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Synergistic Effect of Plant Growth Regulators on Micropropagation of *Eclipta alba*: A Plant with Diverse Medicinal Properties

Vasudha Datta[1], Lalit Sharma[1], Diwakar Aggarwal[1,*], Anil K Sharma[1,*], Kuldeep Dhama[2]

[1]Department of Biotechnology, Maharishi Markandeshwar Engineering College, Maharishi Markandeshwar (Deemed to be University), Mullana-(Ambala) 133207, Haryana, India.
[2]Division of Pathology, ICAR-Indian Veterinary Research Institute, Izatnagar, UP, India.

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Medium strength

Plant growth regulators

RAPD

Shoot multiplication

Tissue culture

ABSTRACT

Synergism between various plant growth regulators is reported to be a key factor for the development of efficient *in vitro* propagation for any plant. Therefore, the current study examines the synergistic impact of plant growth regulators on *in vitro* propagation of *Eclipta alba,* an important medicinal plant possessing diverse medicinal properties. For the establishment of aseptic cultures, nodal segments were employed as explants on MS medium supplemented with 2.5 µM of 6-benzyle adenine (BA). Varying concentrations of BA and Kinetin (KIN)(0.0-5.0 µM), either alone or in combination with α-naphthalene acetic acid (NAA @ 0.0-5.0 µM) and indole 3-acetic acid (IAA@ 0.0-5.0 µM), were found to be effective for promoting shoot proliferation. Compared to KIN, BA was found to promote shoot proliferation and elongation more effectively. Further, the addition of 0.5µM NAA in the MS medium supplemented with 2.5 µM of BA increased shoot multiplication and elongation frequency from 58 and 17 percent to 65 and 21 percent respectively. The rooting frequency was found to be maximum on 1/2 strength MS medium supplemented with 5.0 µM of indole 3-butyric acid (IBA), which was found to be a superior auxin for inducing roots as compared to the NAA and IAA. With a 75% survival rate*, in vitro* raised plantlets were effectively acclimatized first in a poly house and later under greenhouse conditions. Molecular analysis was carried out using RAPD markers, with results indicating that the micropropagated plants were genetically identical to the mother plant. The developed micropropagation protocol for *E. alba* can be used at the commercial level for the mass multiplication of plants.

* Corresponding author

E-mail: diwakaraggarwal@yahoo.co.in (Diwakar Aggarwal);
anibiotech18@gmail.com (Anil K Sharma)

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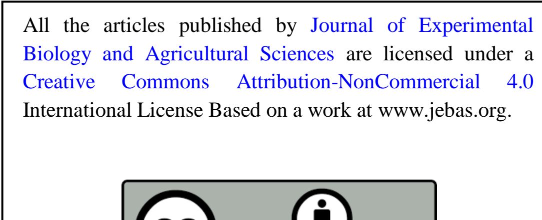

1 Introduction

Eclipta alba (Linn.) Hassk also known as *E. prostrate* belonging to the family Asteraceae, is a well-known plant for its diverse medicinal properties (Timalsina and Devkota 2021). Locally it is known as Bhringraj, Bhringaraja, Kesaraja in Hindi, and false daisy in English. This plant is mainly used to treat jaundice for a long time and incorporated as a major ingredient in several antihepatotoxic drugs in various systems of medicines (John et al. 2014). Active ingredients responsible for anti-hepatoxic activity are wedelolactone and desmethylwedelolactone (Han et al. 2013). It is an important constituent of the dasapuspam, a combination of ten promising herbs used as a general tonic for good health and prosperity (Uthaman and Nair 2017). This plant is considered as an important herbal medicine in Ayurveda, Unani, and Siddha healthcare systems alike. Besides liver disorders, it is also considered useful for the treatment of hepatitis, spleen enlargements, chronic skin diseases, tetanus, and elephantiasis. Its scalp hair growth-promoting activities are also widely documented (Xiong et al. 2021).

