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An efficient micropropagation protocol for *Eclipta alba* (L.) Hassk.: an endangered, medicinally important plant

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ABSTRACT

An in vitro protocol has been established for large-scale clonal propagation and conservation of Eclipta alba. Single nodal segment (NS) was used as an explant source for in vitro culture establishment, in which Murashige and Skoog (MS) medium fortified with 1.0 mgl^{-1} N_6-benzyladenine (BA) and 0.25 mgl^{-1}á-napthalene acetic acid (NAA) supported rapid axillary bud induction. A very elevated rate of shoot multiplication (22 shoots per axillary bud) was attained in the same growth medium within 21 days. A highest number of 15 well-developed roots per shoot were noted in MS medium containing 1.5 mg l^{-1} indole-3-butyric acid (IBA). The in vitro raised plantlets were well acclimatized in coco pith and soil (3:1) with the survival frequency of 91 per cent. From a NS, 88 acclimatized plants were achieved within 60 days. The sustainable potentiality of multiple shoot culture for over 10 months assured the technology towards conservation of genetic resources.

Keywords **:** *Eclipta alba*, field performance, micropropagation, sustained culture

The scientific world has now started taking a vast interest in the *in vitro* regeneration and conservation of plants particularly those which are medicinally as well as economically significant. This is mainly due to the fact of the incredible increase in the requirement for natural herbal drugs for their negligible side effects. Moreover, the exhaustion of natural resources due to urbanization and industrialization made their continued existence susceptible that has made the researchers opt for an alternative technique, which could bring about large-scale proliferation of plants using a trouble-free, speedy and genetically stable technique (Sharma *et al.*, 2013). Micropropagation offers a practicable system of rapidly multiplying the plant material by means of plant tissue culture techniques.

E. alba is one of such medicinally important herb that posses different significant properties. This is used as dyeing agent for hair blackening and also prevents premature hair-fall. The plant is conventionally used in the treatment of skin disorders, liver diseases and enhances the memory. The plant juice is employed as a tonic and diuretic in hepatic and spleen enlargement. The plant extracts have been reported to enclose quite a lot of phytoconstituents such as wedelolactone, dimethyl-wedelolactone, eclalbatin, oleanolic acid, ursolic acid, stigmasterol-3-Oglucoside, ecliptasaponin, *etc.* (Thakur and Mengi, 2005). The plant involves many other biological activities like hepatoprotective, anti-inflammatory, antiviral, antibacterial, hypotensive, spasmogenic, antioxidant, analgesic, cardioprotective and antianaphylactic properties (Ragavendran *et al.*, 2014).

Due to ever increasing pharmaceutical demand, the wild status of *E. alba* has been drastically affected. Consequently, to accomplish the need for pharmaceutical industries and to pass up utilizing raw materials from the wild, there is an imperative need to establish a competent *in vitro* regeneration method. Though some reports are available on the establishment of *in vitro* plant regeneration through organogenesis (Gawde and Paratkar, 2004; Ray and Bhattacharya, 2008; Prakash *et al.*, 2015), but, there is no published information on the assessment of sustainable culture and exhaustive acclimatization protocol which is yet an important step in the successful protocol development. Hence, the aim of this work was to establish a simple and efficient method for speedy *in vitro* propagation of *E. alba* including long-term sustainability and high-frequency *ex vitro* acclimatization.

MATERIALS AND METHODS

Plant material and disinfection

The nodal explant (NS) of 2–3 cm in size was collected from one year old plant growing in the experimental field garden of Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, India. Explants were sterilized following the protocol of Kundu *et al.* (2017). NSs were washed meticulously under running tap water and washed vigorously with 1 per cent (*v/v*) savlon solution made with a 1 per cent (*v/v*) tween 20, and then rinsed multiple times with distilled water. They were then treated with 1 per cent (*w/v*) bavistin for 20 min and subsequently rinsing five times in sterile distilled water. The explants were subsequently treated for 6 min with 4 per cent (*v/v*) sodium hypochlorite. The surface sterilized explants were aseptically trimmed at the cut ends to reduce the size to about 0.5-1.5 cm prior to placement on culture media.

