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AN EFFICIENT MICROPROPAGATION SYSTEM FOR *ECLIPTA ALBA* – A VALUABLE MEDICINAL HERB

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Summary

An efficient rapid and large-scale *in vitro* clonal propagation of the valuable medicinal herb *Eclipta alba* (*Asteraceae*) by enhanced axillary shoot proliferation in cotyledonary node segments was designed. The medium type, various carbon sources, plant growth regulators, and coconut water markedly influenced *in vitro* propagation of *Eclipta alba*. An *in vitro* plantlet production system has been investigated on Murashige and Skoog (MS) medium with the synergistic combination of benzyladenine (4.4 μ*M*), kinetin (4.6 μ*M*), 2-isopentenyladenine (4.9 μ*M*), gibberellic acid (1.4 μ*M*), 5% coconut water, and 3% sucrose which promoted the maximum number of shoots as well as beneficial shoot length. Subculturing of cotyledonary node segments on a similar medium enabled continuous production of healthy shoots with similar frequency. Rooting was highest (94.3%) on full strength MS medium containing 9.8 μ*M* indolebutyric acid. Micropropagated plants established in garden soil, farmyard soil, 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.

Key words: axillary shoot proliferation; *ex vitro*; growth characters; growth regulators; hardening.

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 (Anonymous, 1952). The alcoholic extract of the plant has shown antiviral activity against Ranikhet disease virus. For healthy black and long hair the plant is commonly used in hair oil all over India. The fresh juice of leaves is used as an appetite stimulant, as a digestive, and as a mild bowel regulator. It is commonly used in viral hepatitis to promote bile flow and protect the parenchyma, and it is popularly used to enhance memory and learning. The plant has a reputation as an anti-aging agent in Ayurvedic medicine. *Eclipta alba* is widely used in India as a cholagogue and deobstruent in hepatic enlargement, and used to treat jaundice and other ailments of the liver and gall bladder (Orning et al., 1980). Coumestan-type compounds, wedelolactone and dimethyl wedelolactone, were isolated as the main active principles of *Eclipta alba*, both constituents exhibiting antihepatotoxic activity (Wagner et al., 1986; Franca et al., 1995). *In vivo* tests proved that wedelolactone neutralizes the lethal and myotoxic activities of rattlesnake venom (Mors et al., 1989).

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. 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 (Ajithkumar and Seeni, 1998; Sahoo and Chand, 1998; Prakash et al., 1999). Further, genetic improvement is another approach to augment the drug-yielding capacity of the plant (Tejavathi and Shailaja, 1999). Therefore it is important to develop an efficient micropropagation technique for *Eclipta alba* to rapidly disseminate superior clones once they are identified. There have been few reports to date on micropropagation in the genus using nodal explants (Franca et al., 1995; Zafar and Sagar, 1999; Borthakur et al., 2000).

The purpose of this study was to develop an *in vitro* propagation method from cotyledonary nodes of *E. alba*, an economically important species. In the present work we have, for the first time, established a rapid and reproducible method for high-frequency axillary shoot proliferation from cotyledonary node segments, followed by establishment of regenerated plants in soil. We have also examined the morphological characteristics, growth characteristics, and floral features.

Materials and Methods

Plant material and disinfections. Healthy young cotyledonary nodes of *Eclipta alba* (L.) Hassk. were collected from the Botanical Field Evaluation Garden of the Bharathidasan University at Tiruchirappalli. The explants were taken on October 8, 2002 (7.00 a.m.), for culture initiation. After removing the cotyledonary node segments (1.0–1.5 cm), segments were washed thoroughly under running tap water for 30 min followed by treatment with a solution of 2% (v/v) Teepol (Reckitt Benckiser, India) and 70% (v/v) ethanol for 15 s and thereafter washed three to five times with autoclaved sterilized distilled water. The explants were then surface-disinfected with 0.1% (w/v) aqueous mercuric chloride solution for 5–6 min and finally rinsed with autoclaved distilled water (five to seven changes).

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The cotyledonary node segments were then trimmed at both ends prior to inoculation on culture media.

Culture conditions. Single disinfected cotyledonary node segments were cultured on MS (Murashige and Skoog, 1962) basal medium supplemented with 3% (w/v) sucrose (Himedia, Mumbai, India) for culture initiation and 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 gelling with 0.8% (w/v) agar (Himedia). In all the experiments, the chemicals used were of analytical grade (Himedia, Kelco, Merkard, and Sigma). The medium was dispensed into culture vessels (Borosil, Mumbai, India) and autoclaved at 105 kPa and 121°C for 15 min. The surface-disinfected explants were implanted vertically on the culture medium [test tubes (150 × 25 mm) containing 15 ml medium] and plugged tightly with non-absorbent cotton. All the cultures were incubated at 25 ± 2°C under 16 h photoperiod of 45–50 $\mu mol\, m^{-2} s^{-1}$ irradiance provided by cool white fluorescent tubes (Philips, India) and with 55–60% relative humidity. All subsequent subcultures were done at 4 wk intervals.

