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A Rapid Micropropagation of nodal explants of *Eclipta alba* (L.); A Multipurpose Medicinal Herb

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An efficient *in vitro* regeneration protocol was developed for medicinally important plant *Eclipta alba*. Successful regeneration and multiplication of nodal explants of *E. alba* were obtained in cytokinin enriched B5 medium. Several cytokinins [6-benzylaminopurine (BAP), kinetin (KIN), thidiazuron (TDZ), gibberellic acid (GA_3) and spermidine] were supplemented alone and its combinations for obtaining better results. The best growth frequency response was achieved in the combinations of 1.0 BAP + 0.3 KIN + 1.5 GA_3 (mg/L) concentration (7.4 ± 0.9 cm shoot length & 100 % regeneration). Better roots were developed in half-strength B5 medium along with IBA (1.0 mg/L) hormone and exhibits maximum root length (7.0 ± 0.8cm) along with multiple roots (8.8 ± 0.8) at 92 %. The well-developed Plantlets were successfully acclimatized to plastic-cups containing autoclaved sand and garden soil (1:1) and kept undisturbed with plastic cover for maintaining the humidity. The plantlets were watered regularly and maintained at green house.

Keywords: *In vitro* propagation, *Eclipta alba*, Growth regulators

Abbreviations: BAP- 6-benzylaminopurine; KN – Kinetin; GA_3 - Gibberellic acid; B5- Gamborg (1968); PGR_S - Plant growth regulators; IBA -Indole-3- butyric acid; TDZ - Thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5-yl urea)

Eclipta alba (L.) Hassk. (Asteraceae) is a small, branched, annual herb with white flower heads, native to tropical and subtropical regions of the world. The plant is traditionally used in the treatment of liver diseases, skin disorders, premature graying of hair, and enhance the memory (Dhaka and Kothari, 2005; Banji *et al.*, 2007; Ray and Bhattacharya, 2010). The plant juice is used as a tonic and diuretic in hepatic and spleen enlargement (Baskaran and Jayabalan, 2005; Jadhav *et al.*, 2009; Singh *et al.*, 2010). The plant extracts are reported to contain several phyto-constituents like eclalbatin, alpha-amyrin, ursolic acid, oleanolic acid, ecliptasaponin, daucosterol, stigmasterol-3-Oglucoside etc. (Thakur and Mengi, 2005). The plant possesses many biological properties like anti-inflammatory and bronchodilator (Leal et al., 2000) hepatoprotective and anti-hyperglycemic agent (Ananthi *et al.*, 2003), antiviral, antibactarial, spasmogenic, hypotensive, analgesic, antioxidant(Sharma *et al.*, 2001; Karthikumar *et al.*, 2007; Veeru *et al.*, 2009), cardioprotective (Baliga *et al.*, 2004) and antianaphylactic (Patel *et al.*, 2010) properties. There is an urgent need for development of

rapid and large scale production of this highly valuable medicinal herb using tissue culture technique.

In vitro culture techniques offer a viable tool for bulk multiplication of genetically identical plant material, large scale production and germplasm conservation of valuable plants (Tomar and Gupta, 1998). The *in vitro* technology could be a cost effective means of high-volume production of the elite planting material throughout the year, without any seasonal constrains (Ajithkumar and Seeni, 1998; Prakash *et al.,* 1999). Hence, the study was aimed to establish a rapid and reproducible *in vitro* regeneration system for *E. alba* using nodal explants on modified B5 medium.

Materials and methods

Source of plant material

The fresh and healthy young twigs (15–20cm) of *E. alba* was collected (during November and December months) from Botanical Garden, Department of Biotechnology, Periyar University, Salem. Nomenclature of this plant was identified using standard floras. A voucher specimen was deposited in Natural Drug Research Laboratory (NDRL), Department of Biotechnology, Periyar University, Salem.

Surface sterilization and preparation of explants

The nodal explants were excised aseptically from field grown plants (0.5cm) and the surface sterilization of explants were done by standard procedures: Washed thoroughly (15 minutes) under running tap water to remove the dust particles and treated with 2 % (w/v) fungicide, (for 5–10 min) to reduce the chance of fungal contamination. About 2–3 drops of Tween–20 (Hi–Media, India) was used as surface sterilizing agents of explants followed by washing under running tap water for removal of detergents and disinfectants.

Subsequently, surface sterilization of the explants was carried out under aseptic conditions in laminar air hood. Freshly prepared Mercuric chloride aqueous solution [0.05% (w/v)] was used for 3–5 min. Then, rinsed thoroughly (3–4 times) with sterile distilled water to remove any traces of the disinfectant and edges were trimmed with sterile surgical blade. The explants were kept in sterile filter paper for drying before inoculation.