It's widespread and has diverse medicinal uses that resulted in an increased rate of plant extraction from natural habitats. Therefore, populations of high-value medicinal plant species are decreasing at an alarming rate (Das and Mishra 2021). Continuous, haphazard, and indiscriminate harvesting of medicinal plants in large quantities from many ecosystems, together with habitat damage, is causing irreparable genetic diversity and planting material loss (Aggarwal et al. 2020). Moreover, *E. alba* is emerging as an economically valuable medicinal crop plant due to its utilization in the preparation of a wide array of medical products. Although the plant can grow under diverse environmental conditions, the growth is more profusely in moist or wet areas usually in the surroundings of rice fields, damp areas around wastelands, on the banks of wastewater channels contaminated with residues of toxic materials including chemicals and metals (Singh et al. 2010). Formulations prepared from such low-quality raw materials often results in poor-quality products and have quality control clearance issue also. Furthermore, traditionally *E. alba* is raised through seeds and stem cuttings in moist and wet places, growing plants in moist and wet conditions makes them vulnerable to attack by pests and various diseases, which leads to major crop loss (Singh et al. 2010). This arising the need for high-quality planting material in bulk to meet ever-increasing industrial demand of the plant with a uniform composition of active ingredients.

To overcome these problems, during the past few years, *in vitro* culture techniques have been extensively developed and applied to conserve high-value medicinal plants (Aggarwal et al. 2022). Moreover selection of cell lines with high secondary metabolites of therapeutic value will be significant in improving the pharmaceutical worth of this plant. A potential method of producing enough material for commercial planting is *in vitro* multiplications (Aggarwal et al. 2019). An exogenous supply of phytohormones is reported to be one of the most important variables regulating any plant's *in vitro* propagation, and convincing data suggest that synergism between auxins and cytokinins is one of the primary determinants driving cell differentiation and organogenesis (Alwakil et al. 2022). The effects of synergism on the establishment of effective *in vitro* propagation protocols for diverse plants have been examined in previous research (Rasool et al. 2013; Gupta et al. 2020). Different hormones have different signaling pathways and the ratio of endogenous to exogenous hormone levels can be linked to this behavior of hormones (Gupta et al. 2020). Hence the development of a simple and reproducible micropropagation protocol is essential to ensure the bulk supply of quality planting material for *E. alba* and the present study was carried out to develop an efficient micropropagation protocol for selected plants of *E. alba* by investigating the role of synergism between auxins and cytokinins.

2 Materials and methods

2.1 Plant material, chemicals, glassware

Selected plants of *E. alba* (young, free of any symptoms of diseases) growing at the herbal garden of M.M. college of pharmacy, university campus. Mullana, Ambala (Haryana, India) were selected for the study (Figure 1A). Nodal explants (Fig. 1B) were collected from selected plants and were brought to the laboratory for further processing and culture establishment. Throughout the study, MS medium (Murashige and Skoog 1962) containing 58 mM sucrose, gelled with 0.7% (w/v) agar (MS medium) was used as a basal medium. Before the medium was autoclaved at 121°C for 20 minutes, its pH was adjusted to 5.8. Aseptic cultures (Fig. 1C) were established using the protocol described by Aggarwal et al. (2012). Briefly, explants were inoculated on MS medium supplemented with 2.5 μM benzyl adenine (BA) alone at 25±1°C under cool white fluorescent lamps (Philips India Ltd, Mumbai) with the light intensity of 42 μmolm^{-2}s^{-1} inside the culture vessel in a 16 h light. Nodal explants were surface sterilized with 0.1 % (w/v) mercuric chloride ($HgCl_2$) for 5-7 minutes and rinsed with sterile water for 3-4 times before inoculation into the culture vessel.

2.2 Effect plant growth regulators on shoot multiplication and elongation

To examine how plant growth regulators (auxins and cytokinins) affect shoot multiplication and shoot elongation, newly regenerated shoots were collected from parent explant and inoculated on MS medium that was differentially supplemented with BA and kinetin (KIN) (0.0-5.0 μM), either alone or in combination with NAA and IAA (0.0-5.0 μM). Different combinations and concentrations of cytokinins and auxins used in the study were given in Tables 1 and 2.

