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Nutrient Optimization for Improved *in vitro* Plant Regeneration in *Eclipta alba* (L.) Hassk. and Assessment of Genetic Fidelity using RAPD Analysis

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Abstract

This study highlights the effect of different inorganic micronutrients like copper, cobalt, molybdenum, zinc, boron, iodine, iron and manganese in accelerating and amplifying *in vitro* shoot bud induction and proliferation of a medicinally important plant, *Eclipta alba* (L.) Hassk. Direct shoot bud induction was observed on MS fortified with Kn (2 mg/l). However, maximum number of shoots was achieved when GA_3, 0.5 mg/l was added to induction medium along with 1µM copper sulphate (ten times the normal MS level). Optimization of nutrient level in the basal medium promoted maximum regeneration response from both shoot tips and nodal explants. Elongated shoots were rooted in MS supplemented with IBA, 1.0 mg/l. Healthy, green plantlets with well developed roots, flowered normally in the field. Genetic stability of micropropagated plantlets was evaluated using RAPD markers. The amplification products were monomorphic in micropropagated plantlets and similar to those of mother plant revealing the genetic uniformity of plantlets. The regeneration protocol is highly efficient and reproducible so would be useful for mass multiplication, *ex situ* conservation and genetic transformation of *E. alba* (L.) Hassk.

Introduction

Annual herbaceous plant *Eclipta alba* (L.) Hassk. [synonym- *Eclipta prostrata* (Linn.), family: *Asteraceae*] native to tropics and subtropics during rainy season, is popularly known as 'False Daisy' or 'Bhringraj' and has tremendous medicinal importance since ancient times. Apart from blackening and promoting hair growth; antiageing, hepatoprotective, antihyperglycemic, antidiabetic, anti-inflammatory, antioxidant and antimicrobial activities of the plant have also been

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well documented (Jadhav et al. 2009). Pharmacological investigations have elucidated association of hepatoprotective property mainly due to two active constituents; wedelolactone and desmethylwedelolactone (Franca et al. 1995).

Although, there are several reports on *in vitro* plant regeneration of this medicinally important plant (Franca et al. 1995, Borthakur et al. 2000, Gawde and Paratkar 2004, Dhaka and Kothari 2005, Ray and Bhattacharya 2008, Singh et al. 2012) but attempts have not been made to standardize nutrient requirement to improve regeneration. As, the nutrient requirement of the cultured tissue may vary according to different stages of explant growth and development so an understanding of optimal nutrient concentration in the culture medium could lead to increase growth and evoke efficient *in vitro* morphogenesis. Considering the above facts, the present study was undertaken to optimize the levels of inorganic micronutrients *viz.* copper, cobalt, molybdenum, zinc, boron, iodine, iron and manganese to improve the regeneration potential of *E. alba* and to highlight their individual role in accelerating and amplifying the *in vitro* shoot bud induction and proliferation.

Plant cell culture results in high frequency of variation in regenerated plants (Larkin and Scowcroft 1981). Therefore, proper evaluation of genetic uniformity of regenerants with that of the mother plant for upholding desired traits is critically needed. To fulfill this purpose, molecular markers such as RAPD is widely employed as it is quick, simple, reliable, cost effective and requires only small amounts of DNA (Goyal et al. 2012). So the current study also inculcates RAPD analysis for evaluating genetic stability of the *in vitro* raised plantlets.

Materials and Methods

Young healthy plants of *Eclipta alba* were collected from the National Institute of Ayurveda, Jaipur, India and further grown in the botanical garden of University of Rajasthan, Jaipur. Nodal segments (0.5 - 1.0 cm) and shoot tips (0.2 - 0.5 cm) were excised from young healthy branches, rinsed in 20% (v/v) 'Extran' (liquid detergent; Merck, India) for 3 to 5 min and washed with running tap water for half an hour, immersed in 0.1% aqueous $HgCl_2$ solution for 4 to 5 min, and finally washed in autoclaved distilled water five times. Since the use of sodium hypochlorite did not prevent contamination, mercuric chloride was used as surface sterilizing agent throughout the experiment.

