ROLE OF BASAL MEDIA, CARBON SOURCES AND GROWTH REGULATORS IN MICROPROPAGATION OF *Eclipta alba* – A VALUABLE MEDICINAL HERB

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ABSTRACT

An efficient, rapid and large-scale *in vitro* clonal propagation of the valuable medicinal herb *Eclipta alba* (Asteraceae) by enhanced auxillary shoot proliferation in cotyledonary nodal segments was designed. The medium type, various carbon sources, plant growth regulators markedly influenced *in vitro* propagation of *E. alba*. The i*n vitro* plantlet production system was investigated on Murashige and Skoog (MS) medium with the synergistic combination of BA (4.4µM), Kin (9.2µM), 2iP (2.4µM) and 3% sucrose which induced maximum number of shoots as well as beneficial shoot length. Subculturing of cotyledonary nodal segments on similar medium enabled continuous production of healthy shoots with similar frequency. Rooting was highest (94.3%) on full strength MS medium containing 9.8µM IBA. Micropropagated plants established in a mixture garden soil, farmyard (manure) and sand (2:1:1) were uniform and identical to the donor plant with respect to growth characteristics as well as floral features. These plants grew normally without showing any morphological variation.

KEYWORDS: axillary shoot proliferation; hardening; *ex vitro*; growth regulators; growth characters

ABBREVIATIONS: BA - Benzyladenine, Kin - Kinetin, 2iP - 2-isoPentenyladenine, IAA - Indole-3-acetic acid, IBA - Indole-3-butyric acid, NAA - α -Naphthalene acetic acid

1. INTRODUCTION

Eclipta alba (L.) Hassk. (Asteraceae), a small, branched annual herb with white flower heads, is native to the tropical and subtropical regions of the world. It is used as a tonic and diuretic in hepatic and spleen enlargement. It is also used in catarrhal jaundice and for skin diseases [1]. The alcoholic extract of the plant has shown antiviral activity against Ranikhet disease virus [1]. The plant is commonly used in hair oil all over India for healthy black and long hair. The fresh juice of leaves is used for increasing appetite, improving digestion and as a mild bowel regulator. It is commonly used in viral hepatitis to promote bile flow and protect the parenchyma and popularly used to enhance memory and learning. The plant has a reputation as an antiageing agent in Ayurveda. *Eclipta alba* is used as a general tonic for debility. Externally it is used for inflammation, minor cuts and burns and the fresh leaf-juice is considered very effective in stopping bleeding. Leaf juice mixed with honey is also used for children with upper respiratory infections and also used in eye and ear infections. *Eclipta alba* is a source of coumestan-type compounds used in phytopharmaceutical formulations of medicines prescribed for treatment of cirrhosis of the liver and infectious hepatitis [2]. *Eclipta alba* is widely used in India as a cholagogne and deobstruent in hepatic enlargement, for jaundice and other ailments of the liver and gall bladder [3]. Coumestan-type compounds, wedelolactone and dimethyl wedelolactone,

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have been isolated as the main active principles of *Eclipta alba*, both constituents exhibiting antihepatotoxic activity [4-5]. *In vivo* tests indicate that wedelolactone neutralizes the lethal and myotoxic activities of rattlesnake venom [6]. Wedelolactone (WL) and dimethylwedelolactone (DWL) showed potent activity when were tested in the trypsin inhibition bioassay (*in vitro*) [7]. The roots have emetic and purgative properties and it have been applied externally as an antiseptic to ulcers and wounds in cattle. The shoot extract shows antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* [1]. From the whole plant of *Eclipta alba*, a new triterpene saponin, namely eclalbatin, together with alpha-amyrin, ursolic acid and oleanolic acid have been isolated [8]. In Ayurveda a large number of indigenous drugs have been mentioned possessing analgesic properties. The total ethanol extract of *E. alba* have been shown to possess analgesic properties [9].

Since the harvest of medicinal plants on a mass scale from their natural habitats is leading to a depletion of plant resources, the conservation of these valuable genotypes is imperative. Micropropagation via shoot culture, often utilized to maintain clonal fidelity, would be a specially appropriate in this respect [10]. Large-scale, unrestricted exploitation of this natural resource to meet the ever increasing demand for it by the Indian pharmaceutical industry coupled with limited cultivation and insufficient attempts for its replenishment, this medicinally important and endangered plant species have markedly depleted [11-13]. In recent years, there has been an increased interest in *in vitro* culture techniques which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered and threatened medicinal plants [14-16]. Commercial exploitation and elimination of natural habitats consequent to urbanization has led to gradual extinction of several medicinal plants. Micropropagation is an effective approach to conserve such germplasms. Further, genetic improvement is another approach to augment drug-yielding capacity of the plant [17]. Therefore it is important to develop an efficient micropropagation technique for *E. alba* to rapidly disseminate superior clones once they are identified. Tissue culture techniques can play an important role in the clonal propagation of elite clones and germplasm conservation of this medicinal herb. There have been few reports to date on micropropagation in the genus using nodal explants [5,18]. However, the establishment of a micropropagation protocol for *E. alba* constitutes a useful tool for large scale plant production, assuring continuous availability of plant material appropriate for the study of factors that influence the production of the target secondary metabolites as well as for strategies of *in vitro* culture to increase the yield of these active principles accumulated in cultures of *E. alba*.