Table 1 The effect of different concentrations of BA and KIN on shoot proliferation and elongation of *E. alba* on MS medium

Plant growth regulators (µM)		*Morphogenic Responses*							
		% Explants responded		Average no. of shoots multiplied		Average no. of shoots elongated		Average shoot (cm)	
BA	KIN	*Mean* ± SD	95% CI	*Mean* ± SD	95% CI	*Mean* ± SD	95% CI	*Mean* ± SD	95% CI
0	0	10.7±0.15^{k}	10.22, 11.10	1.1±0.3^{k}	0.76, 1.43	0±0^{j}	0.25, .25	1.5±0.30^{i}	1.14, 1.92
0.1	0	15.7±0.17^{i}	15.25, 16.14	5.5±0.20^{i}	5.23, 5.89	5.8±0.11^{c}	5.60, 6.12	4.5±0.20^{d}	4.14, 4.92
0.5	0	35.4±0.43^{f}	34.95, 35.84	10.4±0.30^{f}	10.10, 10.76	7.3±0.26^{a}	7.04, 7.55	6.4±0.43^{a}	6.01, 6.78
1.5	0	47.2±0.41^{c}	46.82, 47.70	14.2±0.25^{b}	13.90, 14.56	6.1±0.30^{b}	5.91, 6.42	5.1±0.23^{b}	4.74, 5.52
2.5	0	58.2±0.49^{a}	57.82, 58.70	17.4±0.25^{a}	17.10, 17.76	4.7±0.15^{ef}	4.50, 5.023	4.9±0.15^{c}	4.54, 5.32
5	0	52.4±0.404^{b}	52.02, 52.90	13.1±0.25^{c}	12.83, 13.49	4.3±0.20^{g}	4.11, 4.62	4.2±0.15^{ef}	3.84, 4.62
0	0.1	12.1±0.32^{j}	11.72, 12.60	4.3±0.25^{j}	4.00, 4.66	5.2±0.20^{d}	5.01, 5.52	4.4±0.40^{d}	4.07, 4.85
0	0.5	27.3±0.45^{h}	26.92, 27.80	8.5±0.35^{h}	8.20, 8.86	4.8±0.25^{e}	4.6, 5.12	4.2±0.35^{de}	3.84, 4.62
0	1.5	36.6±0.25^{e}	36.22, 37.10	11.6±0.26^{e}	11.26, 11.93	4.6±0.25^{f}	4.41, 4.92	3.8±0.36^{g}	3.41, 4.18
0	2.5	39.1±0.35^{d}	38.72, 39.60	12.3±0.15^{d}	12.03, 12.69	4.1±0.25^{h}	3.91, 4.42	4.1±0.49^{fg}	3.74, 4.52
0	5	32.2±0.41^{g}	31.82, 32.70	10.3±0.37^{g}	10.002, 10.66	3.9±0.17^{i}	3.64, 4.15	4.0±0.26^{h}	3.61, 4.38

Means± SD with the same letter within a column are not significantly different according to Duncan's multiple range test ($P< 0.05$), 95% CI- represents confidence intervals i.e. minimum and maximum values of mean at 95 % confidence level.

2.3 *In vitro* rooting of microshoots and plantlet acclimatization

For induction of *in vitro* roots, elongated shoots were cultured on full strength, 1/2 strength, and 1/4 strength of MS medium supplemented with varying concentrations of NAA, IAA, or IBA, ranging from (0–5 µM) (table 3). After successful root induction, plantlets were acclimatized in a poly house under regulated conditions of temperature (25–28°C) and humidity (90–95 %) using a mixture of soil and agro peat (3:1 ratio) contained in polythene bags. A minimum humidity of 90% was maintained throughout the early periods and then gradually decreased to 40%. Later, plants were moved to conditions with a 50% light reduction (green net).

2.4 Testing of clonal fidelity

After two weeks of hardening, genomic DNA was isolated from the leaves of randomly chosen plants using Doyle and Doyle's (1990) CTAB technique. By loading an aliquot of samples, the quality of the DNA was examined on a 0.7% (w/v) agarose gel (Figure 2A), and the concentration was calculated using a standard spectrophotometer method (Sambrook and Russel 2001). For this, 10 RAPD decamer primers (OPD1-OPD10; Operon Technologies, Alameda, CA) were used for the PCR amplification and 20 ng of genomic DNA, 1.0 U of Taq DNA polymerase (Larova, Teltow, Germany), 100 µm of dNTP mixture, 2.0 µl of reaction buffer (10X), and 10 nmol of primer made up the reaction mixture was used. Mill-Q water was then added to bring the total volume to 20 µl. Amplifications were carried out using the Gene Amp 2700 thermal cycler (Applied Biosystem, San Francisco, USA). Initial denaturation at 94°C for 5 min was followed by 41 cycles of 94 °C for 1 min, 45 °C for 45 sec, and 72 °C for 1.5 min, with a final extinction at 72 °C for 5 min. Following ethidium bromide staining, the amplified products were separated on a 1.5% (w/v) agarose gel and examined under a UV transilluminator (VilberLoumart, France).