Effect of basal media. Three different media including MS, B5 (Gamborg et al., 1968), and SH (Schenk and Hildebrandt, 1972) were evaluated for their effects on *in vitro* growth and development of *Eclipta alba*. All the basal media contained 3% (w/v) sucrose and were solidified with 0.8% (w/v) agar and different concentrations of cytokinins, including 2.2–22.2 μM benzyladenine (BA), 2.3–23.2 μM kinetin (Kin), or 2.4–24.6 μM 2-isopentenyladenine (2iP).

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

Effect of cytokinins. Cotyledonary node segments were cultured on MS medium containing 3% (w/v) sucrose and with 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, and 4.4 μM BA + 2.3–23.2 μM Kin + 2.4 μM 2iP.

Effect of cytokinins and coconut water. Cotyledonary node segments were cultured on MS medium containing 3% (w/v) sucrose with 0.8% (w/v) agar and supplemented with 5% (w/v) coconut water and different combinations and concentrations of plant growth regulators, including 2.2–13.3 μM BA, 9.2–23.2 μM Kin, 9.8–24.6 μM 2iP; 4.4 μM BA + 4.6–13.9 Kin, 4.4 μM BA + 4.9–14.7 μM 2iP; 4.4 μM BA + 4.6–9.2 μM Kin + 2.4 μM 2iP; 4.4 μM BA + 4.6 μM Kin + 4.9–9.8 μM 2iP and addition of 1.4 μM gibberellic acid (GA_3).

Rooting medium. Elongated shoots were excised from each culture passage and transferred to full-strength and half-strength 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), 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 soil, and sand (2:1:1). Each was irrigated with one-eighth strength MS basal salt solution devoid of sucrose and inositol every 4 d for 2 wk. The potted plantlets were covered with porous polyethylene sheets for maintaining high humidity and were maintained inside the culture room conditions. The relative humidity was reduced gradually, and after 30 d the plantlets were transplanted to a botanical evaluation garden and kept under shade in a net house for further growth and development. The morphological characteristics, growth characteristics, and floral features were examined.

Statistical analysis. Experiments were set up in a randomized block design and each experiment usually had 10 replications and was repeated at least three times. Ten to 15 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 detect the significance of differences among the treatment means. The treatment means were compared using Duncan's multiple range test at a 5% probability level according to Gomez and Gomez (1976).

Results and Discussion

Effect of basal media and cytokinins on shoot regeneration. Cotyledonary node explants of *E. alba* were cultured on three types of media and supplemented with various concentrations of BA, Kin, and 2iP individually for shoot regeneration. Among the three different media and growth regulators tested, MS medium was found to support a better response for shoot regeneration than B5 and SH media (data not shown). MS medium was more effective than other media for other medicinal plants as well (Gao et al., 1999; Komalavalli and Rao, 2000; Monsor et al., 2003; Wondyifraw and Surawit, 2004).

Among the three different media used, the explants in MS medium appeared healthy and grew vigorously (Fig. 1*a*). On MS medium supplemented with 4.4 μM BA, 96% of segment explants produced shoots after 65 d with an average of 7.8 shoots per explant. Gawde and Paratkar (2004) reported that the best response was observed on MS medium with 4.4 μM BA. B5 and SH media induced vitrification with an average number of new shoots produced (Fig. 1*b*, *c*). A similar phenomenon was observed by Monsor et al. (2003).

In the present study, higher concentrations of cytokinin reduced the shoot number as well as shoot length. Hu and Wang (1983) reported that higher concentrations of cytokinin reduced the number of micropropagated shoots. 2iP and Kin enhanced shoot proliferation with an increase in the hormone concentration. In the present study, higher concentrations of BA (8.8–22.2 μM) reduced the shoot numbers as well as shoot length. A similar response was observed by Indra and Dha (2000). 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 moderately enhancing shoot length. The media (B5, SH, and MS) containing Kin (23.2 μM) or 2iP (24.6 μM) 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 d of culture (Fig. 1*d*). In all media, shoot sprouting frequency was higher in BA and 2iP than Kin.