The purpose of this study was to develop an *in vitro* propagation method from cotyledonary nodes of *E. alba*. This study also included efforts to improve the secondary metabolism of this medicinal plant. In the present work we have established a suitable medium, carbon source and plant growth regulators for a rapid and reproducible method for high-frequency axillary shoot proliferation from cotyledonary nodal segments followed by successive establishment of regenerated plants in soil and have examined the morphological, growth characteristics and floral features of these plants.

2. MATERIALS AND METHODS

Plant material and Disinfections

Healthy young cotyledonary nodes of *E. alba* (L.) Hassk were collected from the Botanical field evaluation Garden of the Bharathidasan University at Tiruchirappalli. Cotyledonary nodal segments (1.0 – 1.5 cm) were excised and then washed thoroughly under running tap water for 30 minutes followed by treatment with an aqueous solution of 2% (v/v) Teepol (Reckitt Benckiser, India) and 70% (v/v) ethanol for 15 seconds and washing with autoclaved sterile distilled water three to five times. The explants were then surface-disinfected with 0.1% (w/v) aqueous mercuric chloride solution for 5 - 6 minutes and finally rinsed with autoclaved distilled water (five to seven changes). The cotyledonary nodal segments were then trimmed at both ends prior to inoculation on culture media.

Culture conditions

Single disinfected cotyledonary nodal segments were cultured on MS basal medium [19] supplemented with 3% (w/v) sucrose (Himedia, India) and 0.8% (w/v) agar for culture initiation and these served as explant sources for subsequent experiments. The pH of the medium (supplemented with respective growth regulators) was adjusted to 5.8 with 1 N NaOH or 1 N HCl before addition of 0.8% (w/v) agar (Himedia, India). In all the experiments, the chemicals used were of analytical grade (Himedia, Kelco, Merkard and Sigma). The medium was dispensed into culture vessels (Borosil, India) and autoclaved at 105 kPa (121^0 C) for 15 min. The surface-disinfected explants were implanted vertically on the culture medium (test tubes (150x25mm) containing 15 ml medium) and plugged tightly with non-absorbent cotton. All the cultures were incubated at 25 ± 2^0 C under 16 h photoperiod of 45 – 50 µmol m^{-2} s^{-1} irradiance provided by cool white fluorescent tubes (Philips, India) and with 55 – 60% relative humidity (RH). All subsequent subcultures were performed at four weeks intervals.

Effect of Basal media

Three different media including MS [19], B5 [20] and SH [21] were evaluated for their effects on *in vitro* growth and development of *E. alba*. All the basal media contained 3% (w/v) sucrose and solidified with 0.8% (w/v) and different concentrations of cytokinins, including 2.2-22.2 µM of Benzyladenine (BA), 2.3-23.2 µM of Kinetin (Kin) and 2.4-24.6 µM of 2-isoPentenyladenine (2iP).

Effect of Carbon sources

Cotyledonary nodal segments were cultured on MS agar medium supplemented with 4.4 µM BA and different types of carbon sources, including 3% (w/v) of glucose, fructose and sucrose.

Effect of Cytokinins

Cotyledonary nodal segments were cultured on MS medium containing 3% (w/v) sucrose and 0.8% (w/v) agar and supplemented with different combination and concentrations of plant growth regulators, including 4.4 µM BA + 2.3-23.2 Kin; 4.4 µM BA + 2.4-24.6 µM 2iP; 4.4 µM BA + 2.3-23.2 µM Kin + 2.4 µM 2iP.

Rooting medium

Elongated shoots were excised from each culture passage and transferred to full-strength and half-strength (1/2 MS) MS medium containing 3% (w/v) sucrose and 0.8% (w/v) agar. The medium was further supplemented with 2.8-17.1 µM indoleacetic acid (IAA) or 2.5-14.8 µM indolebutyric acid (IBA) or 2.7-16.1 µM naphthaleneacetic acid (NAA) individually.