Media used

Modified B5 medium was used and prepared by adding aliquots of stock solutions along with 3 % sucrose as carbon source and 0.8 % agar for solidification. The pH of hormonal medium was calibrated between 5.5–5.8 using 0.1N NaOH and 0.1N HCl prior to autoclaving at 121 ^{o}C or 15 lbs for 15 minutes. Different concentrations of cytokinins and auxins were added to the modified B5 medium in combinations and alone for development of shoots and roots. The combined effect of growth hormones (BAP, Kin and gibberellic acid), were used for culturing the explants B5 medium supplemented with optimized concentration of BAP (0.5–1.0 mg/L) and different concentrations of Kin (0.3, 0.4 & 0.5 mg/L) or Gibberellic acid (1.0–1.5 mg/L) (Table1) respectively.

Inoculation and incubation

Nodal explants (1.0 cm) were cultured on different hormonal medium aseptically and incubated at 24 ± 2 ^{o}C under 16-h photoperiod, with light intensity of 40 mg/L µmol m^{-2} s^{-1} provided by cool white, fluorescent tubes. The regenerated shoot cultures were maintained (for 4 weeks period) and it was subcultured for multiple shoots formation. The elongation of multiple shoots was observed in same or improved medium (kept for incubation).

***In vitro* rooting and acclimatization**

Well developed and matured shoots were excised and transferred to growth regulator (full and half strength B5 medium) for root induction and monitored regularly for root proliferation. The well-developed plantlets (5–10 cm) were carefully taken out from the medium and washed thoroughly in running tap water for removal of all medium traces. Plants were transferred to acclimatization tray or cups containing soil. Plantlets were covered with polyethylene bags to maintain high humidity and irrigated with tap water regularly and placed in greenhouse (for 7–8 weeks). Then, it was shifted to the nursery and finally transferred to the field and observe the survival rate of individual.

Statistical analysis

All the experiments were performed in triplicates (Number of Explants = 14) for mean and standard deviation (± SD) Values are calculated. The analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were performed using INSTAT or SPSS 20 (IBM SPSS 20 statistics).

Results

Shoot regeneration and multiple shoots proliferation

The micropropagation results of nodal explants of *E. alba*, cultured on modified B5 medium were presented in Table.1 and figure.1. Nodal segments were cultured on B5 medium supplemented with cytokinins, BA, KIN and thidiazuron (0.5, 0.5 BAP + 0.5 KIN, 0.5BAP + 0.5 TDZ, 1.0 BAP + 0.5 S + 0.5 GA_3 and1.0 BAP + 0.3 KIN + 1.5 GA_3 mg/L), reflect varied response for shoot induction. As per the observation, BA was found more effective than Kin and spermidine, (the number and frequencies of shoot formation (after 8 weeks) within two subcultured periods. The multiple hormones (1.0 BAP + 0.3 KIN + 1.5 GA_3 mg/L) were produced better shoot proliferation and elongation rates (7.4 ± 0.9 cm shoot length, 7.7 ± 1.1 multiple shoots) with 100 % regeneration, followed by other hormonal combinations.

Table 1. Micropropagation of nodal explants of *E. alba* using different concentrations and combination of cytokinin and auxin (No. of explants =14)

Hormone (mg/L)					%	Mean no of	Mean no of
BAP	KIN	Spermidine	TDZ	GA_3	regeneration	shoot length (cm)[a]	shoots[a]
0.5	-	-	-	-	42.85	3.1 ± 0.1d	2.6 ± 0.5f
0.5	0.5	-	-	-	71.42	2.8 ± 0.5e	2.6 ± 0.0f
0.5	0.7	-	-	-	57.14	1.9 ± 0.1i	2.0 ± 0.0g
0.5	-	-	0.5	-	85.71	4.3 ± 0.4c	3.5 ± 0.5e
1.0	-	0.5	-	-	50.00	2.8 ± 0.9e	2.2 ± 1.2g
1.0	-	1.0	-	-	64.28	2.8 ± 0.1e	2.6 ± 1.5f
1.0	-	0.5	-	0.5	92.85	4.0 ± 1.0c	4.6 ± 1.5d
0.5	0.5	0.5	-	-	42.85	2.1 ± 0.0f	2.1 ± 0.8g
0.5	0.5	-	-	0.5	57.14	2.2 ± 0.0f	2.3 ± 0.4f
1.0	0.5	-	-	0.5	64.28	2.6 ± 1.0e	2.3 ± 0.5f
2.0	-	-	-	-	64.28	2.4 ± 1.0e	2.0 ± 1.0g
1.0	0.3	-	-	1.5	100	7.4 ± 0.9a	7.7 ± 1.1a
1.0	0.3	-	-	1.4	92.85	6.7 ± 1.8b	6.5 ± 2.0b
1.0	0.3	-	-	1.3	85.71	6.1 ± 1.2b	5.8 ± 1.8c
1.0	0.5	0.5	-	0.5	71.42	3.7 ± 1.0d	4.4 ± 0.8d