2.5 Statistical evaluation

All studies were carried out using three replicates, two explants in each culture vessel, and were then repeated three times. GraphPad Prism 4 software was used for the analysis of variance in the data and to compare the means ± standard deviation using the Duncan Multiple Range Test (DMRT). Confidence intervals analysis, predicting minimum and maximum fluctuation in mean value is also carried out.

3 Results

3.1 Effect of Plant growth regulators on shoot multiplication and shoot elongation

Aseptic cultures were established utilizing mercuric chloride as a surface sterilizing agent as it was found satisfactory for the establishment of aseptic cultures. In the first experiment, the effects of various concentrations of BA and KIN cytokinins (ranging from 0.0 to 5.0 µM) on the proliferation and elongation of *E. alba* microshoots on MS media were examined. Among the selected two cytokinins, BA was found better as compared with

KIN (Table 1). The highest proportion of responded explants (58.2) was reported in MS medium supplemented with 2.5 µM of BA (Table1). Similarly, the maximum number of shoots also proliferated on the same medium (17.4 shoots per culture vessel). Whereas the highest amount of elongated shoots (7.3 per culture vessel) and highest shoot length (6.4 cm) were found on MS medium supplemented with 0.5 µM of BA (Table 1).

The second set of experiments looked at how varying concentrations of auxins i.e. NAA and IAA (0.0-5.0 µM) affected the growth and elongation of micro-shoots of *E. alba* on MS media supplemented with 2.5 µM of BA (Table 2). The addition of auxins increased the shoot multiplication and shoot elongation potential of the *E. alba* microshoots (Figure 1D, E). The percent response of explants increased to 65.3% with the addition of 0.5 µM NAA in

Figure 1 Micropropagation of *Eclipta alba*, A. Selected plants of *E. alba* growing at MMDU Mullana campus, B. Nodal segments of *E. alba* used for initiation of aseptic cultures, C. Establishment of aseptic cultures on MS medium supplemented with 2.5 µM BA, D. Shoot multiplication on MS medium supplemented with 0.5µM NAA in combination with 2.5 µM BA, E. Shoot elongation on MS medium supplemented with 0.1µM NAA in combination with 2.5 µM BA, F. In *vitro* rooting of micro shoots on ½ MS in combination with 5.0 µM IBA, f. Acclimatized *E. alba* plants

combination with 2.5 µM BA. A significant increase was also observed in the shoot multiplication frequency of microshoots of *E. alba,* whereas not much variation was observed in shoot elongation frequency and number of elongated shoots (Table 2).

Table 2 The effect of different concentrations of NAA and IAA on proliferation and elongation of *E. alba* on MS medium supplemented with 2.5 µM BA.