Acclimatization and transfer of plantlets to soil

Plantlets with well-developed roots were removed from the culture medium and after washing the roots gently under running tap water, plantlets were transferred to plastic pots (10 cm diameter) containing autoclaved garden soil, farmyard (manure) and sand (2:1:1). All were irrigated with 1/8 MS basal salt solution devoid of sucrose and inositol every 4 days for two weeks. The potted plantlets were covered with porous polyethylene sheets for maintaining high humidity and were maintained under the culture room conditions. The relative humidity was reduced gradually and after 30 days the plantlets were transplanted to a botanical evaluation garden and kept under shade in a net house for further growth and development. The morphology, growth characteristics and floral features were examined.

Statistical Analysis

Experiments were set up in a Randomized Block Design (RBD) and each experiment usually had 10 replicates and was repeated at least three times. Ten to fifteen explants were used per treatment in each replication. Observations were recorded on the frequency (number of cultures responding for axillary shoot proliferation and root development) and the number of shoots per explant, shoot

length, roots per shoot and root length respectively. The analysis of variance (ANOVA) appropriate for the design was carried out to assess the significance of differences among the treatment means. The treatment means were compared using Duncan's Multiple Range Test (DMRT) at a 5% probability level [22].

3. RESULTS AND DISCUSSION

Effect of basal media and Cytokinins on shoot regeneration

Shoot regeneration from *E. alba* cotyledonary nodal explants cultured on three types of media and supplemented with various concentrations of BA, Kin and 2iP individually was examined. The degree of growth and differentiation varied considerably with the medium constitution [23-24]. As nitrogen is to be a constituent of plant cell components, its deficiency inhibits plant growth. In addition to this total nitrogen content, the ratio of nitrate to ammonium (NH_4^-) is a very important aspect in nitrogen nutrition [25]. This is because the ratio strongly influences the pH of the medium, which in turn determines the absorption of other nutrients [26]. Thus, as in most of the plant species, the relatively higher supply of nitrate-nitrogen within the MS medium could have exerted the profound effect on shoot growth of this plant species. Among the three different media and growth regulators tested, MS was found to be the better medium compared to B5 and SH media (Table 1). MS medium has also been found to be more effective than other media by other investigators [26-29]. In contrast to the report of Bajaj [30] who found SH to be best basal medium for Cardamom, MS medium produced the higher number of shoots in this study on *E. alba*.

Among the three different media used, the explants in MS medium + 4.4 μM BA were healthy and grew vigorously (Fig. 1a). In MS medium, 4.4 μM BA yielded 96% of segment explants bearing shoots after 65 days and explants exhibited on average 7.8 shoots with normal and healthy leaves. B5 and SH media induced vitrification with new number of shoots production (Fig. 1b, c). A similar phenomenon has been observed in *Balanites aegyptiaca* [29]. MS medium supplemented with 4.4 μM BA was more effective for frequency of sprouting shoot, number of shoots and shoot length than other concentrations of BA, Kin and 2iP (Table 1). Comparing the effect of cytokinin type (BA, Kin and 2iP) on shoot production, the best response was achieved on BA in Indian wild strawberry [31]. Cytokinin was to enhance the shoot multiplication response of BA compared to other cytokinins in *Tabebuia* and *Macrotyloma* [32-33]. BA was far more effective than Kin for inducing proliferation of axillary buds in *Dalbergia latiflolia* [34]. BA and Kin in MS medium showed variable responses and BA was more effective than Kin in inducing multiple shoots in *Dalbergia* [35]. MS medium with Kin concentrations did not result in increase in the number of shoots in *Gloriosa superba* [36].

In the present study, higher concentrations of cytokinin reduced the shoot number as well as shoot length (Table 1). This accorded with Hu and Wang who reported that higher concentrations of cytokinin reduced the number of micropropagated shoots [37]. MS medium containing BA was most effective for shoot multiplication. In MS medium, 2iP and Kin showed shoot proliferation with increase in the hormone concentration. In *Vigna radiata*, 2iP was the most effective cytokinin resulting in multiple shoot induction rather than shoot elongation but BA showed better response resulting in multiple shoot induction as well as shoot elongation [38]. 2iP was more effective than BA for shoot formation in *Gloriosa superba* [36]. In the present study, higher concentrations of BA (8.8 – 22.2 μM) reduced the shoot number as well as shoot length. A similar response was observed in Indian wild strawberry [31]. However, in each medium, increased concentrations of Kin (13.9 – 18.5 μM) or 2iP (14.7 – 17.3 μM) enhanced the shoot number as well as moderate shoot length. The explants of media (B5, SH and MS) containing more Kin (23.2 μM) or 2iP (24.6 μM) showed decreased shoot numbers as well as shoot length. Excised explants cultured on MS medium formed white compact callus at the proximal ends of the node after 30 days of culture (Fig.1d). Similar results were also observed in *Peganum harmala* [39] and *Holostemma ada – kodien* [40]. The callus formation might be due to the accumulated auxin at the basal cut ends, which stimulates cell proliferation especially in the presence of cytokinins [41]. The callus formation at the basal cut ends of node explants on cytokinin enriched

medium is frequent in species with strong apical dominance [42]. B5 and SH media slowly development of basal callus shows in B5 and SH media when compared to MS medium. A comparison of cytokinin activity, for shoot sprouting in each medium showed that BA and 2iP resulted in higher frequency of shoot sprouting and shoot number than Kin (Table 1).