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Culture medium and culture conditions

Murashige and Skoog's (1962) (MS) basal medium was used all through this study. This was prepared by adding 3 per cent (*w/v*) sucrose (Hi-Media, India) and different concentrations of plant growth regulators (PGRs) to MS basal salts and vitamins. MS medium devoid of PGRs served as control. The pH of the medium was adjusted to 5.7 before adding 0.8 per cent (*w/v*) agar. Molten medium (10 ml) was dispensed into each culture tube, plugged with nonabsorbent cotton plugs and autoclaved at 121ºC for 15 min. The cultures were incubated at 24 ± 2ºC in light with 16 h photoperiod supplied by white fluorescent tube lights (Phillips, India), with a light intensity of 50 µmol.$m^{-2}s^{-1}$ photosynthetic photon flux density (PPFD).

Shoot induction and multiplication

For shoot induction, the nodal segments (NSs) were cultured on MS medium containing 6-benzyladenine (BA) and kinetin (Kin) at different concentrations (0, 0.1, 0.5, 1.0, 1.5 and 2.0 mgl^{-1}), either individually or in combination with a-naphthalene acetic acid (NAA) (0, 0.1, 0.25, 0.5, 0.75 and 1.0 mgl^{-1}). The frequency with which the explants produced shoots, the number of shoots per explant and average shoot length were recorded after 21 days of culture.

Sustained culture of multiple shoots

In vitro generated shoots were sub-cultured for further multiple shoot proliferation in a sustainable approach. The identified best-performing medium for multiple shoot proliferation was used for this sustained culture. Five subcultures were executed at an interval of two months, for a period of 10 months in the same medium. The performance of these subcultures was evaluated on the basis of response frequency, number, and length of multiple shoots. The selected shoots from the sustained culture could be exploited for further root induction and acclimatization procedure.

Root induction

The proliferated shoots from the culture tubes were separated, excised and inoculated for root induction. The root induction medium consisted of $^1/_2$MS supplemented with different concentrations (0, 0.5, 1.0, 1.5 and 2.0 mgl^{-1}) of indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) alone. Data concerning rooting percentage, mean number of roots per shoot and root length were recorded after 21 days of transfer onto the rooting medium.

Acclimatization

Rooted plants were carefully removed from the culture tubes, washed with sterile water to remove agar media and transferred to paper cups filled with autoclaved garden soil and sand (1:1), coco pith and soil (3:1) and saw dust and soil (3:1) (*w/w*). It was supplied regularly with $^1/_4$MS salt solution devoid of sucrose to supply required nutrients and prevent fungal contamination. The plants were covered with transparent polythene bags to prevent desiccation and maintain high humidity. These plants were maintained in the culture room at 24 ± 2ºC and 16 h photoperiod with a light intensity of 50 µmol.$m^{-2}s^{-1}$ PPFD. The relative humidity was gradually reduced and after 4 weeks the plants were removed from the cups and transferred to pots filled with 1:1 mixture of garden soil and farmyard manure (*w/w*). The acclimatized plants were successfully transferred and established in the field. The *ex vitro* survival percentage of plants was evaluated after 8 weeks and any variation in morphological characters was noted.

Experimental design and statistical analysis

All of the experiments were conducted with a minimum of 20 replicates per treatment. The experiments were repeated five times. The cultures were observed periodically and morphological changes were recorded at regular intervals. The results were analyzed statistically using SPSS (Version 11, SPSS Inc. Chicago, USA). The significant differences among the means were analyzed using Duncan's multiple range test (Duncan, 1955) at P = 0.05.

RESULTS AND DISCUSSION

Shoot induction and multiplication

The influence of cytokinin on the rate of *in vitro* shoot multiplication of *E. alba* single NS are summarized in table 1. Explants on MS medium without any cytokinin (control) showed condensed rate of germination (74.6±1.20%) and produced very low number of shoots (6.4±0.45) per explant. But explants cultured on MS medium fortified with cytokinins at various concentrations showed significant disparity in the regeneration frequency and number of shoots. Among the cytokinins analyzed, the best response (96.6±1.10%) was obtained in the presence of 1.0 mgl^{-1} BA which was significantly higher than other concentrations of BA and KN. Also the number and length of shoots (16.5±0.62%; 6.4±1.15 cm) was highest in 1 mgl^{-1} BA supplemented medium after 21 days. The superiority of BA has been well confirmed in a number of earlier studies (Siddique and Anis, 2008; Saha *et al.*, 2016; Thokchom and Maitra, 2017). The naturally occurring ribosides and nucleotides in BA are comparatively more stable than other cytokinins (Letham and Palni, 1983), one of the probable explanations for its enhanced response. It has been assumed that BA is the most commonly preferred cytokinin for micropropagation, because it influences cell division, cell elongation, RNA synthesis, protein synthesis and enzyme activity. But, if concentration of BA is raised above the optimum level (>1 mgl^{-1}), the