In each medium, Kin was more effective than BA and 2iP for shoot length. MS medium fortified with 4.6 μM Kin attained the highest shoot length of 16.8 cm after 65 d of culture in the subsequent subculture. All the media and hormones induced little white compact calluses at the base of the explants after 30 d of culture. In each medium, the shoots produced roots at the basal callus after 45 d 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 numbers of roots. However, the shortest roots were produced in B5 medium followed by SH medium. Similar responses were observed in *Korarima* (Wondyifraw and Surawit, 2004). MS medium supplemented with Kin promoted greater rooting than BA and 2iP. A similar result was observed by Franca et al. (1995). It was difficult to isolate a single shoot with root from each culture passage because of the possibility of damaging roots. In each media, the cultures, which were maintained for a long time (after 4 wk), resulted in gradual browning and defoliation of leaves of the shoot. A similar phenomenon was observed in *Eclipta alba* (Borthakur et al., 2000). The successive subculture was carried out at 4-wk intervals, and plants exhibited normal appearance. The observations indicate that these media are at concentrations favorable for promoting shoot proliferation in *Eclipta alba*. All further experiments were conducted on MS medium.

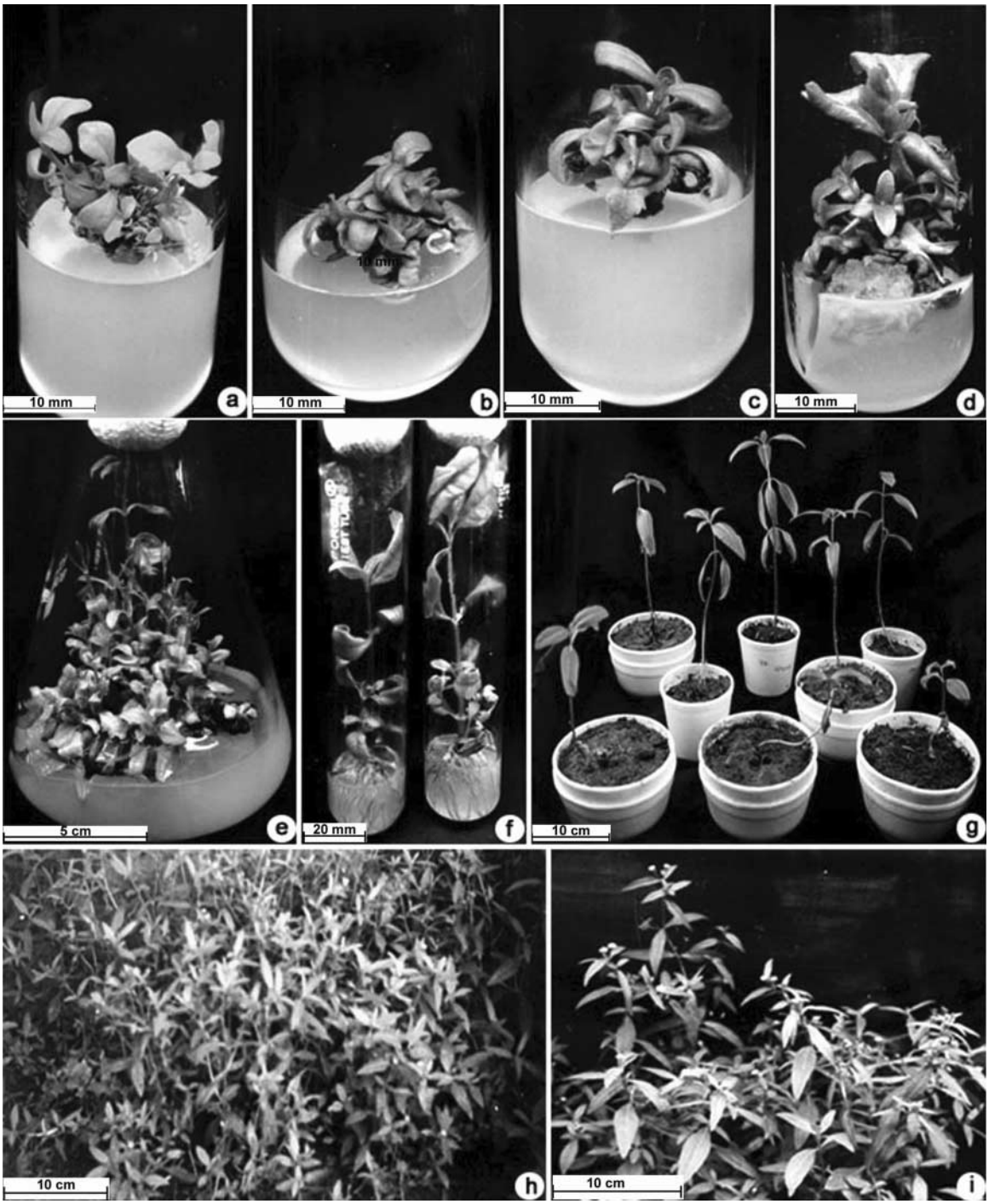

FIG. 1. *In vitro* plantlet formation and growth characteristics of *Eclipta alba*. *a*, Induction of axillary branching from cotyledonary node segment on MS + 4.4 μ*M* BA (after 4 wk). *b*, Induction of axillary branching from cotyledonary node segment on B5 + 4.4 μ*M* BA (after 4 wk). *c*, Induction of axillary branching from cotyledonary node segment on SH + 4.4 μ*M* BA (after 4 wk). *d*, Regeneration of shoots from cotyledonary node segment showing callus formation. *e*, Proliferation of axillary shoots from subcultured cotyledonary node segments on MS medium containing 4.4 μ*M* BA + 4.6 μ*M* Kin + 4.9 μ*M* 2iP + 1.4 μ*M* GA_3 + 5% coconut water. *f*, Regeneration of roots from shoots cultured on full-strength MS medium containing 9.8 μ*M* IBA. *g*, *In vitro*-regenerated plantlets transferred to plastic cups containing garden soil, farmyard soil, and sand (2:1:1). *h*, Acclimatized micropropagated plants (after 6 wk). *i*, Flowering of acclimatized micropropagated plants (after 10 wk).