Plant growth regulators (µM)		*Morphogenic Responses*							
		% Explants responded		Average no. of shoots multiplied		Average no. of shoots elongated		Average shoot (cm)	
NAA	IAA	*Mean* ± SD	95% CI	*Mean* ± SD	95% CI	*Mean* ± SD	95% CI	*Mean* ± SD	95% CI
0	0	11.1±0.25[k]	10.84, 11.42	1.5±0.15[k]	1.27, 1.78	0±0[h]	0.21, 0.21	1.9±0.15[i]	1.70, 2.15
0.1	0	28.3±0.25[i]	28.07, 28.65	17.6±0.25[i]	17.38, 17.88	7.8±0.20[a]	7.65, 8.07	6.76±0.15[a]	6.54, 6.99
0.5	0	65.3±0.25[a]	65.04, 65.6	21.2±0.28[f]	21.01, 21.52	6.8±0.05[b]	6.65, 7.07	6.2±0.28c	6.00, 6.45
1.5	0	47.33±0.11[b]	47.04, 47.62	18.2±0.15[b]	18.01, 18.52	4.8±0.11[e]	4.62, 5.04	5.4±0.15[d]	5.20, 5.65
2.5	0	44.23±0.15[d]	43.94, 44.52	14.4±0.26a	14.14, 14.65	4.1±0.26[f]	3.88, 4.31	5.1±0.23[de]	4.94, 5.39
5	0	34.7±0.30[g]	34.47, 35.05	13.7±0.15[c]	13.47, 13.98	3.9±0.15[f]	3.72, 4.14	4.4±0.20[g]	4.24, 4.69
0	0.1	26.4±0.28[j]	26.17, 26.75	15.2±0.15[j]	15.01, 15.52	6.3±0.1[c]	6.08, 6.51	6.4±0.20[b]	6.20, 6.65
0	0.5	45.5±0.20[c]	45.24, 45.82	16.4±0.21[h]	16.14, 16.65	5.6±0.22[d]	5.38, 5.81	5.1±0.15[e]	4.94, 5.39
0	1.5	41.4±0.20[e]	41.14, 41.72	15.8±0.26[e]	15.54, 16.05	4.6±0.20[e]	4.45, 4.87	4.8±0.1[f]	4.57, 5.02
0	2.5	36.4±0.35[f]	36.14, 36.72	14.6±0.17[d]	14.34, 14.85	4.3±0.27[f]	4.13, 4.56	4.5±0.1[f]	4.34, 4.79
0	5	32.1±0.15 [h]	31.87, 32.45	12.7±0.20[g]	12.51, 13.02	3.8±0.15[g]	3.65, 4.07	4.1±0.2[h]	3.87, 4.32

Means± SD with the same letter within a column are not significantly different according to Duncan's multiple range test ($P < 0.05$), 95% CI- represents confidence intervals i.e. minimum and maximum values of mean at 95 % confidence level.

Table 3 Effect of different auxins and MS media strength on rooting of *E. Alba* micro-shoots

MS medium strength	Auxin (µM)	Percentage of microshoots showing rooting		Average no. of roots per shoot		Average root length (cm)	
		Mean ± SD	95% CI	Mean ± SD	95% CI	Mean ± SD	95% CI
Full strength	0	0±0[i]	0.23,0.23	0±0[i]	0.17, 0.17	0±0[f]	0.21, 0.21
Full strength	1.0 NAA	55.7±0.15[k]	55.53, 55.99	2.4±0.2[de]	2.22, 2.57	1.1±0.15[de]	0.95,1.38
Full strength	2.5 NAA	61.3±0.15[h]	61.10, 61.56	2.1±0.0 5[f]	1.96, 2.30	1.4±0.15[c]	1.21,1.64
Full strength	5.0 NAA	63.4±0.35[f]	63.20,63.66	2.1±0.05[ef]	1.99, 2.33	1.3±0.20[cd]	1.11,1.54
Full strength	1.0 IBA	64.5±0.05[e]	64.33, 64.79	2.5±0.15[cd]	2.39, 2.73	1.16±0.25[c]	0.951,1.38
Full strength	2.5 IBA	67.5±0.3[d]	67.26,67.73	2.6±0.1[c]	2.42, 2.77	1.2±0.15[cd]	1.05,1.48
Full strength	5.0 IBA	71.3±0.2[c]	71.06,71.53	3.2±0.15[b]	3.06, 3.40	2.4±0.05[b]	2.21,2.64
Full strength	1.0 IAA	58.4±0.11[j]	58.23, 58.69	2.1±0.25[f]	1.99, 2.33	1.13±0.25[e]	0.91,1.34
Full strength	2.5 IAA	60.4±0.20[l]	60.23,60.69	1.7±0.15[g]	1.59, 1.93	1.3±0.20[cd]	1.11,1.54
Full strength	5.0 IAA	62.7±0.11[g]	62.53, 62.99	1.5±0.15[h]	1.36, 1.70	1.1±0.11[de]	0.95,1.38
½ MS	5.0 IBA	78.5±0.11[a]	78.33, 78.79	3.5±0.15[a]	3.36, 3.70	2.9±0.15[a]	2.71,3.14
¼ MS	5.0 IBA	72.3±0.25[b]	72.13,72.59	3.3±0.1[b]	3.12, 3.47	2.4±0.26[b]	2.18,2.61

Means± SD with the same letter within a column are not significantly different according to Duncan's multiple range test ($P < 0.05$), 95% CI- represents confidence intervals i.e. minimum and maximum values of mean at 95 % confidence level.