Table 1: Effect of PGR on shoot regeneration from nodal segments of *E. alba* in MS medium

BA (mgl^{-1})	KIN (mg l^{-1})	NAA (mg l^{-1})	Regeneration (%)	No. of shoot	Shoot length (cm)
0	0	0	74.6±1.20	6.4±0.45	4.2±0.67
0.1	0	0	79.5±0.75	8.6±0.36	5.6±0.33
0.5	0	0	86.5±0.98	11.2±0.78	6.2±0.78
1.0	0	0	96.6±1.10	16.5±0.62	6.4±1.15
1.5	0	0	88.6±1.12	14.5±1.12	5.6±0.86
2.0	0	0	78.2±0.56	9.6±0.98	5.5±0.56
0	0.1	0	81.5±0.85	6.8±0.56	4.5±0.36
0	0.5	0	83.2±0.6	7.2±0.75	6.8±0.45
0	1.0	0	85.6±0.76	7.8±0.84	7.8±0.95
0	1.5	0	94.6±0.50	10.5±0.36	8.2±0.33
0	2.0	0	73.6±1.20	7.6±0.67	7.6±0.45
1.0	0	0.1	79.6±0.86	11.6±0.33	5.8±0.36
1.0	0	0.25	98.8±0.96	22.5±0.56	7.2±1.15
1.0	0	0.5	92.5±1.30	18.6±0.67	6.8±0.56
1.0	0	0.75	87.6±1.15	13.5±0.88	6.5±0.78
1.0	0	1.0	83.1±0.95	8.6±1.20	6.2±0.74
LSD (0.05)			**12.4**	**2.5**	**1.7**

Table 2: Effect of subsequent subculture on the performance of *in vitro* multiple shoot proliferation

No. of subculture	Response (%)	No of shoots	Shoot length (cm)
Subculture 1	100.00±00.00	22.60±0.33	7.63±0.33
Subculture 2	100.00±00.00	21.20±0.66	7.10±0.25
Subculture 3	100.00±00.00	22.30±0.45	6.86±0.46
Subculture 4	100.00±00.00	22.46±0.36	6.95±0.67
Subculture 5	100.00±00.00	23.10±0.33	7.22±0.36
LSD (0.05)	**NS**	**NS**	**0.56**

Table 3: Effect of auxins on root formation from microshoots of *E. alba* in ½MS medium

IAA (mg l^{-1})	IBA (mg l^{-1})	Frequency of explant inducing root (%)	No. of Root shoot^{-1}	Root length shoot^{-1} (cm)
0	0	54.6±0.56	4.5±0.45	2.5±0.98
0.5	0	66.5±0.67	6.7±0.67	2.8±0.58
1.0	0	76.5±0.88	7.2±0.68	3.6±0.67
1.5	0	88.5±1.20	13.5±0.86	4.2±0.66
2	0	72.6±0.78	11.2±0.33	3.8±0.52
0	0.5	68.5±0.67	6.8±0.36	3.2±0.36
0	1.0	82.6±0.33	11.5±0.75	4.1±0.45
0	1.5	91.5±0.64	15.6±0.86	4.5±0.56
0	2.0	78.6±0.78	10.2±1.15	3.7±0.76
LSD (0.05)		**11.8**	**2.8**	**0.9**

Fig. 1: *In vitro* culture establishment of *E. alba.*

(a) *Ex vitro* plant of *E. alba*; (b) Regeneration of multiple shoots from nodal explants; (c) Multiplication of *in vitro* culture; (d) Jar full culture; (e) Root induction in microshoots; (f) Acclimatized plants of *E. alba* after two months.