Carbon sources and shoot regeneration. The responses of *in vitro* cultures to different carbon sources added to the medium were frequently tested. Although carbohydrates are of prime importance for *in vitro* organogenesis, carbon metabolism *in vitro* is still not clearly understood (Kozai, 1991). It is well established that carbohydrate requirements depend upon the stage of culture and may show differences according to the species (Thompson and Thorpe, 1987). Cotyledonary node segments were cultured on MS medium containing 3% (w/v) glucose, fructose, and sucrose, respectively. These media were supplemented with 4.4 μ*M* BA and 0.8% (w/v) agar. Among the three carbon sources, sucrose proved to be better for shoot regeneration than fructose and glucose (data not shown). Similar results were obtained in micropropagation of Cork oak (Romano et al., 1995) and *Kaempferi* (Shirin et al., 2000). However, sucrose and glucose induced the highest frequency of organogenesis in *Bixa orellana* (De Paiva Neto et al., 2003).

Satisfactory shoot proliferation from cotyledonary node explants was obtained on sucrose (3% w/v), whereas glucose and fructose resulted in fewer shoots; shoot lengths also varied. However, on sucrose medium, leaf chlorosis occurred after 10 wk, and shoots gradually declined. A similar response was observed by Hisashi and Yasuhiro (1996). In *Malus robusta* 'Rehd. No 5', fructose yielded the lowest number of shoots (Pua and Chong, 1984). 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 yielded 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. Carbohydrates control morphogenesis by acting as energy sources 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 (Pritchard et al., 1991). MS medium fortified with fructose attained a shoot length of 16.4 cm after 65 d of culture.

We observed that glucose and fructose promoted root induction slowly even at 30 d of culture. However, sucrose readily promoted the light green silky roots as well as lengthy roots. All the roots turned white after 45 d of culture. It was difficult to isolate shoots with roots from each carbon source containing shoot multiplication medium (MS + 4.4 μ*M* BA). The carbon sources appeared to damage the roots, which were difficult to harden. All further experiments were conducted on sucrose.