In each medium, Kin was more effective than BA and 2iP for shoot length. MS medium fortified with 4.6 μM Kin attained highest shoot length of 16.8 cm after 65 days of culture. All the media and hormones induced callus at the base of the explant after 30 days of culture. In each media, the shoots produced roots at the basal callus after 45 days of culture. The type of basal media affected root number and root length. The highest numbers of roots were obtained in MS, while both B5 and SH gave the lowest number of roots (data not shown). However, the shortest roots were produced in B5 medium followed by SH medium. Similar response was observed in *Korarima* [26]. The MS medium supplemented with Kin promoted more root formation than BA and 2iP. Similar result was observed in *Eclipta alba* [5]. It was difficult to isolate a single shoot with root from each culture passage because of damage to the roots. In each media, when the cultures were maintained for a long time (after 4 weeks), there was gradual browning and defoliation of leaves. A similar phenomenon was observed in by Borthakur *et al.* [18]. Successive subculture was carried out at four week intervals. The observations indicate that these media are at concentrations favorable for promoting shoot proliferation in *E. alba*. All the further experiments were conducted on MS medium.

Carbon sources and shoot regeneration

The responses of *in vitro* cultures to different carbon sources added to the medium were also tested. Although carbohydrates are of prime importance for *in vitro* organogenesis, carbon metabolism *in vitro* is still not clearly understood [43]. It is well established that carbohydrate requirements depend upon the stage of culture and may show differences according to the species [44]. Cotyledonary nodal segments were cultured on MS medium containing 3% (w/v) of glucose, fructose or sucrose respectively. These media were supplemented with 4.4 μM BA and 0.8% (w/v) agar. Among the three carbon sources, sucrose proved to be better for shoot regeneration than fructose or glucose (Table 2). Similar results were obtained in micropropagation of Cork oak [45] and Kaempferia [46]. Sucrose has been commonly used as a carbon source in tissue culture media [47]. This is due to its efficient uptake across the plasma membrane [48]. However, glucose was most effective for shoot proliferation in Prunus [49]. The shoot multiplication was greater in the presence of sorbitol than sucrose [50]. Sucrose and glucose gave a similar rate of proliferation in sour cherry [48]. However, sucrose and glucose induced highest frequency of organogenesis in *Bixa orrellana* [51].

Satisfactory shoot proliferation from cotyledonary nodal explants was obtained on sucrose (3% (w/v)) whereas glucose and fructose resulted in less number of shoots and shoot lengths also varied (Table 2). However, on sucrose medium, leaf chlorosis occurred after ten weeks and shoots gradually declined. A similar response was observed in Prunus [49]. In *Malus robusta* 'Rehd. No 5' fructose gave the lowest number of shoots [52]. However, the same authors showed that shoot cultures of the apple Scion cultivar Macspur showed no differences in shoot multiplication between sucrose, fructose and glucose. MS medium supplemented with sucrose produced moderate shoot length (13.6 cm) as well as healthy shoots. However, shoot elongation was best on fructose, followed by glucose but the shoots were not healthy. This difference could not be directly linked to the carbohydrate nutritional aspects, but with carbohydrate osmotic contribution. Pritchard, Wyn-Jones and Tomos who reported that Carbohydrates control morphogenesis by acting as energy source and also by altering the osmotic potential of the culture medium, which alters such cell wall properties as extension, hardening and composition, followed by subsequent modification in morphogenesis in wheat [53]. MS medium fortified with fructose attained shoot length of 16.4 cm after 65 days of culture.

Whereas, glucose and fructose promoted root induction slowly even at thirty days of culture. However, sucrose readily promoted light green silky as well as lengthy roots. All the roots turned white after forty-five days of culture. It was difficult to isolate shoots with roots from each carbon source containing shoot multiplication medium (MS + 4.4 μM BA) because of

damage to the roots which were difficult to harden. All the further experiments were conducted on sucrose.

