Research Article

An Efficient Protocol for Shoot Regeneration in *Eclipta alba*: A Plant with Multiple Medicinal Properties

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ABSTRACT

Aims/Background: *Eclipta alba* (Linn.) Hassk. (Asteraceae) frequently referred to as bhringoraj in Indian Subcontinent and false daisy in other parts of the world is regarded as an herb of high medicinal value by practitioners of Ayurveda, Unani and Siddha medicine and plays a protective role for several liver ailments. The present study was dedicated on the establishment of a shoot regeneration protocol for *Eclipta alba*. **Materials and Methods:** Aseptic cultures were established through conventional tissue culture techniques, utilizing 0.1% mercuric chloride as a disinfectant on MS medium supplemented with 2.5 µM BA. Effect of PGRs, including cytokines and auxins, were studied on shoot regeneration potential of *E. alba*. Further effect of leaf maturity and different antibiotics were also evaluated on shoot regeneration efficacy of *E. alba*. Finally, regenerated plants were assessed for their true to type nature using RAPD and ISSR markers. **Results:** Shoot regeneration took place using Murashige and Skoog (MS) media supplemented with 15.0 mM of NAA and 1.0 mM of BA using leaves as explants. Furthermore, antibiotics and leaf position been shown to alter the shoot regeneration capability of leaf explants. Regenerated plantlets were found true to type using RAPD and ISSR markers. **Conclusion:** An efficient shoot regeneration protocol was developed for *E. alba*, which can be further used for trait specific genetic transformations of *E. alba*.

Keywords: 6S rRNA, Antibiotics, Auxins, ISSR, Leaf Position, RAPD.

INTRODUCTION

From the beginning of Earth's history, plants have supplied a significant amount of the essential requirements for all forms of life, like shelter, oxygen, food and medicine.[1] Throughout the course of human history, a wealth of knowledge has been collected to facilitate the uses of plants based on their ability to satisfy our most essential needs. The historical accounts of literature, including oral traditions and written records, provide evidence of the effectiveness of various plants for medicinal purposes.[2] According to Rates,[3] the predominant mode of therapy in conventional indigenous medicine involved utilization of medicinal flora preparations for treatment of several ailments. Despite advancements in synthetic drug, traditional medicine and synthetic medications continue to serve as significant sources of drugs and therapies.[4] Consequently, these medicinal plants are currently being utilized in a more extensive and improved manner than in previous times. Therefore researchers have become more enthusiastic in screening various plant species and revisiting old cures in search of new and improved pharmaceuticals.[5] Moreover, in terms of sales most important drugs have plant origins.[6]

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As a consequence, more plants are being taken from their native environments, resulting in declines in their population sizes due to which numerous species of high-value medicinal plants have gone from being threatened to critically endangered.[7]

Eclipta alba (Linn.) Hassk. belonging to the Asteraceae family commonly referred to as false daisy and/or bhringoraj/bhringraj. It is regarded as a plant of high medicinal value by practitioners of Ayurveda, Unani and Siddha medicine. In the Ayurvedic medical tradition, it plays a protective role for the liver.[8] In addition to its hepatoprotective, hair-strengthening, anti-aging and immunomodulator properties, it is also regarded as a nerve tonic, as mentioned in ancient literature.[9] Further, it has been shown to reduce inflammation and acts a bronchodilator.[10] As, *E. alba* is used in the production of many different medicines leading to destroying its natural habitats, the continuous, random and indiscriminate harvesting of huge amounts of plants across various ecosystems is leading to a catastrophic loss of genetic variety and planting material.[11] Because of the therapeutic importance of its contents, researchers have deemed a biochemical characterization of *E. alba* to be significant. Thus arises need to conserve and genetically transform this important plant.

Now-a-days, substantial advancement has been made in practical application of *in vitro* culture techniques to address the aforementioned challenges in safeguarding of valuable medicinal plants.[12] Further various *in vitro* propagation techniques have been proposed as a viable approach for generating sufficient material for commercial cultivation of valuable medicinal plants.[13] Therefore the present was taken up to formulate plant specific shoot regeneration method for *E. alba*. Further genetic uniformity analysis of regenerated plants was done by means of RAPD and ISSR markers.