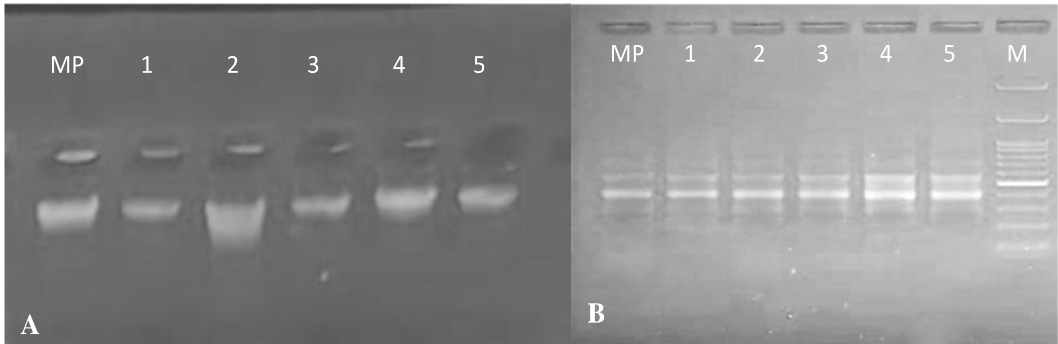

Figure 2, DNA quality check and RAPD profiles of micropropagated plantlets and mother plant of *E. alba*plants. A.Lane MP: Genomic DNA from Mother plant, Lane 1-5: Genomic DNA from micropropagated plant, B. RAPD profile with primer no 3, Lane MP: Mother Plant; Lane 1-5: micropropagated plants; Lane M: 1 kb molecular weight markers

3.2 Influence of auxins and medium strength on rooting of microshoots

On the rooting of microshoots, the impact of various auxins such as NAA, IBA, and IAA as well as medium strength (MS, 1/2 MS, and 1/4 MS) was also investigated. Among the tested auxins, IBA was found superior as compared to the NAA and IAA in the induction of *E. alba* microshoots root (Table 3). Maximum rooting frequency of microshoots i.e.78.5 % was observed on ½ strength MS medium containing 5.0µM IBA (Fig. 1F). Further, the maximum number of roots per shoot (3.5) and maximum root length (2.90 cm) was also observed on same medium (Table 3).

3.3 Acclimatization of plantlets

First, rooted *E. alba* microshoots were successfully acclimatized in polyhouse conditions with a survival rate of 80 %. After 15 days of exposure in the polyhouse, the plants were moved to a net house with a 50% light reduction, here the microshoots have a survival percentage of 75 % (Figure 1 G). After successful acclimatization plants were cultivated under field conditions. A total of 75 rooted microshoots from ½ strength MS medium containing 5.0 µM IBA were used in the study (3 experiments with 25 rooted micro- shoots). A potting mix containing soil, vermicompost, and sand in a ratio of 2:1:1 was used for the acclimatization of plantlets.

3.4 Assessment of clonal fidelity using RAPD analysis

Among the used ten RAPD primers, six were produced scoreable bands with sizes ranging from 250 to 2000 bp. With an average of 4 bands per RAPD primer, the number of bands for each primer ranged from a minimum of 3 to a maximum of 6. Bands had a monomorphic nature. The clonal nature of these shoots was demonstrated by the similarity between the RAPD banding profiles in plants generated from micropropagation and the mother plant (Figure 2B).

4 Discussion

The major problem faced by industries engaged in the production of plant-based medicinal formulations is the uninterrupted and uniform supply of quality planting material throughout the year. The problem can be overcome with the development of rapid, efficient, simple, and most importantly reproducible micropropagation protocol for the selected plants (Moraes et al. 2021). A similar issue arose with *E. alba* as well, while there are reports on its *in vitro* multiplication, they may be genotype-dependent and their techniques lack consistency (Dhaka and Kothari 2005; Singh et al. 2010; Ragavendra et al. 2014; Yesmin et al. 2015). Thus arises the need for the development of a genotype-specific micropropagation protocol for *E. alba* to meet ever increasing demand of the industry.