percent response and number of shoots were significantly reduced. Nevertheless, BA in combination with NAA (Figs. 1b-c) noticeably improved the percent regeneration (98.8±0.96%), number of shoots (22.5±0.56) and shoot length (7.2±1.15 cm). The elevated concentration of NAA (>0.5 $mg1^{-1}$) resulted in slight callusing at the basal cut end thus dropping the rate of shoot regeneration and number of shoots. The

Table 4: Effect of different organic potting mixture for acclimatization of micropropagated plants

Treatment	Primary hardening		Secondary hardening		
	Survival %	Survival %	No. of roots shoot^{-1}	Root length (cm)	Plant height (cm)
Garden soil and sand (1:1)	75.64±0.45	78.66±0.45	10.56±0.46	4.45±0.28	13.52±0.67
Coco pith and soil (3:1)	82.52±0.36	91.65±0.33	11.56±0.86	5.20±0.46	15.62±0.33
Saw dust and soil (3:1)	73.52±0.56	82.55±0.67	9.92±0.22	4.56±0.36	14.12±0.45
LSD (0.05)	**8.5**	**7.2**	**1.7**	**0.42**	**1.2**

present results corroborate with the earlier reports where low level of auxin assisted cytokinin in promoting enhanced shoot multiplication in numerous species such as *Citrullus colocynthis* (Meena and Patni, 2007), *Cornus alba* (Ilczuk and Jacygrad, 2016), *etc*.

Effect of sub-culturing on shoot regeneration

At the proliferation phase, the consistency in shoot formation over repetitive subcultures was noted. Shoot cultures were maintained by sub-culturing (Fig. 1d) the regenerated shoots to fresh shoot induction medium (MS added with 1 mgl^{-1} BA and 0.25 mgl^{-1} NAA) after every two months interval (Table 2). The shoot regeneration ability was tested up to five subculture passages. After every cycle, the rate of shoot multiplication was absolute (20 out of 20) with a similar number of shoots and shoot length, showing no significant variation as per DMRT. A similar approach to sustainable culture up to several passages was adopted earlier for high-frequency multiplication of other plant species (Gantait *et al.*, 2010; Saha *et al.*, 2014). The constancy in shoot number may be because of the suppression of apical dominance during subculture that stimulated basal meristematic cells to form new shoots.

Root induction

Root initiation from the basal cut end of the shoots was noticed one week after relocating to the root induction medium. The presence of an auxin (IAA or IBA) at a low concentration in $^1/_2$MS medium was found to be effectual for rooting (Fig. 1e), and the best rooting (91.5±0.64%) was achieved in this medium supplemented with 1.5 mgl^{-1} IBA with fairly superior root numbers (15.6±0.86) and root lengths per shoot (4.5±0.56 cm) (Table 3). Rooting frequency amplified gradually over time and reached a maximum after 21 days of culture. Optimal root formation using IBA has also been employed for other plant species, including and *Teucrium stocksianum* (Bouhouche and Ksiksi, 2007), *Talinum triangulare* (Swarna and Ravindhran, 2012) and *Viola pilosa* (Soni and Kaur, 2014).

Acclimatization

In all the various types of planting substrates examined, 91.65±0.33% survival of the plantlets was recorded (Table 4) under *ex vitro* conditions. All the plantlets transferred for hardening, grew well and appeared healthy (Fig. 1f). The plants acclimatized in coco pith and soil showed the best response in terms of average plant height (15.62±0.33 cm), a number of roots (11.56±0.86) and root length (5.20±0.46) plant^{-1}. There was no detectable physiological variation among the potted regenerants and mother plants with respect to growth characteristics.

A competent *in vitro* propagation protocol with well-acclimatization strategy has been developed for *E. alba* in the present study. There is a great reduction in time, energy and production cost of micropropagated plantlets and augmented the survival rate in the field condition. The procedure could be employed for the conservation of this precious medicinal plant and also for raising disease free better-quality plants that could be supplied to various pharmaceutical industries as and when required. Also, the method has great prospect for enhancement of this crop using other biotechnological advances such as genetic transformation and enhancement of secondary metabolites. This can be attained by adapting *in vitro* culture strategies to increase the biomass yield of active ingredients which has gained considerable interest in the pharmaceutical world.

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REFERENCES

Bouhouche, N. and Ksiksi, T. 2007. An efficient *in vitro* plant regeneration system for the medicinal plant *Teucrium stocksianum* Boiss. *Pl. Biol. Rep*., **1**: 179-84.