MATERIALS AND METHODS

Plant material, chemicals, glassware and culture establishment

The plants utilised in this study were grown in M. M. (Deemed to be) University's herbal garden in Mullana, Ambala, Haryana, India (Latitude-30.275285 and Longitude -77.04758). HiMedia Laboratories (Mumbai, India) supplied all frequently used chemicals, while Sigma Aldrich Chemical Company (USA) supplied growth regulators, antibiotics and speciality chemicals. Unless otherwise specified, all experiments have been carried out in 250 ml glass culture vessels (Life Technologies (India) Pvt Ltd, New Delhi) containing 50 mL of medium. Before autoclaving at 121°C for 20 min, the pH of the medium was adjusted to 5.8. Clean contamination free cultures were established under *in vitro* conditions as previously described by Datta *et al.*[14] and grown at 25±1°C under a standard 16 hr light/8 hr dark cycle (cool white fluorescent lamps; 40 μm/m²/s). Explants for regeneration and transformation investigations were *in vitro* leaves from elongated microshoots grown on MS agar media[15] supplemented with 2.5 μM Benzyladenine (BA) and 0.1 μM α-Naphthaleneacetic Acid (NAA).

Shoot regeneration

Young *in vitro* leaves measuring between 1.5 and 2.0 centimetres in length were cut from microshoots were subjected to treatment with varying amounts of NAA, IAA and 2,4-D (0-25.0 μM), with their adaxial surface being inoculated. Within another experiment, impact of different quantities of BA, Kin and TDZ (0-5 μM) along with 15.0 μM of NAA was tested for shoot regeneration.

Influence of leaf maturity on shoot regeneration potential of *E. alba*

Shoot regeneration potential was assessed for diverse degrees of maturation shown by leaf. The experimental procedure involved the excision of the third to seventh leaves from the apex of the plant. Subsequently, leaves at different developmental stages were inoculated on MS agar medium with 15.0 μM of NAA and 1.0 μM of BA.

Influence of different antibiotics and there concentration on shoot regeneration potential of *E. alba*

Furthermore, an examination was conducted on the impact of various antibiotics, such as cefotaxime, carbenicillin and cephalexin, across a spectrum of concentrations from 0 to 500 mg/L, on the development of shoot regeneration originating from leaf segments. Following autoclaving, the MS medium that had been augmented with 15.0 μM of NAA and 1.0 μM of BA was supplemented with varying quantities of antibiotics (0 to 500 mg/L) that had undergone filter sterilization.

Assessment of genetic fidelity

To conduct the genetic analysis of the regenerated plants, RAPD and ISSR was carried out. Genomic DNA was extracted from *in vitro* raised plants and the maternal (control) plant using CTAB procedure as reported by.[16] RAPD and ISSR primers based amplification was performed in a reaction volume of 20 μL (25 ng of extracted DNA, 2.0 μL reaction buffer (10X), 1.0 U Taq DNA polymerase, 100 μM dNTPs mixture, 10 nM primer and autoclaved double distilled water to make

up the volume 20 µL). 20 arbitrary decamer primers for RAPD and 16-20 nucleotide long ISSR primers (20 in number) were used in the study. Both RAPD and ISSR primers were synthesized from Genni, Bangalore, India. The amplification reaction consisted of an initial denaturation of 94ºC for 5 min was followed by 41 cycles of 94ºC for 1 min, 36ºC for 45 sec (55ºC in the case of ISSR) and 72ºC for 1.5 min, with a final extinction of 72ºC for 5 min using Gene Amp 2700 thermal cycler (Applied Biosystems, CA). The amplified products were resolved by electrophoresis on a 1.0% agarose gel in tris-acetate EDTA buffer, stained with ethidium bromide and visualized under UV light. The number of bands was recorded and examined using a Gel Doc System Gel Doc Mega; Biosystematica, USA).

Statistical analysis

The trials were conducted three times, each with triplicate repetitions. Statistical analysis involved performing an analysis of variance on the data, followed by mean comparisons through the Duncan's multiple range test (where significance was set at $p<0.05$). All these computations were carried out using GraphPad Prism 4 software by GraphPad located in San Diego, CA.

RESULTS

Fresh *in vitro* leaves measuring approximately 0.5 and 1.0 centimetres in length were used to induce shoot regeneration. These leaves were subjected to treatment with varying conc. of NAA, IAA and 2,4-D (0.0-25.0 µM). Out of three tested auxins for shoot regeneration NAA was found to be best as compared with IAA and 2,4-D. Maximum shoot regeneration frequency of 42.6% was seen with 15 µM of NAA. Mean shoots per explant i.e., 12.3% was also seen and highest callus formation frequency of 41.2% was observed with 15.0 µM of 2,4-D (Table 1). NAA was suitable for shoot regeneration whereas 2,4-D was found to be better for callus formation. To examine action of cytokinin's and auxins another independent experiment was conducted where the effect of various cytokinin's namely BA, Kin and TDZ (0.0 to 5.0 µM) was studied on MS media enriched with 15.0 µM of NAA (Table 2). Shoot regeneration frequency was increased to 47.3% from 42.6% with 1.0 µM of BA and 15.0µM of NAA.TDZ and KIN were also found to be effective for increase in shoot regeneration potential, but not as much as effective as BA. Mean number of shoots also increased from 12.3% to 14.5% in MS media supplemented with 1.0 µM of BA combination with 15.0 µM of NAA. Overall, it was observed that addition of cytokinin's leads to enhancement in shoot regeneration frequency as compared with MS media supplemented with only auxins.