Shoot tips and nodal segments were cultured in 100 ml Erlenmeyer flasks containing 40 ml MS supplemented with 0.8% agar (Merck, Bacteriological Grade) and 3% (w/v) sucrose. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C and 1.2 - 1.3 kg cm^{-2} pressure for 20 min. All cultures were

maintained at 26 ± 1°C with a photoperiod of 16 hrs provided by white fluorescent tubes and incandescent bulbs (40 W, Philips, India).

Shoot tips and nodal segments were cultured separately on MS containing various cytokinins: Kn (0.5 - 5.0 mg/l), BAP (0.5 - 5.0 mg/l) or TDZ (0.1 - 2.0 mg/l), 3% sucrose and 0.8% agar. After 15 - 20 days, the cluster of shoot buds proliferated in primary cultures was sectored and transferred onto proliferation medium supplemented with varying concentrations of Kn (0.5 - 5.0 mg/l) in combination with GA_3 (0.1 - 3 mg/l). Healthy green shoots (> 2 cm) were transferred to rooting medium and smaller ones were subcultured for further proliferation. Shoot tips and nodal segments of *in vitro* elongated shoots were also used as explants. Each treatment consisted of six replicates and the experimental unit was four explants per flask.

In another set of experiments, MS supplemented with Kn (2 mg/l) was modified with different concentrations of cobalt chloride (up to 50×), manganese sulphate (up to 10×), zinc sulphate (up to 10×), copper sulphate (up to 50×), sodium molybdate (up to 50×), potassium iodide (up to 5×), boric acid (up to 10×), iron disodium ethylene diaminotetracetic acid (up to 5×), one nutrient at a time, to study the effect of a particular nutrient on shoot bud induction from shoot tips and nodal segments. After 3 weeks of culture, all the shoot buds were transferred to the medium with a modified concentration of a particular nutrient used in the induction medium.

Individual young healthy shoots (> 2 cm), obtained at the end of 30 days, were excised from shoot clusters and transferred to MS and half MS containing 3% (w/v) sucrose, 0.8% (w/v) agar and supplemented with 0.5 - 2.0 mg/l each of IBA, IAA, phenylacetic acid (PAA) and NAA individually.

The plantlets with well developed roots obtained after 20 days of culture on rooting media were removed from cultures, washed gently under running tap water and transferred to earthen pots containing mixture of farmyard manure, autoclaved garden soil and sand (1 : 2 : 1). The pots were covered with porous polythene bags for maintaining humidity and irrigated every fourth day with one-eighth strength MS basal salt solution devoid of sucrose for 2 weeks. The relative humidity was reduced gradually, and after 15 days of acclimatization the plantlets were transferred to field conditions and kept under shade in a net house for further growth and development. The morphological characteristics, growth parameters and floral features were examined. After 2 weeks, 20 regenerants from each explant (shoot tip and node) were tested for per cent survival.

The plantlets used to assess clonal fidelity were obtained from a single seedling which served as the mother plant (Mo). Genomic DNA was isolated

from the leaves of 12 randomly selected micropropagated plants and the Mo using the standard CTAB method (Doyle and Doyle 1990). A total of 40 random decamer primers (Operon Technologies, Almeda, USA) were used for RAPD analysis, out of which 18 were selected on the basis of reproducibility of bands. RAPD amplification was carried out in 20 μl reaction volume containing 25 ng DNA, 1× PCR buffer (Bangalore Genei, India), 200 μM dNTPs (Bangalore Genei, India), 0.8 μM of RAPD primer, 0.3 μl of Taq DNA polymerase (Bangalore Genei, India) in a thermal cycler (BioRad, UK). PCR amplification conditions were an initial denaturation at 94°C for 4 min followed by 39 cycles of denaturation at 92°C for 30 s, annealing at 37°C for 45 s, extension at 72°C for 2 min and a final extension at 72°C for 7 min. The PCR products were separated on 1.5% agarose (Himedia, India) gel through electrophoresis using 100 bp and 1 kb ladder as 'the band size standard' and photographed using Gel Documentation System (Bio-Rad, UK). RAPD analysis was repeated twice using each primer to establish reproducibility of the banding pattern.