Effect of cytokinins on shoot regeneration. Various concentrations of Kin (2.3–23.2 μ*M*) and 2iP (2.4–24.6 μ*M*) with BA (4.4 μ*M*) were tried for shoot induction and to determine the multiplication potential of cotyledonary nodes. In combination with different concentrations of Kin, 2iP and 4.4 μ*M* BA induced axillary shoots. Shamsudeen Varisai et al. (1999) reported that BA alone or in combination with other cytokinins induced shoot organogenesis from shoot tips and cotyledonary node explants of *Macrotyloma uniflorum*. Greater numbers of morphologically distinct multiple shoots were developed from the cotyledonary nodes in MS medium containing BA combined with Kin or 2iP. Multiple shoots developed with a combination of BA (4.4 μ*M*) and 2iP (14.7 μ*M*) grew faster, while those initiated in the BA (4.4 μ*M*) and Kin combinations grew slower. BA and Kin combinations developed fewer multiple shoots in some species (Sivakumar and Krishnamurthy, 2000). The minimum number of roots were produced at the base of shoots at low concentrations of Kin (2.3–4.6 μ*M*) or 2iP (2.4–4.9 μ*M*) and with BA (4.4 μ*M*). Rooting was completely arrested at Kin concentrations of 9.2–23.2 μ*M* or 2iP concentrations of 9.8–24.6 μ*M*, both with BA (4.4 μ*M*). White-greenish compact callus developed directly from the cut ends of multiple shoots containing MS medium fortified with BA (4.4 μ*M*) and Kin in the range 4.6–23.2 μ*M* and 2iP in the range 4.9–24.6 μ*M* within 4 wk of culture. A significant shoot sprouting frequency and number of shoots was obtained in MS medium supplemented with BA (4.4 μ*M*) + Kin (13.9 μ*M*) and BA (4.4 μ*M*) + 2iP (14.7 μ*M*) but the shoot lengths differed (Table 1). The combined effect of cytokinins (BA and Kin) enhanced multiple shoot bud regeneration in *Arachis hypogaea* (Venkatachalam and Jayabalan, 1997). However, the combination of cytokinins (BA and Kin) failed to improve shoot multiplication (Purohit and Dave, 1996). The MS medium containing BA (4.4 μ*M*) and a low concentration of Kin (2.3–9.2 μ*M*) or 2iP (2.4–9.8 μ*M*) increased the shoot length but caused a low frequency of axillary shoot formation. With an increase in concentration of Kin (13.9 μ*M*) or 2iP (14.7 μ*M*) in the medium, the number of axillary shoots also increased significantly (Table 1). But the higher the concentration of Kin (23.2 μ*M*) or 2iP (24.6 μ*M*) in the medium revealed a decrease in the number of axillary shoots as well as shoot length. However, the shoot length was best with BA (4.4 μ*M*) + Kin (2.3 μ*M*) (Table 1). The maximum number of shoots was obtained in MS

TABLE 1

INFLUENCE OF CYTOKININS ON SHOOT REGENERATION FROM COTYLEDONARY NODE EXPLANTS OF *ECLIPTA ALBA*

Growth regulators (μ*M*)	Shoot sprouting frequency (%)	Number of shoots per explant (mean ± SE)	Shoot length (cm) (mean ± SE)
BA (4.4) + Kin			
2.3	72.2 de	8.3 ± 0.36 d	12.9 ± 0.94 a
4.6	79.6 e	8.8 ± 0.34 cd	10.5 ± 0.74 b
9.2	82.0 b	9.6 ± 0.78 c	8.3 ± 0.63 c
13.9	90.5 a	14.0 ± 0.52 a	4.5 ± 0.35 d
18.5	80.3 bc	13.5 ± 0.58 ab	4.1 ± 0.32 de
23.2	73.1 d	11.5 ± 0.46 b	3.3 ± 0.20 e
BA (4.4) + 2iP			
2.4	70.4 f	8.4 ± 0.58 ef	10.8 ± 0.78 a
4.9	78.2 de	8.9 ± 0.60 e	10.1 ± 0.61 ab
9.8	83.1 bc	11.9 ± 1.40 d	8.0 ± 0.44 c
14.7	95.2 a	16.5 ± 1.56 a	4.9 ± 0.29 d
17.3	84.0 b	15.8 ± 1.25 ab	3.8 ± 0.27 de
24.6	79.0 d	14.0 ± 1.31 c	3.2 ± 0.32 e
BA (4.4) + 2iP (2.4) + Kin			
2.3	77.0 d	12.6 ± 0.38 bc	8.7 ± 0.87 a
4.6	88.4 b	14.0 ± 1.02 b	6.9 ± 0.63 b
9.2	98.7 a	25.8 ± 1.21 a	4.9 ± 0.40 c
13.9	86.0 bc	16.4 ± 0.64 ab	3.4 ± 0.18 d
18.5	84.2 c	12.5 ± 0.58 c	3.0 ± 0.21 de
23.2	80.1 cd	8.5 ± 0.45 d	2.8 ± 0.26 e

Data were recorded after 65 d of culture.

Treatment means followed by different *letters* within PGR and response variable combinations are significantly different from each other ($P < 0.05$); comparison by Duncan's multiple range test.

medium supplemented with BA (4.4 μ*M*) + Kin (9.2 μ*M*) and 2iP (2.4 μ*M*), but the shoots were small when the concentrations of Kin or 2iP were raised. Initially, white-greenish compact callus was formed directly at the cut ends of the cotyledonary node containing MS medium fortified with BA (4.4 μ*M*) + 2iP (2.4 μ*M*) + Kin (2.3–23.2 μ*M*), but this callus later turned deep black and the shoots declined after 10 wk of culture. The shoot length was strongly affected by high concentrations of cytokinins (4.4 μ*M* BA + 23.2 μ*M* Kin, 4.4 μ*M* BA + 17.3–24.6 μ*M* 2iP and 4.4 μ*M* BA + 18.5–23.2 μ*M* Kin + 2.4 μ*M* 2iP). A similar phenomenon was observed by De Paiva Neto et al. (2003) in *Bixa orellana*.