The shoot regeneration response was also assessed using novel methodologies like diverse degrees of maturation shown by leaf. The experimental procedure involved the excision of the third to seventh leaves from the apex of the plant. Leaves of different maturation index i.e., third to seventh from top to bottom were inoculated on MS medium supplemented with 15.0 µM of NAA and 1.0 µM of BA, leaf at fifth position showed the maximum result (50.2%), followed by leaves at sixth and seventh position respectively. Maximum shoots per explant was also seen on 5th position leaf (15.2%) (Table 3). Effect of antibiotics like carbenicillin, cefotaxime and cephalexin (0 to 500 mg/L), which will be subsequently used during the genetic transformation experiment for killing of residual *Agrobacterium tumefaciens* cells was assessed on shoot regeneration potential of leaf explants of *E. alba*. Addition of cefotaxime at 300 mg/L led to enhancement of shoot regeneration potential of leaf segments to 53.4%. The mean number of shoot/explants also increased to 15.7. Out of the three tested antibiotics as mentioned above, cefotaxime was found to be promontory, carbenicillin was found to be neutral and cephalexin was found to be inhibitory for leaf segments of *E. alba* (Table 4).

Table 1: Effect of different auxins at variable concentration on shoot regeneration frequency in *E. alba*.

Auxin (mM)	Frequency of leaves showing shoot regeneration (%)	Frequency of leaves showing callus formation (%)	Mean no. of shoots/ explants
NAA 0.0	00.0±00.0^{o}	00.0±0.00^{j}	0.00±0.00^{i}
2.5	13.4±0.3^{k}	00.0±0.00^{j}	4.3±0.4^{h}
5.0	22.7±0.2^{i}	8.9±0.55^{i}	7.1±0.36^{e}
10.0	36.4±0.40^{c}	13.4±0.35^{g}	9.8±0.32^{b}
15.0	42.6±0.25^{a}	18.7±0.4^{e}	12.3±0.66^{a}
25.0	38.1±0.40^{b}	15.5±0.30^{f}	10.2±0.30^{b}
IAA 2.5	9.6±0.26^{n}	00.0±0.00^{j}	3.8±0.30^{h}
5.0	11.4± 0.45^{m}	00.0±0.00^{j}	5.6±0.36^{g}
10.0	25.3±0.32^{f}	9.7±0.20^{i}	6.8±0.30^{f}
15.0	30.1±0.40^{d}	13.5± 0.35^{g}	9.6±0.20^{c}
25.0	24.6±0.40^{gh}	11.5± 0.35^{h}	8.7±0.36^{d}
2,4-D 2.5	12.6±0.3^{l}	15.5±0.35^{f}	5.1±0.35^{g}
5.0	20.3±0.5^{j}	22.5±0.35^{d}	6.4±0.35^{f}
10.0	23.6±0.85^{h}	32.5±0.4^{c}	8.3±0.30^{d}
15.0	28.7±0.30^{e}	41.2±0.55^{a}	0.0±0.00^{j}
20.0	24.6±0.36^{g}	33.4±0.55^{b}	0.0±0.00^{i}

Statistically validated with Duncan's multiple range test ($p<0.05$), Values sharing a common letter within the column are not significant at $p<0.05$

Table 2: Effect of cytokinins on shoot regeneration frequency of *E. alba* on MS media supplemented with 15 μM NAA.