The data on initial explants establishment, shoot bud formation, shoot proliferation and rooting were collected for 4 weeks. All the experiments were repeated thrice and the values were represented as mean ± standard error. Data were subjected to one-way analysis of variance by Fischer's least significant difference ($p < 0.05$) (Gomez and Gomez 1984). For RAPD analysis, consistent and well-resolved bands were scored manually as '1' if present and '0' if absent in the gel.

Results and Discussion

Both shoot tips and nodal segments were used as initial explants for aseptic establishment of cultures on MS supplemented with various cytokinins. The best initial morphogenic response (6 - 8 shoots/explant) was observed after 15 days when shoot tips and nodal segments were cultured on medium with Kn (2 mg/l) (Fig. 1a, Table 1). The degree of growth and differentiation varied considerably with the medium constitution. Significant variation in number of shoots was observed when Kn was increased to 2 mg/l and a further increase to 5 mg/l resulted in reduction of shoots.

Shoot tips and nodal segments excised from the *in vitro* raised axenic shoots formed on Kn (2 mg/l) were also cultured on various concentrations of Kn (0.5 - 5 mg/l) for further shoot bud induction. Explants taken from *in vitro* propagated shoots of *E. alba* responded better as compared to field explants. Similar, observations were also reported earlier (Dhaka and Kothari 2005).

It was also observed that at various concentrations of cytokinins simultaneous root induction from nodes also occurred which inhibited further shoot

multiplication. The shoots obtained in the medium with Kn (0.5 - 5 mg/l), showed significant increase in leaf size, internode elongation and stem thickness but at the optimum concentration of kinetin (2 mg/l) simultaneous rooting was minimized and the increase in leaf size, internode elongation and stem thickness was also negligible. Kinetin was also found to be more effective than BAP for promoting shoot length as reported earlier (Baskaran and Jayabalan 2005) (Table 2).