- = No growth regulators added. The values are mean within a column followed by the same letter are not significantly different by Duncan's multiple range test ($p > 0.05$). [a] Values corresponds to means [± standard error (SE)] of three independent experiments. For each experiment 14 cultures were used.

Rooting and acclimatization

In vitro elongated shoots of *E. alba* (2.5–3.0cm) were transferred to half-strength B5 medium supplemented with different concentrations of auxins (IBA) and combine with IAA. Different concentrations of IBA and IAA yielded different degree of roots. The B5 medium containing half strength 1.0 mg/L IBA produced better roots (92 %) (7.0 ± 0.8cm) and multiple and branching roots (8.8 ± 0.8). Plantlets having well-developed roots (5–6cm) were taken out from the culture vials and washed with running tap water. Plantlets were transferred to plastic cups containing autoclaved sand and garden soil (1:1) and kept undisturbed with plastic cover to maintain the moisture. The plantlets were watered regularly and it was periodically acclimatized to green house (Table 2, Fig 1d).

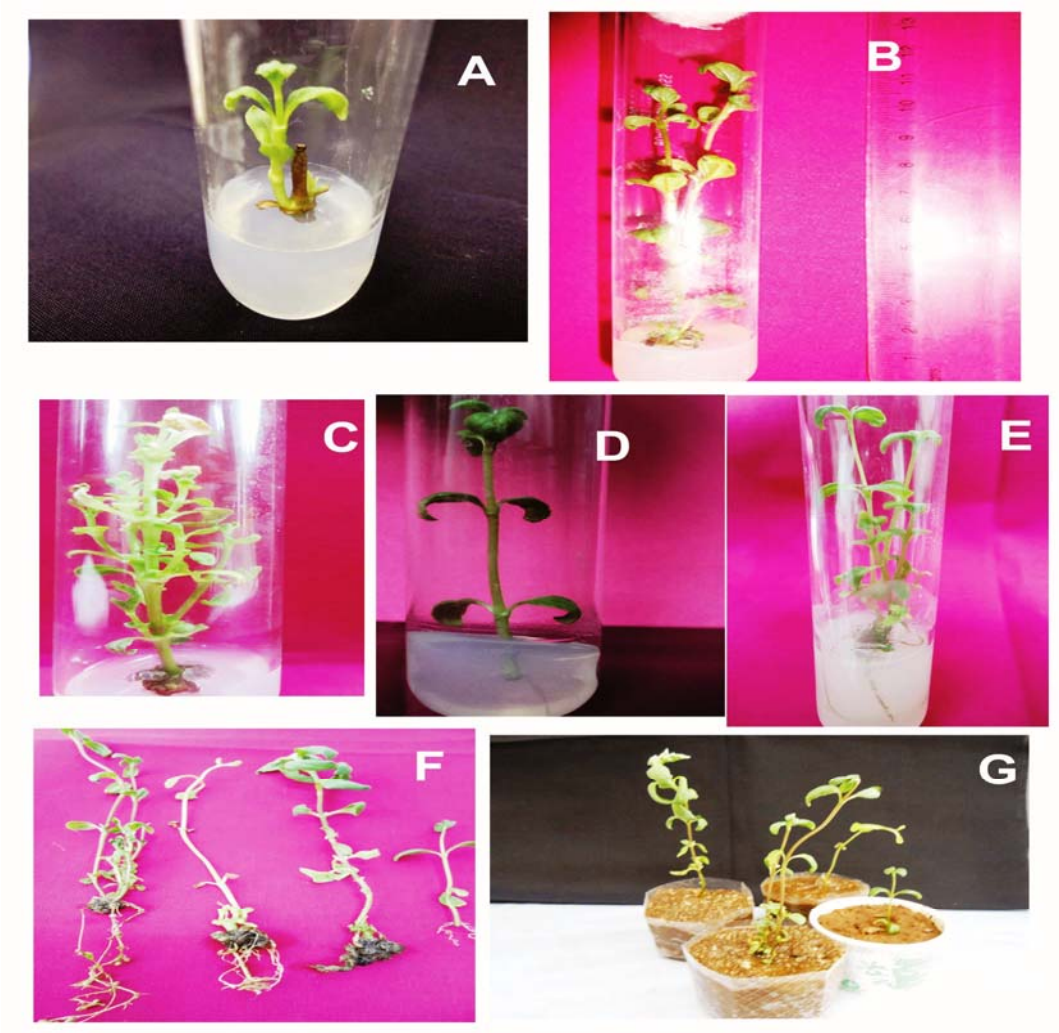

Fig. 1 Micropropagation of *E. alba* using nodal explants. (A) Shoot initiation on B5 medium supplemented with 0.5 BAP +0.5KIN mg/L (B); Shoot elongation on B5 medium fortified with (1.0 BAP + 0.3 KIN + 1.4 GA_3 mg/L) (C); Induction of multiple shoots on supplemented with 1.0 BAP + 0.3 KIN + 1.5 GA_3 mg/L (D and E); Root formation on half strength B5 medium (F); *In vitro* roots developed on B5 medium supplemented with (1.0 IBA mg/L) (G); Acclimatized plant in garden soil.

Table 2. Rooting response of *E. alba* in ½ strength B5 medium

PGR concentrations (mg/L)		% response	Mean no. of roots[a]	Mean root length (cm)[a]
IBA	**IAA**			
-	-	71	3.0 ± 0.8b	2.3 ± 8.0b
0.5	-	85	3.6 ± 1.2b	7.1 ± 1.2a
1.0	0.5	50	2.3 ± 0.4c	2.8 ± 0.8b
1.0	-	92	8.8 ± 0.8a	7.0 ± 0.8a
0.5	-	42	3.0 ± 0.1b	2.3 ± 0.1b

- = No growth regulators added. The values are mean within a column followed by the same letter are not significantly different by Duncan's multiple range test ($p > 0.05$). Values are corresponds to mean (± SE). For each experiment 14 cultures were used.

Discussion