PGRs (μM)		Frequency of leaves showing shoot regeneration (%)	Mean no. of shoots/explants
0.0	0.0	00.0±0.00^{i}	00.0 hr±0.00^{f}
NAA 15.0	BA 0.5	42.5±0.4^{d}	12.9±0.30^{c}
	1.0	47.3±0.20^{a}	14.5±0.35^{a}
	2.5	43.4±0.32^{c}	12.3±0.25^{c}
	5.0	40.2±0.25^{f}	12.7±0.26^{c}
	Kin 0.5	38.7±0.3^{h}	10.9±0.30^{e}
	1.0	39.4±0.26^{g}	11.2±0.30^{d}
	2.5	40.3±0.25^{f}	11.8±0.26^{d}
	5.0	38.9±0.32^{h}	10.9±0.30^{e}
	TDZ 0.5	42.4±0.3^{d}	12.3±0.36^{c}
	1.0	45.6±0.28^{b}	13.7±0.25^{b}
	2.5	42.3±0.65^{d}	13.1±0.35^{b}
	5.0	41.5±0.4^{e}	13.5±0.3^{b}

Statistically validated with Duncan's multiple range test (p<0.05), Values sharing a common letter within the column are not significant at p<0.05

Table 3: Effect of leaf maturity on shoot regeneration frequency of *E. alba* on MS media supplemented with 15 .0 μM of NAA and 1.0 μM BA.

Leaf number (from top to bottom)	Frequency of leaves showing shoot regeneration (%)	Mean no. of shoots/explants
Third	40.3±0.25^{d}	13.7±0.25^{c}
Fourth	43.1±0.35^{c}	14.1±0.2^{b}
Fifth	50.2±0.35^{a}	15.2±0.4^{a}
Sixth	44.6±0.26^{b}	14.6±0.36^{b}
Seventh	43.7±0.25^{c}	14.7±0.20^{b}

Statistically validated with Duncan's multiple range test (p<0.05), Values sharing a common letter within the column are not significant at p<0.05

Out of the selected 40 primers, 16 RAPD and 12 ISSR primers facilitated amplification of DNA fragments (Tables 5 and 6). These primers developed 7 bands at the highest and two bands at the lowest. Out of 123 markers found, 55 markers were for ISSR primers and 68 markers were for RAPD primers. The size ranged from 250-2,000 bp. similar banding profile with both the markers established the clonal nature of in *vitro* raised plants with mother plants.

DISCUSSION

Increasing interest in medicinal plants has opened avenues to develop shoot regeneration and genetic

Table 4: Effects of various antibiotics on shoot regeneration frequency of *E. alba* on MS media supplemented with 15 .0 μM of NAA and 1.0 μM BA.

Antibiotic	Concentration (mg/L)	Frequency of leaves showing shoot regeneration (%)	Mean no. of shoots/ explants
Cefotaxime			
	0	46.2±0.30^{f}	14.3±0.20^{b}
	100	48.9±0.35^{e}	15.1±0.25^{a}
	300	53.4±0.36^{a}	15.7±0.25^{a}
	500	52.6±0.25^{b}	14.7±0.26^{b}
Carbenicillin			
	100	46.7±0.20^{f}	14.3±0.20^{b}
	300	49.7±0.20^{d}	14.9±0.4^{b}
	500	50.6±0.40c	13.9±0.30^{c}
Cephalexin			
	100	22.3±0.20^{g}	9.1±0.25^{d}
	300	14.1±0.35^{h}	4.2±0.5^{e}
	500	0.0±0.00^{i}	0.0±0.00^{f}

Statistically validated with Duncan's multiple range test (p<0.05), Values sharing a common letter within the column are not significant at p<0.05

Table 5: RAPD primer sequences used in the profiling of regenerated plants and the number of bands amplified with their size range.

Primer no.	Primer sequence (5'-3')	Band number	Range size (bp)
OPD-1	ACCGCGAAGG	4	500-1500
OPD -2	GGACCCAACC	5	500-2500
OPD -3	GTCGCCGTCA	3	500-1000
OPD -4	TCTGGTGAGG	--	--
OPD -5	TGAGCGGACA	3	250-1000
OPD -6	ACCTGA ACGG	3	500-1500
OPD -7	TTGGCACGGG	--	--
OPD -8	GTGTGCCCCA	3	250-1500
OPD -9	CTCTGGAGAC	4	500-1500
OPD -10	GGTCTACACC	3	250-1000
OPD -11	AGCGCCATTG	--	--
OPD -12	CACCGTATCC	5	500-2500
OPD -13	CTTCCCCAAG	5	250-1500
OPD -14	CATCCGTGCT	5	250-1500
OPD -15	AGGGCGTAAG	4	250-1000
OPD -16	TTTCCCACGG	5	250-1500
OPD -17	GAGAGCCAAC	5	250-2500
OPD -18	CTGGGGACTT	4	250-2500
OPD -19	CTGCGGTCAG	--	--
OPD -20	ACCCGGTCAC	7	250-1500

Table 6: ISSR primer sequences used in the profiling of regenerated plants and the number of bands amplified with their size range.