Effect of cytokinins and coconut water on shoot regeneration .Synergistic effects of cytokinin and coconut water on shoot regeneration were assayed. MS medium was supplemented with different combinations and concentrations of shoot multiplication medium [2.2–13.3 μ*M* BA + 5% coconut water; 9.2–23.2 μ*M* Kin + 5% coconut water; 9.8–24.6 μ*M* 2iP + 5% coconut water; 4.4 μ*M* BA + Kin (4.6–13.9 μ*M*) + 5% coconut water; 4.4 μ*M* BA + 2iP (4.9–14.7 μ*M*) + 5% coconut water; 4.4 μ*M* BA + Kin (4.6–9.2 μ*M*) + 2iP (2.4 μ*M*) + 5% coconut water; 4.4 μ*M* BA + Kin (4.6 μ*M*) + 2iP (4.9–9.8 μ*M*) + GA_3 (1.4 μ*M*) + 5% coconut water]. *In vitro* growth and development of *Eclipta alba* was highly influenced by concentrations of growth regulators and coconut water added to the culture medium. Addition of coconut water (5% w/v) increased the shoot number as well as shoot length (Table 2). Several reports have confirmed the beneficial effect of coconut water for micropropagation (Reghunath and Bajaj, 1992; Bajaj et al., 1993; Brain and Richard, 1993; Sajina et al., 1997; Wondyifraw and Surawit, 2004). The most significant shoot sprouting frequency and number of shoots was obtained in MS medium supplemented with BA (4.4 μ*M*) + 5% coconut water; Kin (13.9 μ*M*) + 5% coconut water; 2iP (17.3 μ*M*) + 5% coconut water; BA (4.4 μ*M*) + Kin (9.2 μ*M*) + 5% coconut water; BA (4.4 μ*M*) + 2iP (9.8 μ*M*) + 5% coconut water and BA (4.4 μ*M*) + Kin (6.9 μ*M*) + 2iP (2.4 μ*M*) + 5% coconut water (Table 2). The MS medium containing BA (2.2–4.4 μ*M*), Kin (9.2–13.9 μ*M*), 2iP (9.8–14.7 μ*M*), BA (4.4 μ*M*) + Kin (4.6 μ*M*) and BA (4.4 μ*M*) + 2iP (4.9 μ*M*) and inclusion of coconut water (5% w/v) resulted in higher shoot length as well as lower numbers of roots. However, an increase in the concentration of BA, Kin, or 2iP in the medium resulted in a decrease in shoot number as well as shoot length. Green compact basal callus was formed from the cut ends and later turned deep black. The exudates on the medium completely inhibited multiple shoot growth and shoots declined vigorously. MS medium supplemented with the BA (4.4 μ*M*) + Kin (4.6–9.2 μ*M*) + 2iP (2.4 μ*M*) combinations increased shoot numbers as well as reducing the shoot length. The maximum number of shoots were obtained in MS medium supplemented with BA (4.4 μ*M*) + Kin (4.6 μ*M*) + 2iP (4.9 μ*M*) + 5% coconut water, and the addition of GA_3 (1.4 μ*M*) increased the shoot number and moderately increased shoot length (Fig. 1*e*). A combination of cytokinin and GA_3 promoted multiple shoot formation (Sahoo and Chand, 1998). GA_3 has been shown to promote differentiation of shoot buds in *Ocimum* species (Pattnaik and Chand, 1996; Sahoo et al., 1997). The shoot length varied significantly in all combinations and concentrations of shoot multiplication medium. The shoot length was best with BA (4.4 μ*M*) + 5% coconut water, Kin (9.2, μ*M*) + 5% coconut water, and 2iP (9.8 μ*M*) + 5% coconut water (Table 2). Addition of coconut water (10–15%) significantly affected the axillary shoot bud formation, except for shoot length (data not shown). Similar responses were observed by Wondyifraw and Surawit (2004) in Krawan. Several reports have revealed the positive effects of coconut water for *in vitro* multiplication (Nadganda et al., 1983; Bajaj et al., 1993; Shirgurkar et al., 2003). In contrast, it has been reported to suppress shoot bud differentiation in *Begonia* (Heide, 1969) and *Duboisia myoporoides* (Kukreja and Mathur, 1985). Thus the role of GA_3 in shoot bud induction in plant species is controversial.