One of the foremost difficulties with the development of micropropagation protocol for any plant is the establishment of aseptic cultures from mature explants. Due to the problem of infestation by various microbes and enhanced frequency of contamination in explants selected from plants growing under field conditions as compared with explants chosen from plants growing under protected areas like a nursery, it is difficult to establish aseptic cultures (Bhadaneand Patil 2016). Field-grown plants are found to be more infected with bacteria and fungi both exogenously and endogenously. So it becomes important to properly disinfect explants for the establishment of aseptic cultures. In the present study mercuric chloride ($HgCl_2$) at the concentration of 0.1% (w/v) was used as a surface sterilizing agent and was seen to be satisfactory for the establishment of aseptic cultures. Mercuric chloride was the choice of surface disinfecting agent for the establishment of aseptic cultures in various micropropagation studies (Kajla et al. 2018, Samala et al. 2022).

In the present investigation both BA and KIN increased the frequency of explant regeneration as well as the number of shoots that proliferated and elongated, in comparison to the MS medium

without any PGR (Table 1). These results are in agreement with earlier studies where BA was found to be beneficial for *in vitro* shoot multiplication of various plant species including *E. alba* (Bhaskaran and Jayabalan 2005; Choudhary et al. 2021; Iiyama and Cardoso 2021).

Auxins are a group of naturally occurring and synthetically produced plant hormones. They assume a significant role in plant development and are known as master growth regulators in terms of various plant physiological activities like morphogenesis including cell division, differentiation, and elongation (Bishopp et al. 2011). Furthermore, other plant hormones, such as cytokinins, abscisic acid, gibberellins, and polyamines cooperate synergistically or antagonistically with auxin to trigger cascades of events prompting morphogenesis and other development processes in the plant (Hu et al. 2019). Therefore in the present study, the effect of NAA and IAA was investigated in combination with BA on various plant development processes (Table 2). The addition of NAA led to enhanced explant regeneration frequency including enhancement in the number of shoots proliferated and elongated per explant in comparison to MS medium with BA only (Table 2). NAA is reported to play an important role in cytokinin metabolism and stability (Saini et al. 2013) which may help in obtaining higher shoot regeneration frequency in explants including the number of shoots proliferated and elongated.

The success of any *in vitro* propagation protocol depends upon the induction of efficient *in vitro* rooting. Keeping in mind the importance of *in vitro* rooting, ample work has been carried out to develop efficient rooting protocols for various plants, and for this, auxins are the first choice as plant growth regulators (Aggarwal et al. 2020; Oanh et al. 2022). Therefore in the present study, also the effect of auxins i.e. NAA, IBA, and IAA (0-5.0 μM) was examined for efficient in vitro rooting of *E. alba* microshoots (Table 3). Furthermore, it has been shown that plants grown *in vitro* conditions occasionally experience nutritional stress and exhibit restricted root system growth to maximize nutrient absorption. Therefore, it's important to maximize nutrient concentrations since they frequently affect root growth by activating local and systemic signaling pathways (Elmaataoui et al. 2020). Therefore the impact of medium strength (full strength MS, ½ MS, and ¼ MS) on the rooting efficiency of *E. alba* microshoots was also investigated (Table 3). Lowering of basal medium composition in combination with IBA resulted in enhanced rooting of microshoots (Table 3). The rooted plantlets were finally acclimatized under polyhouse and greenhouse conditions and showed healthy growth and survival upon transfer to the potting mixture.

Clonal fidelity is one of the most important aspects of the micropropagation industry. Molecular markers (like RAPD) are found to be suitable for generating DNA profiles and have proved to be an effective tool in assessing the genetic stability of plants propagated through *in vitro* methods (Williams et al. 1990). The use of RAPD and ISSR markers to identify genetic similarities or differences in micropropagated material from distinct plants has proved successful (Aggarwal et al 2012, Singh et al. 2012). The remarkable degree of genetic homogeneity displayed by the micropropagated plants in the current study (Figure 2), which attributable to the genome's resistance to aseptic manipulations and cultural stresses throughout the various stages of micropropagation.

Conclusion

Conclusively, the present study presents a simple, efficient, and reproducible micropropagation protocol for selected plants of *E. alba.* The interaction among auxins and cytokinins is found to be critical for the optimization of micropropagation protocol. Lowering of basal medium composition was found suitable for efficient rooting of microshoots. The developed protocol can be successfully used for large-scale production of *E. alba* plants for a uniform and stable supply of quality plantlets for the pharmaceutical industry.

Conflict of Interest

There are no conflicts of interest among the authors.

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