The *in vitro* propagation of *E. alba* was achieved in B5 medium supplemented with different combinations or alone of BA (0–2.0 mg/L), combined with kinetin, spermidine, thiadiazuron and gibberellic acid (1.0 BAP + 0.5 S + 0.5 GA_3 mg/L, 1.0 BAP + 0.5 KIN + 1.5 GA_3 mg/L, 0.5S + 0.5 KIN + 0.5 GA_3 mg/L). The better results of multiple shoots were observed in medium containing 1.0 BAP +0.3 KIN + 1.5 GA_3 mg/L and produced maximum number of multiple shoots (7.0/per explants) (100 %). The other combinations and concentration of BAP, spermidine, thidiazuron found to be moderate rate of shoots. The earlier studies highlights better micropropagation response of *E. alba* was reported by Shashi *et al.,* (2012) observed the maximum number of shoots in MS medium containing 13.2 mg /L BAP and 4.6 mg/L Kin (100 % with an average of 32.6 shoot buds/explant). Similarly, Baskaran *et al.,* (2004) who also reported BAP, KIN and 2iP stimulate the shoot elongation in same plant. Our results were also comparable with earlier studies of shoot regeneration of *E. alba* using various explants (Franca *et al.,* 1995; Borthakur *et al.,* 2000; Gawde and Paratkar, 2004; Dhaka and Kothari, 2005). None of them use multiple combinations of hormones in the development of an efficient propagation of *E. alba*.

The *in vitro* raised multiple shoots were excised and transferred individually to half strength B5 medium 1.0 IBA mg/L and 0.5 IBA mg/L for better root formation. Half strength B5 medium 1.0 mg/ L IBA were found to be more effective than other concentrations. Similarly, Baskaran *et al.,* (2004) also achieved multiple roots (94.3 %) on full strength MS medium containing 9.8mg/L IBA. Likewise, Mohammad *et al.,* (2012) reported that better root formation was achieved in *Rauvolfia serpentina* using half-strength MS medium containing 0.5 mg/L (IAA).

Based on the above observation of present study, we conclude that the efficient protocol was developed for direct regeneration of *E. alba* using nodal explants. The rooted plantlets were successfully acclimatized and established in soil with survival frequency rate of 70% during hardening process (Fig l g).

Conclusion

To conclude, the outcome of results will encourage large-scale micropropagation of this important medicinal herb in a short period of time to increase the biomass and yield of active principles (wedelolactone, dimethylwedelolactone) or other secondary metabolites of pharmaceutical importance occurred in the plant of *E. alba.* This protocol informs a successful and rapid technique that can be developed for the commercial propagation and conservation of this plant and for future phytomedicine production.

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