Effect of Cytokinins on shoot regeneration

Various concentrations of Kin (2.3-23.2 µM) and 2iP (2.4-24.6 µM) with BA (4.4 µM each) were tried for shoot induction and to determine the multiplication potential of cotyledonary nodes. Combined effect of Kin, 2iP and 4.4 µM BA increased the axillary shoots (Table 3). Axillary shoots were promoted in BAP alone or in combination with other cytokinins in *Macrotyloma uniflorum* [33]. More morphologically distinct multiple shoots were developed from the cotyledonary node in MS medium containing BA combined with Kin or 2iP. Multiple shoots developed with a combination of BA (4.4 µM) and 2iP (14.7 µM) grew faster, while those initiated in BA (4.4 µM) and Kin combination grew slower. A similar response was observed in *Gloriosa* [36]. The minimum number of roots was produced at base of shoots at low concentrations of Kin (2.3-4.6 µM) or 2iP (2.4-4.9 µM) and with BA (4.4 µM). Rooting was completely arrested at the concentrations of Kin (9.2-23.2 µM) and 2iP (9.8-24.6 µM) with BA (4.4 µM each). White-greenish compact callus developed directly from the cut ends of multiple shoots containing MS medium fortified with BA (4.4 µM) and Kin in the range 4.6 – 23.2 µM and 2iP in the range 4.9 – 24.6 µM within four weeks of culture. Significant shoot sprouting frequency and number of shoots was obtained in MS medium supplemented with BA (4.4 µM) + Kin (13.9 µM) and BA (4.4 µM) + 2iP (14.7 µM) but the shoot lengths differed (Table 3). The combined effect of cytokinins (BA and Kin) enhanced multiple shoot bud regeneration in *Arachis hypogaea* [54]. However, the combination of cytokinins (BA and Kin) failed to improve shoot multiplication [55]. The MS medium containing BA (4.4 µM) and low concentrations of Kin (2.3 – 9.2 µM) or 2iP (2.4 – 9.8 µM) increased the shoot length but caused a low frequency of axillary shoot formation. With an increase in concentration of Kin (13.9 µM) or 2iP (14.7 µM) in the medium, the number of axillary shoots also increased significantly (Table 3). But the higher the concentration of Kin (23.2 µM) or 2iP (24.6 µM) in the medium, the number of axillary shoots as well as shoot length decreased. However, the shoot length was best with BA (4.4 µM) + Kin (2.3 µM) (Table 3). The maximum number of shoots was obtained in MS medium supplemented with BA (4.4 µM) + Kin (9.2 µM) and 2iP (2.4 µM) but the shoots were small, when the concentrations of Kin or 2iP were raised (Table 3; Fig 1e). Initially, white-greenish compact callus was formed directly at cut ends of cotyledonary node containing MS medium fortified with BA (4.4 µM) + 2iP (2.4 µM) + Kin (2.3 – 23.2 µM) and later turned dark black and the shoots declined after ten week of culture. The shoot length was strongly affected by high concentrations of cytokinins (4.4 µM BA + 23.2 µM Kin, 4.4 µM BA + 17.3 µM – 24.6 µM 2iP and 4.4 µM BA + 18.5 – 23.2 µM Kin + 2.4 µM 2iP). A similar phenomenon was observed in *Bixa orellana* [51].

Effect of auxins on rooting of shoots

Excised shoots were rooted on half-strength or full-strength MS medium with different types of auxins. The promotory effect of reducing the salt concentration of MS on *in vitro* rooting of shoots has been described [56]. We have found that in *E. alba* reducing MS salt strength to ½ enhanced rooting frequency but also reduced callusing. Half strength and full strength MS medium supplemented with all concentrations of auxins induced roots from shoots within 30 days of culture. Among the three auxins tested, the number of roots and root length varied in both medium (Table 4). Full strength MS medium fortified with 9.8 µM IBA shows better root formation when compared to half strength MS medium with 9.8 µM IBA. Full strength MS medium significantly promoted lengthy roots and strengthened root induction within twenty days of culture. In half strength MS medium, IBA was found to be more effective for root induction than IAA and NAA. Full strength MS medium supplemented with IBA (9.8µM) was more effective for root induction than IAA and NAA (Table 4; Fig.1f). However, IAA and NAA formed slender roots in both medium. Less amount callus formation occurred in all the types of auxins in full strength MS media. IBA was more effective for root induction in both types of

medium than IAA or NAA. Similar responses were observed in different plant species [15, 28, 36].

Hardening of regenerated plants and examination of morphological characteristics

Plantlets were successfully acclimatized without growth chamber facility. 100% plantlet survival was seen after hardening on garden soil, farmyard (manure) and sand (2:1:1) for three weeks (Fig.1g). The percentage of survival was decreased to 96.0 and 74.6%, respectively after four and ten weeks of acclimatization (Table 5). The initial growth rates of plant height were respectively 9.3 ± 0.27 cm after first two weeks of acclimatization. On the other hand, in the following three to ten weeks substantial increase of plant height was observed (Table 5; Fig.1h). Initially, two to three healthy branches each bearing an average of two to three leaves developed adjacent to the main shoot. Thereafter, the number of branches per plant increased to 5.4 ± 0.11 and 15.04 ± 0.22, after four and ten weeks of acclimatization respectively. Flowering occurred at the apical portion of the main shoot initially but after six weeks each branch developed flowering at the terminal region of the branches. The number of flowers per plant increased to 3.9 ± 0.21 and 23.8 ± 0.28, after six and ten weeks of acclimatization (Table 5; Fig.1i). There was no detectable variation among the acclimatized plants with respect to morphological growth characteristics and floral features. All the micropropagated plants were free from external defects.

