle furnace was set to allow a gradual increase (over a period of 2 hours) to reach ashing temperature (500-550 0C) and maintained for 4-8 hours. The furnace with samples was allowed to cool. The ash samples were dissolved in 5 ml of 2N HCl and allowed to stand for 20 minutes. Using Whatman 42 filter paper, the above solution was filtered with repeated washings of distilled water and volume was maintained to 50 ml. Prepared samples were analysed for heavy metal Copper (Cu) using AAS ECAC.

D. Estimation of Bio-Concentration Factor
The Bio-concentration factor was calculated by dividing the metal concentration in plant tissue (mg/kg) by the initial concentration (mg/l) of the metals in the nutrient solution [21].

\[
\text{BCF} = \frac{(P/E)_{i}}{E}
\]

Where \(i\) denotes the heavy metal, and BCF is the Bio-concentration factor and is dimensionless. \(P\) represents the trace element concentration in plant tissues (mg/kg dry wt); \(E\) represents the trace element concentration in the nutrient solution (mg/l). A larger ratio implies better phytoaccumulation capability.

E. Bacoside Content:
For bacoside content evaluation the plant material was washed properly under tap water and fresh weight was recorded. The sample was air dried for two days and then dry weight was recorded. The dried plant material was ground into a fine powder and subjected to Soxhlet extraction and then to HPLC analysis for the evaluation of bacoside content in the heavy metal treated Bacopa monnieri plants as per method described [22].

F. Chlorophyll Estimation
The chlorophyll content (chl a and chl b) of fresh leaves of Bacopa monnieri was extracted using 80% acetone and absorptions were read on a spectrophotometer at 645 nm, 652 nm and 663 nm. Using absorption coefficients the amount of chlorophyll was calculated by applying the formula

\[
\text{Total Chlorophyll } \left( \frac{mg}{g} \right) = \frac{A_{665} \times 10000 \times V}{34.5 \times 1000 \times W}
\]

\[
\text{Chlorophyll a } \left( \frac{mg}{g} \right) = \frac{12.7 \times A_{665} - 2.69 \times A_{652}}{1000 \times W}
\]

\[
\text{Chlorophyll b } \left( \frac{mg}{g} \right) = \frac{22.9 \times A_{663} - 4.68 \times A_{645}}{1000 \times W}
\]

III. RESULTS AND DISCUSSION

A. Morphogenetic response of cultured explants of B. monnieri at various CuSO4 concentrations
As shown in Table 1, the mean number of shoots of B. monnieri cultured in MS media + BAP supplemented with various concentrations of CuSO4 was maximum in the treatment 20 mg/l (10±1.46) followed by 80 mg/l (7.7±0.46), 40 mg/l (6.4±0.640) and control (6.625±1.006). In the treatment 80 mg/l, mean number of leaves was maximum (9.95±0.424) followed by 40 mg/l (8.7±0.266), control (8.1±0.59) and 20 mg/l (8.1±0.233). The maximum mean length of the treated explants was (3.5±0.330cm) in control explants, followed by 40 mg/l (2.925±0.155cm), 20 mg/l (2.825±0.124 cm) and 80 mg/l (2.25±0.177 cm).

B. Observations of FW, DW and Chlorophyll Estimation

![Fig. 1: Morphology of 4 week old cultures of Bacopa monnieri. A-mother culture growing in Herbal Garden at SMVDU, B-Mother culture, C-Control treatment, D-Treatment with 20 mgl⁻¹ copper concentration, E-Treatment with 40 mgl⁻¹ copper concentration, F- Treatment with 80 mgl⁻¹ copper concentration.](image_url)

### Table 1: Morphogenetic Response of Cultured Explants of Bacopa Monnieri Treated with Various Copper Sulphate Concentrations

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean no. of shoots*</th>
<th>Mean no. of leaves</th>
<th>Mean length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.625±1.006</td>
<td>8.3±0.49</td>
<td>3.056±0.330</td>
</tr>
<tr>
<td>20 mg/l</td>
<td>10±0.356</td>
<td>8.1±0.223</td>
<td>2.825±0.124</td>
</tr>
<tr>
<td>40 mg/l</td>
<td>6.4±0.640</td>
<td>8.7±0.266</td>
<td>2.925±0.155</td>
</tr>
<tr>
<td>80 mg/l</td>
<td>7.7±0.46</td>
<td>9.95±0.424</td>
<td>2.25±0.177</td>
</tr>
</tbody>
</table>