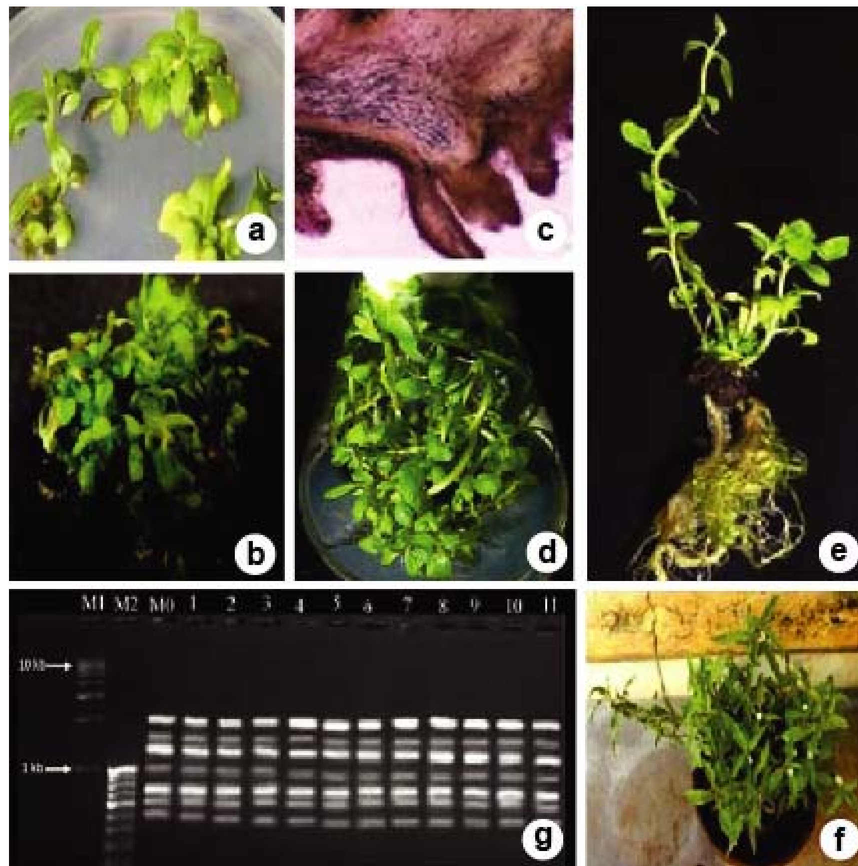

Fig. 1a-g: (a) Direct induction of shoot buds from nodal explants cultured on MS + Kn (2.0 mg/l). (b) Multiplication of shoots from nodal explants cultured on MS + Kn (2.0 mg/l) + GA_3 (0.5 mg/l). (c) Adventitious shoot buds in a section. (d) Shoot buds proliferation and elongation from nodal explants cultured on MS + Kn (2.0 mg/l) + GA_3 (0.5 mg/l wih 1.0 μM $CuSO_4$. (e) Rooting on MS supplemented with IBA (1.0 mg/l). (f) Field transferred plants. (g) RAPD profile of regenerated plantlets (Primer-OPF10) (Lanes: M1 and M2 = Molecular weight markers, Mo = mother plant, 1 - 11= *In vitro* regenerated plantlets.

For shoot proliferation, shoot bud induced from shoot tip and nodal explants on Kn (2 mg/l) was sectored into clumps (2 - 3 shoots each) and subcultured on the medium containing Kn/ BAP in combination with GA_3 (0.5 mg/l) at an

Table 1. Effect of cytokinins on shoot bud induction from shoot tips and nodal explants of *Eclipta alba*.

Cytokinin	Concentration (mg/l)	Shoot tip		Nodal segment	
		% response	Shoot buds mean ± SE	% response	Shoot buds mean ± SE
BAP	0.5	100	4.6±0.3[d]	80	5.2±0.3[e]
	1.0	100	5.2±0.3[f]	100	5.4±0.2[e]
	2.0	100	4.8±0.3[de]	100	6.0±0.4[f]
	3.0	80	4.4±0.4[cd]	100	5.4±0.5[e]
	5.0	80	3.8±0.3[b]	80	4.4±0.4[d]
Kn	0.5	95	4.4±0.8[cd]	100	6.0±0.4[f]
	1.0	100	5.2±0.4[f]	100	6.4±0.5[g]
	2.0	100	6.6±0.5[gh]	100	7.6±0.4[i]
	3.0	100	6.4±0.5[g]	90	7.2±0.3[h]
	5.0	90	4.2±0.8[c]	90	6.4±0.3[g]
TDZ	0.1	85	3.4±0.5[a]	90	3.6±0.2[b]
	0.2	90	3.8±0.4[b]	90	4.0±0.3[c]
	0.5	95	4.2±0.8[c]	100	3.8±0.4[bc]
	1.0	80	3.2±0.4[a]	95	3.6±0.5[b]
	2.0	80	3.2±0.8[a]	90	3.0±0.4[a]

Means (± SE) within a column followed by different letters are significantly different according to Fischer's LSD $p < 0.05$.

Table 2. Effect of adding GA_3 in combination with cytokinins on shoot proliferation and elongation from cultured shoot tips and nodal explants of *E. alba*.

Growth regulators (mg/l)		Percentage response		No. of shoot buds elongated (>2cm)		Shoot buds mean ± SE	
GA_3	BAP	Shoot tip	Node	Shoot tip	Node	Shoot tip	Node
0.5	0.5	80	100	2	2	5.2±0.3[a]	6.2±0.3[c]
0.5	1.0	100	100	2	3	5.6±0.2[b]	7.8±0.3[d]
0.5	2.0	100	100	2	3	8.2±0.3[e]	8.4±0.2[e]
0.5	3.0	90	90	3	2	6.2±0.3[c]	5.8±0.3[b]
0.5	5.0	90	80	2	2	5.4±0.2[ab]	5.6±0.4[b]
GA_3	Kn						
0.5	0.5	90	90	2	3	7.8±0.3[d]	8.4±0.3[e]
0.5	1.0	100	100	3	4	8.2±0.3[e]	8.8±0.2[f]
0.5	2.0	100	100	4	5	10.6±0.2[g]	12.4±0.3[h]
0.5	3.0	100	100	3	5	8.8±0.3[f]	9.8±0.3[g]
0.5	5.0	95	100	2	3	6.0±0.3[c]	5.0±0.4[a]

Means (± SE) within a column followed by different letters are significantly different according to Fischer's LSD $p < 0.05$.

interval of 10 - 15 days. Best proliferation and elongation of shoots was achieved on medium containing Kn (2 mg/l) and GA_3 (0.5 mg/l) where 30 - 40 shoots were formed from each explants after 3 passages. Without GA_3, retarded shoot growth

was observed which depicts the significance of GA_3 in shoot proliferation and elongation as reported for other plants (Dhaka and Kothari 2002). Continuous shoot proliferation could also be sustained through subcultures for consequent 3 years as also reported earlier (Dhaka and Kothari 2005).