TABLE 2

INFLUENCE OF CYTOKININS AND 5% COCONUT WATER ON SHOOT REGENERATION FROM COTYLEDONARY NODE EXPLANTS OF *ECLIPTA ALBA*

Growth regulators (μ*M*)	Shoot sprouting frequency (%)	Number of shoots per explant (mean ± SE)	Shoot length (cm) (mean ± SE)
BA			
2.2	80.2 bc	6.5 ± 0.28 cd	14.1 ± 1.03 ab
4.4	98.6 a	9.7 ± 0.26 a	15.4 ± 1.58 a
8.8	92.1 ab	8.7 ± 0.26 ab	11.1 ± 0.93 c
13.3	86.3 b	7.5 ± 0.59 c	8.7 ± 0.68 d
Kin			
9.2	83.8 b	5.5 ± 0.29 cd	12.7 ± 0.82 a
13.9	90.4 a	7.0 ± 0.60 a	9.9 ± 0.30 b
18.5	85.3 ab	6.4 ± 0.76 b	7.4 ± 0.44 c
23.2	74.0 c	5.7 ± 0.58 c	6.2 ± 0.35 cd
2iP			
9.8	85.3 bc	4.9 ± 0.65 d	13.2 ± 0.98 a
14.7	87.2 b	6.5 ± 0.53 ab	10.3 ± 0.73 b
17.3	94.5 a	6.9 ± 0.40 a	8.5 ± 0.81 bc
24.6	82.1 c	5.6 ± 0.44 c	7.6 ± 0.63 c
BA (4.4) + Kin			
4.6	89.2 b	11.5 ± 0.93 bc	10.4 ± 0.91 a
9.2	93.5 a	16.9 ± 1.24 a	8.0 ± 0.96 ab
13.9	87.7 bc	13.8 ± 1.06 b	5.5 ± 1.23 b
BA (4.4) + 2iP			
4.9	88.3 c	12.6 ± 0.78 c	9.3 ± 0.82 a
9.8	95.5 a	18.3 ± 1.06 a	6.7 ± 0.93 b
14.7	92.6 b	15.9 ± 0.72 ab	4.6 ± 0.90 bc
BA (4.4) + 2iP (2.4) + Kin			
4.6	90.2 bc	18.4 ± 1.52 c	7.3 ± 1.25 a
6.9	97.0 a	29.5 ± 2.14 a	6.8 ± 0.93 ab
9.2	93.6 b	25.5 ± 1.68 ab	5.6 ± 0.74 c
BA (4.4) + Kin (4.6) + GA_3 (1.4) + 2iP			
4.9	99.1 a	43.7 ± 2.18 a	9.7 ± 0.80 a
7.3	98.2 ab	39.2 ± 2.07 ab	6.7 ± 0.63 b
9.8	96.4 c	28.9 ± 1.63 c	5.8 ± 0.54 bc

Data were recorded after 65 d of culture.

Treatment means followed by different *letters* within PGR and response variable combinations are significantly different from each other ($P < 0.05$); comparison by Duncan's multiple range test.

Effect of auxins on rooting of shoots. Excised shoots were rooted on half-strength or full-strength MS medium with different types of auxin. The promotory effect of reducing the salt concentration of MS on *in vitro* rooting of shoots has been described in several reports (Constantine, 1978; Skirvin et al., 1980). We have found in *Eclipta alba* that reducing MS salt strength to one-half normally 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 d of culture. Among the

three auxins tested, the number of roots and root lengths varied in both media (Table 3). Plants rooted in full-strength MS medium significantly developed lengthy roots, and root induction was strengthened within 20 d of culture. Half-strength MS medium fortified with IBA was found to be more effective for root induction than IAA and NAA. Full-strength MS medium supplemented with IBA (9.8 μM) was more effective for root induction than IAA and NAA (Table 3; Fig. 1*f*). However, IAA and NAA formed slender roots in both media. Mild callus formation occurred with all types of auxin in full-strength MS medium. IBA was more effective for root induction in both types of media than IAA and NAA. Similar responses were observed by Sahoo and Chand (1998), Komalavalli and Rao (2000), and Sivakumar and Krishnamurthy (2000).

Hardening of regenerated plants and examination of morphological characters. Plantlets were successfully acclimatized without a growth chamber facility. Plantlet survival was 100% after hardening on garden soil, farmyard soil, and sand (2:1:1) for 3 wk (Fig. 1*g*). However, the survival decreased to 96.0 and 74.6%, respectively, after 4 and 10 wk of acclimatization (Table 4). The initial growth rates of plant height were 9.3 ± 0.27 cm during the first 2 wk of acclimatization. However, in the following 3–10 wk, a substantial increase in plant height was observed (Table 4; Fig. 1*h*). Initially, two to three healthy branches, each bearing an average of two to three leaves, developed adjacent to the main shoot. However, the number of branches per plant increased to 5.4 ± 0.14 and 15.04 ± 0.22, respectively, after 4 and 10 wk of acclimatization. Flowering initially occurred at the apical portion of the main shoot and after 6 wk each branch developed flowering at the terminal region of the branches. However, the number of flowers per plant increased to 3.9 ± 0.21 and 23.8 ± 0.28 respectively, after 6 and 10 wk of acclimatization (Table 4; Fig. 1*i*). 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.