Table 1 Influence of different types of media supplemented with BA, Kin and 2iP on shoot bud induction from cotyledonary node explants of *Eclipta alba*

Growth Regulators (μM)	Shoot sprouting frequency (%)			Number of shoots/explant			Shoot length (cm)		
	B5	MS	SH	B5	MS	SH	B5	MS	SH
BA									
2.2	61.0^{d}	77.4^{c}	54.4^{de}	4.2^{d}	5.8^{cd}	3.2^{cd}	9.1^{c}	11.1^{b}	8.3^{c}
4.4	79.1^{b}	96.0^{a}	60.1^{c}	5.8^{a}	7.8^{a}	3.9^{b}	11.5^{a}	13.7^{a}	10.7^{a}
8.8	86.8^{a}	84.2^{b}	71.5^{a}	5.4^{ab}	7.2^{ab}	4.3^{a}	11.2^{ab}	10.7^{bc}	9.8^{ab}
13.3	77.5^{bc}	78.6^{bc}	64.0^{b}	5.0^{b}	5.9^{c}	4.1^{ab}	8.7^{cd}	9.8^{c}	8.3^{c}
17.7	61.3^{d}	62.1^{d}	60.2^{c}	4.8^{bc}	5.6^{d}	3.9^{b}	5.4^{e}	6.8^{d}	5.6^{d}
22.2	57.2^{de}	60.2^{de}	56.7^{d}	3.6^{e}	4.7^{e}	3.3^{c}	4.2^{ef}	5.7^{de}	5.0^{de}
Kin									
2.3	60.2^{d}	55.0^{e}	50.6^{f}	2.1^{e}	3.0^{de}	1.8^{e}	11.6^{b}	14.5^{b}	10.9^{b}
4.6	68.7^{de}	68.0^{d}	55.4^{de}	2.6^{cd}	3.4^{d}	2.4^{cd}	13.4^{a}	16.8^{a}	12.2^{a}
9.2	70.0^{b}	73.2^{cd}	63.0^{b}	3.0^{c}	4.1^{bc}	2.7^{c}	9.5^{c}	12.1^{c}	11.4^{ab}
13.9	75.1^{a}	86.8^{a}	68.2^{a}	3.8^{ab}	5.0^{a}	3.1^{bc}	6.4^{d}	7.5^{d}	10.1^{bc}
18.5	72.3^{ab}	85.3^{ab}	61.1^{bc}	4.0^{a}	4.9^{ab}	3.9^{a}	6.0^{de}	7.2^{de}	8.0^{d}
23.2	68.4^{bc}	74.1^{c}	56.3^{d}	3.6^{b}	4.5^{b}	3.4^{b}	5.6^{e}	5.8^{e}	6.6^{e}
2iP									
2.4	57.2^{ef}	66.5^{ef}	56.2^{de}	1.4^{de}	2.7^{de}	1.7^{ef}	10.2^{bc}	12.0^{ab}	9.4^{b}
4.9	62.1^{e}	68.3^{e}	59.5^{d}	2.0^{d}	3.1^{d}	1.8^{e}	12.1^{a}	13.2^{a}	10.5^{a}
9.8	68.5^{d}	76.0^{d}	64.0^{c}	3.2^{c}	4.0^{c}	2.7^{d}	10.3^{b}	11.3^{b}	9.1^{bc}
14.7	74.3^{c}	84.7^{b}	68.3^{b}	4.0^{b}	4.7^{bc}	3.3^{bc}	8.2^{d}	9.4^{c}	8.6^{c}
17.3	84.0^{a}	90.1^{a}	71.8^{a}	5.2^{a}	5.2^{b}	4.3^{a}	6.7^{de}	8.0^{cd}	6.5^{d}
24.6	83.1^{ab}	82.4^{bc}	69.2^{ab}	4.6^{ab}	6.0^{a}	3.7^{b}	5.1^{e}	5.3^{e}	4.8^{e}

Treatment means followed by different letters in their superscript are significantly different from each other ($p<0.05$); comparison by DMRT.
Data recorded after 65 days of culture.