For every micropropagation protocol, successful rooting of microshoots is a prerequisite to facilitate their establishment in soil and to sustain their field survival. *In vitro* raised shoots (> 2 cm) were excised from the cluster and transferred to MS (half-strength or full-strength) supplemented variety of auxins for rooting. Best rooting response (100%) was observed on full strength MS supplemented with IBA (1.0 mg/l) (Fig 1e). Similar response was observed by Dhaka and Kothari (2005). IBA is the most commonly used auxin for root formation not only in herbs but also from shoots of woody trees (Goyal et al. 2012). Multiple roots (average 3 - 5 cm in length) developed in 2 - 3 weeks. At higher concentration of IBA the percentage of rooting decreased and callus formation occurred at basal cut end as also reported by Goyal et al. (2012). A low survival frequency was observed when rooted plants were directly transferred from agar containing medium to soil (Dangi et al. 2012). Yu et al. (2000) suggested that aeration was an important factor in the formation of adventitious roots. Therefore, plantlets with well developed root system were washed gently with running tap water to remove the traces of agar and then transferred to porous thermocol pots containing autoclaved garden soil, farmyard manure and sand (2 : 1 : 1) and regularly irrigated with one-eighth strength MS basal salt solution devoid of sucrose every 4th day for 2 weeks. Similar approach was also reported earlier (Baskaran and Jayabalan 2005). The potted plantlets were covered with porous polyethylene bags for maintaining high humidity and kept inside the controlled culture room conditions. The relative humidity was gradually reduced, and after a month the plantlets were transplanted to field conditions where 80% of the micropropagated plantlets after attaining full maturity flowered normally (Fig. 1f).

The regeneration potential of plants is known to be influenced by extrinsic supply of nutrients in the tissue culture media. Inorganic nutrients are essential components of MS and hence offer to be the best variable to study their effect on the morphogenic potential of the plant under study (Kothari et al. 2008). They act as secondary messengers and help in regulating and controlling plant tissue growth (Niedz and Evens 2007).

Realizing the significance of inorganic micronutrients in plant regeneration, effect of eight micronutrients (H_3BO_3, $ZnSO_4$, $CuSO_4$, $CoCl_2$, Fe-EDTA, Na_2MoO_4, $MnSO_4$ and KI) was studied on shoot bud induction and proliferation (Figs 2,3). Reduction in the regeneration capacity of shoot tips and nodal explants was

observed in response to increasing concentration of Zn, I, Fe and Bo, while an increase in concentration of Cu, Mb, Mn, and Co in MS showed improved regeneration response initially up to a threshold beyond which there was decline (Fig. 2). When the concentration of $CuSO_4$ was increased (1 μM), a maximum of