In conclusion, a reproducible, quick and large-scale micropropagation protocol was established from cotyledonary nodes of *E. alba*.

TABLE 3

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 per shoot (mean ± SE)	Root length (cm) (mean ± SE)
Full-strength MS + IAA			
2.8	56.6 d	5.08 ± 0.66 de	3.04 ± 0.39 c
5.7	60.0 bc	7.32 ± 0.47 d	3.61 ± 0.40 bc
8.5	62.5 b	11.38 ± 0.74 bc	4.14 ± 0.83 a
11.4	67.4 a	15.96 ± 0.46 a	3.86 ± 0.78 ab
17.1	54.3 de	12.30 ± 0.67 b	3.62 ± 0.25 b
Full-strength MS + IBA			
2.5	78.0 de	14.64 ± 0.50 d	5.28 ± 0.46 e
4.9	80.4 d	26.32 ± 0.73 bc	6.16 ± 0.25 c
7.4	87.0 bc	28.46 ± 0.65 b	7.04 ± 0.20 a
9.8	94.3 a	34.54 ± 1.31 a	6.72 ± 0.43 ab
14.8	89.5 b	30.70 ± 0.62 ab	5.94 ± 0.36 cd
Full-strength MS + NAA			
2.7	65.7 de	10.48 ± 0.55 d	4.0 ± 0.20 de
5.4	76.0 c	17.04 ± 0.66 bc	4.7 ± 0.38 b
8.1	82.6 ab	21.02 ± 0.95 ab	5.2 ± 0.45 a
10.7	88.0 a	24.36 ± 1.07 a	4.5 ± 0.20 bc
16.1	69.2 d	20.30 ± 0.76 b	4.1 ± 0.16 d
Half-strength MS + IAA			
2.8	48.6 d	3.22 ± 0.28d	2.77 ± 0.17 d
5.7	57.0 b	3.95 ± 0.36cd	3.17 ± 0.23 bc
8.5	58.0 ab	5.62 ± 0.35c	3.42 ± 0.26 b
11.4	60.0 a	10.02 ± 0.62a	3.77 ± 0.22 a
17.1	52.3 c	8.83 ± 0.39ab	3.09 ± 0.18 c
Half-strength MS + IBA			
2.5	64.0 cd	12.05 ± 0.78 e	4.66 ± 0.30 c
4.9	69.0 c	16.80 ± 1.06 d	5.05 ± 0.46 b
7.4	73.2 b	22.48 ± 1.09 ab	5.29 ± 0.39 ab
9.8	84.0 a	23.06 ± 1.20 a	5.32 ± 0.38 a
14.8	70.0 bc	20.98 ± 0.79 c	4.87 ± 0.47 bc
Half-strength MS + NAA			
2.7	57.0 d	8.75 ± 0.88 d	3.22 ± 0.22 de
5.4	60.0 c	11.24 ± 0.69 bc	3.32 ± 0.16 d
8.1	65.4 b	13.91 ± 0.76 ab	4.17 ± 0.29 bc
10.7	74.0 a	14.24 ± 1.21 a	4.88 ± 0.13 a
16.1	58.7 cd	12.06 ± 0.87 b	4.26 ± 0.19 b

Data were recorded after 30 d of culture.

Treatment means followed by different *letters* within medium treatment and response variable combinations are significantly different from each other ($P < 0.05$); comparison by Duncan's multiple range test.

TABLE 4

THE FREQUENCY OF *EX VITRO* SURVIVAL, GROWTH AND FLOWERING OF ACCLIMATIZED MICROPLANTS OF *ECLIPTA 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 per plant	0.0 ± 0.00	3.5 ± 0.11	5.4 ± 0.14	8.0 ± 0.19	13.0 ± 0.57	15.04 ± 0.22
Number of flowers per plant	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	3.9 ± 0.21	9.16 ± 0.18	23.8 ± 0.28

Values shown were proportions of three replicates of 25 microplants.

The protocol reported here could be used for the conservation of this valuable medicinal herb. Axillary shoot proliferation from cotyledonary node was dependent on the interaction between plant growth regulator concentrations in the medium. A rapid multiplication rate could be obtained by a reduced cytokinin ratio associated with coconut water. This protocol has great potential for improvement of this crop by biotechnological approaches such as genetic transformation and production of secondary metabolites.

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