Table 2 Effect of carbon source on shoot proliferation and shoot length of *E. alba* on MS with 4.4 μM BA

Sugar	Concentration (%)	Number of shoots/explant	Shoot length (cm)
Glucose	3	2.1^{bc}	14.2^{b}
Fructose	3	3.9^{b}	16.4^{a}
Sucrose	3	7.8^{a}	13.6^{bc}

Treatment means followed by different letters in their superscript are significantly different from each other ($p<0.05$); comparison by DMRT. Data recorded after 65 days of culture.

Table 3 Influence of cytokinins on shoot regeneration from cotyledonary node explants of *E. Alba*

Growth regulators (μM)	Shoots sprouting frequency (%)	Number of shoots/ explant	Shoot length (cm)
BA(4.4) + Kin			
2.3	72.2^{de}	8.3^{d}	12.9^{a}
4.6	79.6^{e}	8.8^{cd}	10.5^{b}
9.2	82.0^{b}	9.6^{c}	8.3^{c}
13.9	90.5^{a}	14.0^{a}	4.5^{d}
18.5	80.3^{bc}	13.5^{ab}	4.1^{de}
23.2	73.1^{d}	11.5^{b}	3.3^{e}
BA(4.4)+2iP			
2.4	70.4^{f}	8.4^{ef}	10.8^{a}
4.9	78.2^{de}	8.9^{e}	10.1^{ab}
9.8	83.1^{bc}	11.9^{d}	8.0^{c}
14.7	95.2^{a}	16.5^{a}	4.9^{d}
17.3	84.0^{b}	15.8^{ab}	3.8^{de}
24.6	79.0^{d}	14.0^{c}	3.2^{e}
BA(4.4) + Kin + 2iP(2.4)			
2.3	77.0^{d}	12.6^{bc}	8.7^{a}
4.6	88.4^{b}	14.0^{b}	6.9^{b}
9.2	98.7^{a}	25.8^{a}	4.9^{c}
13.9	86.0^{bc}	16.4^{ab}	3.4^{d}
18.5	84.2^{c}	12.5^{c}	3.0^{de}
23.2	80.1^{cd}	8.5^{d}	2.8^{e}

Treatment means followed by different letters in their superscript are significantly different from each other ($p<0.05$); comparison by DMRT.
Data recorded after 65 days of culture.

Table 4 Influence of different auxins and MS medium strength on rooting of *in vitro* – formed shoots of *Eclipta alba*

Growth regulators (μM)	Percentage shoots showing root regeneration	Number of roots/shoot	Root length (cm)
Full strength MS + IAA			
2.8	56.6^{d}	5.0^{de}	3.0^{c}
5.7	60.0^{bc}	7.3^{d}	3.6^{bc}
8.5	62.5^{b}	11.3^{bc}	4.1^{a}
11.4	67.4^{a}	15.9^{a}	3.8^{ab}
17.1	54.3^{de}	12.3^{b}	3.6^{b}
Full strength MS + IBA			
2.5	78.0^{de}	14.6^{d}	5.2^{e}
4.9	80.4^{d}	26.3^{bc}	6.1^{c}
7.4	87.0^{bc}	28.4^{b}	7.0^{a}
9.8	94.3^{a}	34.5^{a}	6.7^{ab}
14.8	89.5^{b}	30.7^{ab}	5.9^{cd}
Full strength MS + NAA			
2.7	65.7^{de}	10.4^{d}	4.0^{de}
5.4	76.0^{c}	17.0^{bc}	4.7^{b}
8.1	82.6^{ab}	21.0^{ab}	5.2^{a}
10.7	88.0^{a}	24.3^{a}	4.5^{bc}
16.1	69.2^{d}	20.3^{b}	4.1^{d}
Half strength MS + IAA			
2.8	48.6^{d}	3.2^{d}	2.7^{d}
5.7	57.0^{b}	3.9^{cd}	3.1^{bc}
8.5	58.0^{ab}	5.6^{c}	3.4^{b}
11.4	60.0^{a}	10.0^{a}	3.7^{a}
17.1	52.3^{c}	8.8^{ab}	3.0^{c}
Half strength MS + IBA			
2.5	64.0^{cd}	12.0^{e}	4.6^{c}
4.9	69.0^{c}	16.8^{d}	5.0^{b}
7.4	73.2^{b}	22.4^{ab}	5.2^{ab}
9.8	84.0^{a}	23.0^{a}	5.3^{a}
14.8	70.0^{bc}	20.9^{c}	4.8^{bc}
Half strength MS + NAA			
2.7	57.0^{d}	8.7^{d}	3.2^{de}
5.4	60.0^{c}	11.2^{bc}	3.3^{d}
8.1	65.4^{b}	13.9^{ab}	4.1^{bc}
10.7	74.0^{a}	14.2^{a}	4.8^{a}
16.1	58.7^{cd}	12.0^{b}	4.2^{b}

Treatment means followed by different letters in their superscript are significantly different from each other ($p<0.05$); comparison by DMRT.
Data recorded after 30 days of culture.