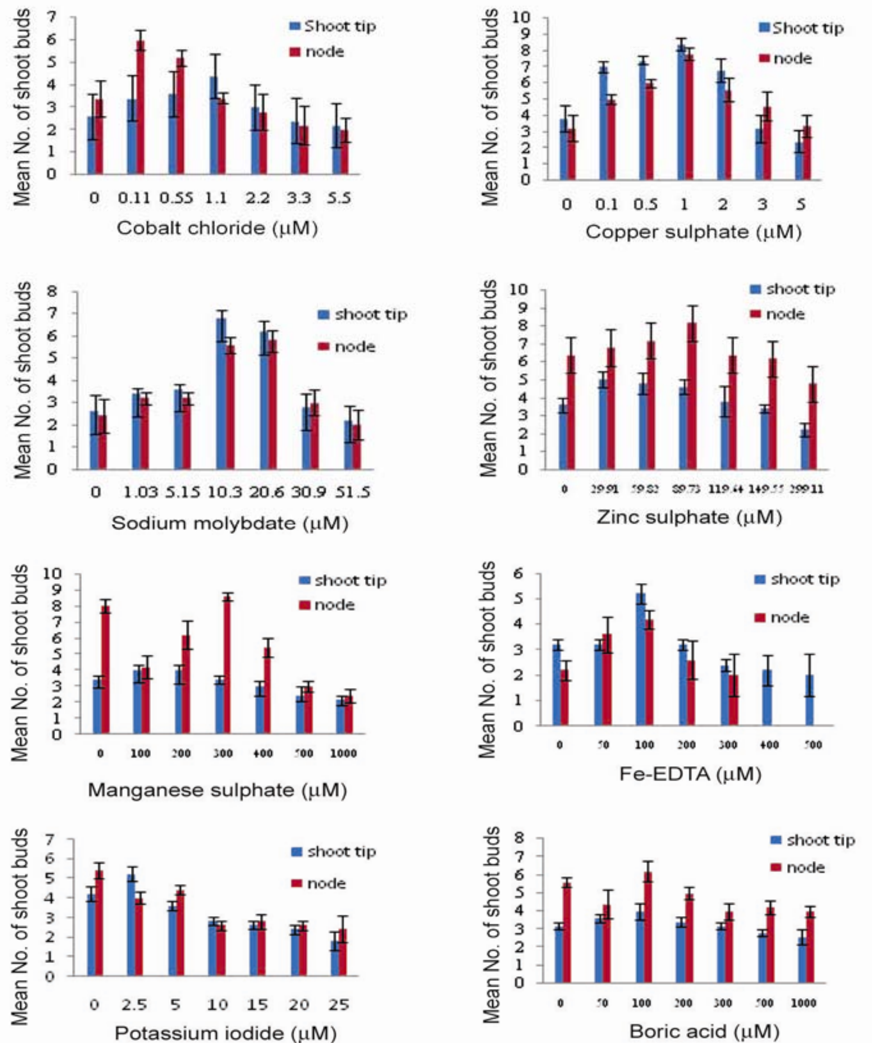

Fig. 2. Effect of different concentrations of inorganic miconutrients on shoot bud induction from shoot tip and nodal explants of *Eclipta alba*. (a) cobalt chloride, (b) copper sulphate, (c) sodium molybdate, (d) zinc sulphat, (e) manganese sulphate, (f) Fe-EDTA, (g) potassium iodide, (h) boric acid.

16 - 17 shoot buds were formed from shoot tips and nodal explants (Figs 1d, 3). Shoots formed on the medium devoid of copper were pale green. Copper is an essential component of several enzymes involved in electron transport, protein,

and carbohydrate synthesis (Kothari et al. 2008). Increase in the regeneration response in the presence of elevated levels of copper was also reported by several workers in various plants (Joshi and Kothari 2007, Kothari et al. 2008, Khurana-Kaul et al. 2010). A maximum of 13 shoots were formed on medium with 0.55 $CoCl_2$ µM when nodal segments were used as an explant as compared to 9 shoots on medium with the basal concentration of cobalt (Figs 2a, 3). Increased $CoCl_2$ has been shown to greatly improve regeneration in other plant species also (Dangi et al. 2012). Co^{2+} inhibits ethylene production by blocking the conversion of l-aminocyclopropane-1-carboxylic acid to ethylene (Yang and Hoffman 1984). Thus, the enhancement of shoot regeneration with increased Co^{2+} is hypothesized to be due to a decrease in the level of ethylene (Dangi et al. 2012). Zinc is essential for protein synthesis, IAA synthesis and nitrogen metabolism but it is toxic at high levels and produces adverse effects on plant growth and development (Wang et al. 2009). The number of shoot buds formed from shoot tip and nodal explants increased initially when the concentration of Zn was less but at higher Zn levels number of shoot buds formed *in vitro* decreased (Fig. 2d). A maximum of 14 shoots were formed when nodal segments were cultured on basal MS concentration of Zn in the medium. However, Ghnaya et al. (2010) reported that elevation in amino acid and carbohydrate (glucose and sucrose) content of *in vitro* cultured plantlets occur due to concomitant zinc variation in culture medium. The 2x Mn concentration was the best for regeneration in case of *Paspalum* (Kothari et al. 2008). Similarly, in the present study an improvement of *in vitro* response has been discerned when manganese concentration was increased to 300 µM (Fig. 2e). Sahasrabudhe et al. (1999). found an increase in the embryogenic response of indica rice on higher concentrations of boric acid which is contradictory to the present study where maximum response was observed at basal concentration of boric acid (100 µM), when both shoot tips and nodal segments were used as explants (Fig. 2h). It was observed that 100 µM H_3BO_3 was advantageous for further multiplication and elongation of shoots in both the explants (Fig. 3). Boron is required for normal plant growth and development of all higher plants and considered to be involved in nucleic acid metabolism, carbohydrate and protein metabolism and cell wall synthesis (Sotiropoulos et al. 2006). In plants, iron is mainly bound to chelators and complex compounds. It was observed that absence of iron in culture medium resulted in the formation of pale green leaves. This is attributed mainly due to absence of heme- and iron sulphur-proteins. Best response was observed at basal concentration of iron which is in conformity with the results obtained earlier. Molybdenum is directly involved in reduction of nitrogen as it acts as a cofactor for the proper functioning of two most crucial enzymes in plants; nitogenase and nitrate reductase. Nitrogenase is needed for nitrogen fixation and nitrate reductase