*Values represent mean ± SE
Table 2 shows that the initial biomass (FW) was maximum in control (13.450 g) followed by 20 mg/l (10.652 g), 80 mg/l (10.225 g) and 40 mg/l (9.855 g). The FW of the plants harvested after 1 month was maximum in control conditions (68.39 g) followed by 20 mg/l (57.71 g), 40 mg/l (49.69 g) and 80 mg/l (40.37 g), respectively. The final biomass (DW) was maximum in 20 mg/l (7.55 g) followed by 40 mg/l (6.63 g), control (5.84 g) and 80 mg/l (4.98 g). The value of Growth index of the cultured Bacopa monnieri after 4 weeks was minimum for treatment 80 mg/l (294) and maximum for 20 mg/l (441) (Table 2 and Fig. 1a). The total chlorophyll content mg/g of FW of the harvested plant samples was maximum at 80 mg/l (0.95) followed by control (0.89), 40 mg/l (0.80) and 20 mg/l (0.70), respectively (Table 2).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Treatment (mg/L)</th>
<th>Initial Biomass (FW in gms.)</th>
<th>Final Biomass (FW in gms.)</th>
<th>Final Biomass (DW in gms.)</th>
<th>Growth Index*</th>
<th>Total Chlorophyll Content mg/g FW</th>
<th>Chl a mg/g FW</th>
<th>Chl b mg/g FW</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>13.450</td>
<td>68.39</td>
<td>5.84</td>
<td>408</td>
<td>0.89</td>
<td>0.084</td>
<td>0.393</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>10.652</td>
<td>57.71</td>
<td>7.55</td>
<td>441</td>
<td>0.70</td>
<td>0.278</td>
<td>0.218</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>9.855</td>
<td>49.69</td>
<td>6.63</td>
<td>404</td>
<td>0.80</td>
<td>0.527</td>
<td>0.213</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>10.225</td>
<td>40.37</td>
<td>4.98</td>
<td>294</td>
<td>0.95</td>
<td>0.533</td>
<td>0.268</td>
</tr>
</tbody>
</table>

*Growth Index = Final weight-Initial weight X 100 Initial weight

Table 2: Fresh Weight (Fw), Dry Weight (Dw), Total Chlorophyll, Chlorophyll A and Chlorophyll B of Bacopa Monnieri Measured After Extraction with 80% Acetone (V/V)

C. Copper Accumulation:
Copper accumulation by the explants cultured in MS media + BAP spiked with different concentrations of CuSO4 was maximum in treatment 80 mg/l (13.450 mg/kg) followed by 40 mg/l (12.085 mg/kg), 20 mg/l (54.05 mg/kg) and control (2 mg/kg) (Table 3 and Fig. 1b).

D. Bacoside Content:
Bacoside content measured by HPLC was found to be maximum in the treatment 40 mg/l (12.405 mg/g DW), 80 mg/l (8.091 mg/g DW), control (8.047 mg/g DW) and 20 mg/l (1.127 mg/g DW) (Table 4 and Figs. 1a and 1b).

E. Elicitation Effect of Copper on Plant Growth
It was observed that the copper added to the media in the form of CuSO4 acted as an elicitor for plant growth at a concentration of 20 mg/l. But when the concentration was increased from 20 mg/l to 80 mg/l, it resulted in the suppression plant growth which can be correlated with maximum growth index value at 20 mg/l and minimum at 80 mg/l (Table 2). It is pertinent to mention here that multiple shoot cultures with a highest average number of 10±0.536 shoots were recorded in the treatment with 20 mg/l CuSO4 concentration indicating the forcing effect of the treatment on shoot proliferation.

F. Copper Accumulation by Bacopa Monnieri
Table 3 and Fig. 1b shows Copper accumulation by B. monnieri at different concentrations and it increased with the background concentration of Copper in the treatment but the response was not linear at higher concentration i.e 80 mg/l. BCF calculated for all the treatments was found to be maximum (3.03) at 40 mg/l followed by 20 mg/l (2.7) and 80 mg/l (1.91) respectively.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conc. of Copper(mg/kg)</th>
<th>BCF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.242</td>
<td>-</td>
</tr>
<tr>
<td>20 mg/l</td>
<td>54.05</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Table 3: Heavy Metal Analysis of Different Culture Treatments by Atomic Absorption Spectrophotometer

Table 4: Bacoside Content of Experimental Plants Treated with Different Copper Sulphate Concentrations by HPLC

Fig. 1a

Fig. 2a: Graphical representation of the Bacoside A content and Growth Index of different culture treatments
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Fig. 1b

Fig. 2b: Graphical representation of the Bacoside A content and Concentration of Copper accumulated by different culture treatments

G. Effect of Copper Accumulation on Bacoside Content

Surprisingly the results of the experiment did not result in a significant increase in the bacoside production rates in the shoot cultures, although, the cultures treated with 40 mg/l of copper showed the maximum bacoside production (12.405 mg/g DW) indicating a positive correlation between degree of growth and metabolite production. This can be attributed to the fact that these types of bio chemicals are released under stress conditions.

Although copper acts as micronutrient for the plant growth yet at high concentrations it is toxic metal for the growth and development of plants. In order to evaluate the hyperaccumulating capacity of Bacopa monnieri for copper following study was undertaken. Phytoremediation studies, using different plant species have already been carried out by earlier workers from India and abroad [23,24,25,26,27,16] but little knowledge is available on the evaluation of plant species as hyperaccumulant using in vitro strategies [28,20,29,30]. In the present study effect and accumulation of copper was seen on Bacopa monnieri grown on MS media under in vitro conditions.

IV. CONCLUSION

From the above results, it can be concluded that Bacopa monnieri has maximum removal efficiency for copper at a concentration of 40mg/l. Therefore, this plant can be used for the removal of copper from the soils having comparable concentrations. Further studies on the recovery of metals by B. monnieri using improved methods can be extended, from laboratory level experiments to the field level removal systems, especially for the removal of copper from industrial sites.

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REFERENCES

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