Duncan, D. B. 1955. Multiple range and multiple F test. *Biometrics,* **11**: 1-42.

Gantait, S., Mandal, N., Bhattacharyya, S. and Das, P.K. 2010. An elite protocol for accelerated quality-cloning in *Gerbera jamesonii* Bolus cv. *Sciella*. *In Vitro Cell. Dev. Biol.-Pl.,* **46**: 537-48.

Gawde, A.J. and Paratkar, G.T. 2004. Micropropagation of *Eclipta alba* Hassk.: an approach to shorten the protocol. *Indian J. Biotech.,* **3**: 128-32.

Ilczuk, A. and Jacygrad, E. 2016. In vitro propagation and assessment of genetic stability of acclimated plantlets of *Cornus alba* L. using RAPD and ISSR markers. *In Vitro Cell. Dev. Biol.-Pl.,* **52** : 379-90.

Kundu, S., Salma, U., Ali, M.N. and Mandal, N. 2017. Factors influencing large-scale micropropagation of *Sphagneticola calendulacea* (L.) Pruski and clonality assessment using RAPD and ISSR markers. *In Vitro Cell. Dev. Biol.-Plant,* **53**: 167-77.

Letham, D.S. and Palni, L.M.S. 1983. The biosynthesis and metabolism of cytokinins. *Annu. Rev. Pl. Physiol*., **34**: 163-97.

Meena, M.C. and Patni, V. 2007. Determination of extractive value of *Citrullus colocynthis* (Linn.) Schrad. (Cucurbitaceae) with different solvents. *Indian J. Env. Sci.,* **11**: 135-6.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Pl*., **15**: 473-95.

Prakash, P., Sharumathy, D., Sunkar, S., Nandagopal, D. and Narendrakumar, G. 2015. Micropropagation of *Eclipta alba* using humic acid as media component. *Pl. Archives,* **15** : 181-5.

Ragavendran, C., Kamalanathan, D. and Natarajan, D. 2014. A Rapid Micropropagation of nodal explants of *Eclipta alba* (L.); A Multipurpose Medicinal Herb. *Res. Biotech.,* **5**: 6-12.

Ray, A. and Bhattacharya, S. 2008. An improved micropropagation of *Eclipta alba* by *in vitro* priming with chlorocholine chloride. *Pl. Cell Tiss. Organ Cult.,* **92**: 315-9.

Saha, S., Adhikari, S., Dey, T. and Ghosh, P. 2016. RAPD and ISSR based evaluation of genetic stability of micropropagated plantlets of *Morus alba* L. variety S-1. *Meta Gene,* **7**: 7-15.

Saha, S., Roy, S., Sengupta, C. and Ghosh, P.D. 2014. Micropropagation and analysis of genetic stability in regenerated plantlets of *Ocimum canum* Sims. *Ind. J. Pl. Physiol.,* **19**: 174-83.

Sharma, A., Bhansali, S., and Kumar, A. 2013. *In vitro* callus induction and shoot regeneration in *Eclipta alba* (l.) Hassk. *Int. J. Life Sci. Pharma Res.,* **3**: 47-51.

Siddique, I. and Anis, M. 2008. An improved plant regeneration system and *ex vitro* acclimatization of *Ocimum basilicum* L. *Acta Physiol. Pl*., **30**: 493-9.

Soni, M and Kaur, R. 2014. Rapid *in vitro* propagation, conservation, and analysis of genetic stability of *Viola pilosa*. *Phys. Mol. Biol. Pl,* **20**: 95-101.

Swarna, J. and Ravindhran, R. 2012. *In vitro* propagation and assessment of genetic integrity of *Talinum triangulare* (Jacq.) Willd: a valuable medicinal herb. *Acta Physiol. Pl.,* **34** : 1987-96.

Thakur, V.D. and Mengi, S.A. 2005. Neuropharmacological profile of *Eclipta alba* (Linn.) Hassk. *J. Ethnopharmacol.,* **102**: 23-31.

Thokchom, R. and Maitra, S. 2017. Micropropagation of *Anthurium andreanum* cv. Jewel from leaf explants. *J. Crop Weed,* **13**: 23-7.