Table 5 The frequency of *ex vitro* survival, growth and flowering of acclimatized microplants of *E. alba*

Parameters	Weeks after transfer (mean ± SE)					
	2	3	4	6	8	10
Survival (%)	100	100	96.0±1.58	82.7±1.50	76.0±1.40	74.6±0.98
Plant height (cm)	9.3±0.27	13.6±0.24	19.4±0.57	28.4±1.00	34.6±1.31	39.5±1.42
Number of branches/plant	0.0±0.00	3.5±0.11	5.4±0.14	8.0±0.19	13.0±0.57	15.0±0.22
Number of flowers/plant	0.0±0.00	0.0±0.00	0.0±0.00	3.9±0.21	9.1±0.18	23.8±0.28

Values shown were proportions of three replicates of 25 microplants.

4. CONCLUSION

In the present study revealed that the *in vitro* plantlet production system was achieved on MS medium with the best combination of BA (4.4 µM), Kin (9.2 µM), 2iP (2.4 µM) and 3% sucrose. The highest root was achieved on full strength MS medium containing 9.8 µM IBA and it was very suitable for hardening. In conclusion it may be stated that the protocol presented in this study yields efficient shoot and root regeneration for cotyledonary nodes. These results will encourage large scale micropropagation of this important medicinal plant. The protocol reported here could also be used for conservating it.

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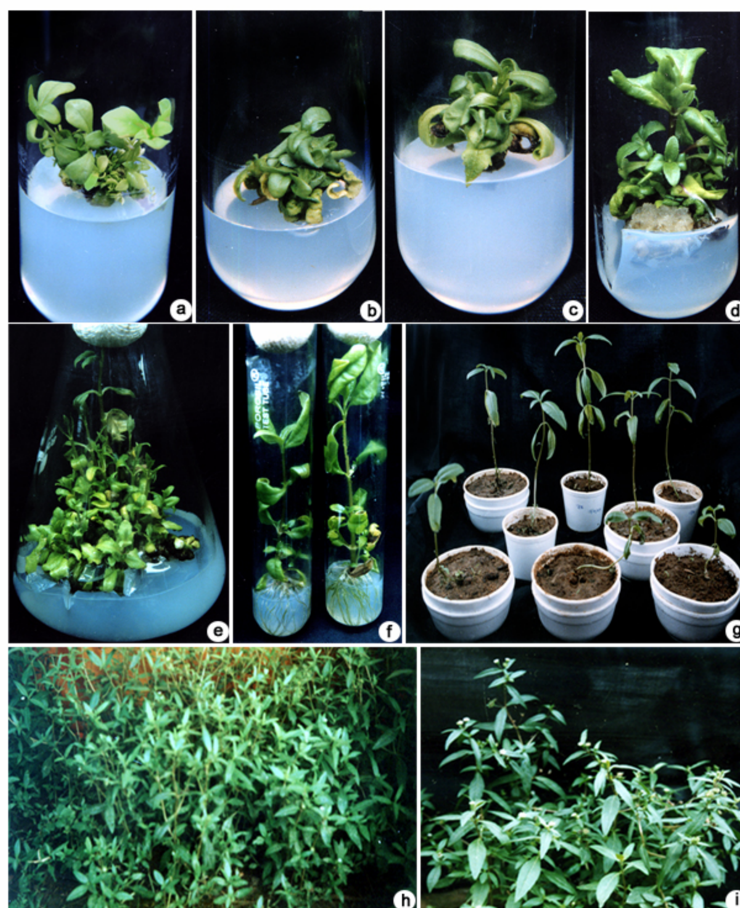

Figure 1 ***In vitro*** plantlet formation and growth characteristics of ***Eclipta alba:***

(a) Induction of axillary branching from cotyledonary nodal segment on MS + 4.4 µM BA (after four weeks).
(b) Induction of axillary branching from cotyledonary nodal segment on B5 + 4.4 µM BA (after four weeks).
(c) Induction of axillary branching from cotyledonary nodal segment on SH + 4.4 µM BA (after four weeks).
(d) Regeneration of shoots from cotyledonary nodal segment showing callus formation.
(e) Proliferation of axillary shoots from subcultured cotyledonary nodal segments on MS medium containing 4.4 µM BA + 9.2 µM Kin + 2.4 µM 2iP.
(f) Regeneration of roots from shoots of *E.alba* cultured on full strength MS medium containing 9.8 µM IBA
(g) *In vitro* regenerated plantlets of *E.alba* transferred plastic cup containing garden soil, sand and farmyard manure (2:1:1)
(h) Acclimatized micropropagated plants of *E.alba* (after six weeks)
(i) The flowering of acclimatized micropropagated plants of *E.alba* (after ten weeks)

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