reduces nitrate to nitrite in nitrogen assimilation process of the plant cell thereby making nitrogen readily available to the plant in usuable form. In this study, best response was observed when molybdenum concentration was 10 times the normal MS level in shoot tip explants and 20 times the normal MS level in nodal explants (Fig. 3).

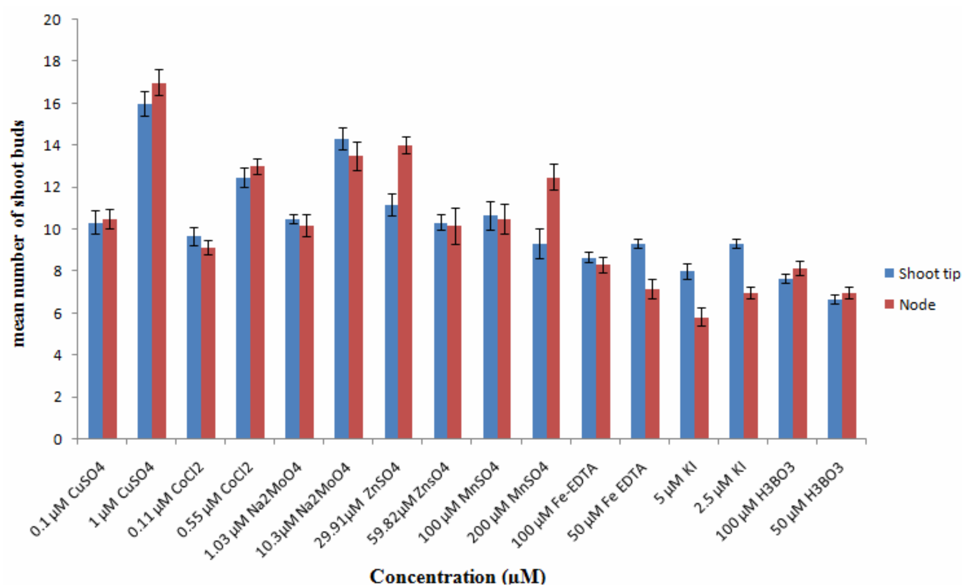

Fig. 3. Effect of different concentrations of inorganic micronutrients on shoot proliferation in *Eclipta alba*.

The regenerated plants were checked for their stability using RAPD primers. Of the 40 RAPD primers used for preliminary screening of the control plants, only 18 gave clear and distinct scorable bands. These primers were further used for the analysis of the micropropagated plants. The 18 primers generated 83 scorable bands. The number of scorable bands varied from 2 (OPT-01) to 7 (OPF-10) with an average of 4.6 bands per primer. The size range for the bands varied from 300 to 3,000 bp. DNA fingerprinting profiles of regenerants revealed that there were no polymorphic DNA fragments and no variation among mother plant and plantlets raised through micropropagation. Comparison of the banding pattern between the mother and micropropagated plants revealed the absence of any polymorphic bands (Fig. 1g), thus, confirming true-to-type nature of regenerated plants. RAPD has been widely used in genetic variation studies in tissue cultured derived plantlets as has been also reported earlier in various plants (Dangi et al. 2012, Goyal et al. 2012).

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