Primer number	Sequence (5'-3')	Band number	Range size (bp)
ISSR-1	CACACACACACACACG	6	500-2000
ISSR-2	GAGAGAGAGAGAGAG	--	--
ISSR-3	GAGAGAGAGAGAGAGATC	4	250-1000
ISSR-4	ACACACACACACACGCGC	--	--
ISSR-5	ACACACACACACAC	5	250-1000
ISSR-6	CACACACACACACATG	7	500-1500
ISSR-7	CACACACACACACAGC	5	500-1500
ISSR-8	GAGAGAGAGAGAGAGATA	6	500-1500
ISSR-9	GCGCGCGCGCGCGCGCT	--	--
ISSR-10	GCGCGCGCGCGCGCGCA	--	--
ISSR-11	GCGCGCGCGCGCGCGCAT	5	250-1500
ISSR-12	(CTCTCTCTCTCTCTCTG	5	500-2000
ISSR-13	CTCTCTCTCTCTCTCTA	3	500-2500
ISSR-14	CTCTCTCTCTCTCTCTAG	2	500-1000
ISSR-15	GTGTGTGTGTGTGTGTA	3	500-1500
ISSR-16	GTGTGTGTGTGTGTGTC	--	--
ISSR-17	ATATATATATATATATC	--	--
ISSR-18	ATATATATATATATATG	--	--
ISSR-19	ATATATATATATATATGC	4	500-2500
ISSR-20	ATATATATATATATAT	--	--

transformation system in order to transfer novel traits into the plants. Furthermore, the widely recognized limitation to genetic engineering in various plant species, including *E. alba*, is the absence of an efficient shoot regeneration protocol.[17] *Eclipta alba*, a plant renowned for its distinct medicinal properties and utilization in various medical formulations, serves as a prominent example. Shoot regeneration is the one of the most critical and first step towards development of any genetic transformation protocol. Hence, the current research concentrates on the establishment of an efficient shoot regeneration protocol for *E. alba*.

First study was aimed to investigate the impact of various Plant Growth Regulators (PGRs) on the establishment of a shoot regeneration method for *E. alba*. The initial phase of investigations focused on exploring the effects of several auxins, specifically NAA, IAA and 2,4-D, over a range of concentrations (ranging from 0.0 to 25.0 µM). Upon comparison, NAA emerged as the most effective among the three tested auxins for inducing shoot regeneration, surpassing the effects of both IAA and 2, 4-D (Table 1). The exogenous application of auxins holds paramount importance within plant tissue culture systems to facilitate successful plant regeneration. Earlier also auxins, especially NAA, have been documented for their capacity to stimulate organogenesis in different plants.[18] Moreover, they play a pivotal role in a multitude of developmental processes by triggering cellular and molecular changes encompassing cell expansion, proliferation and transcription.[19] Significantly, among the various auxins, NAA is recognized for its heightened stability, persisting within tissues for extended durations.[20] This attribute could account for the observed phenomenon of achieving the highest regeneration frequency with NAA. Auxins have garnered recognition as pivotal regulators, organizing a multitude of cellular processes within plants. Their influence on cellular activities is primarily linked to the concentration of auxins present in plant cells, a factor intricately regulated by processes such as auxin metabolism, homeostasis and transport.[21] Maintaining an optimal auxin-to-cytokinin ratio holds paramount importance for the proper growth of undifferentiated plant cells.[22] Consequently, a separate experiment was conducted to examine the combined impact of auxins and cytokinins. Therefore next experiment focused on evaluating the effects of three cytokinins-namely BA, Kin and TDZ, at varying concentrations (ranging from 0.0 to 5.0 µM) on shoot regeneration potential of *E. alba*. These evaluations were carried out on MS medium in combination with 15 µM of NAA, as outlined in the Table 2. The inclusion of cytokinins in conjunction with auxins was found to enhance the frequency of shoot regeneration in *E. alba*, surpassing the outcomes observed with auxin alone (Table 2, Figure 1). The establishment of a suitable auxin-cytokinin ratio has been demonstrated to exert influence over auxin-induced organogenesis by regulating the transport-dependent intercellular distribution of auxin.[23] NAA's involvement in cytokinin metabolism and stability has been established[24] and this could potentially account for its contribution to the heightened frequency of shoot organogenesis in leaf explants. These findings are in line with prior research that emphasized the importance of the cytokinin-to-auxin ratio regarding shoot organogenesis[14] and provide new insights on role of optimal auxin-to-cytokinin ratio in achieving shoot regeneration in variety of plants.

Furthermore, the research uncovered that the location of leaf tissue (from top to bottom) significantly impacts the shoot regeneration potential of explants. Among the leaves, those positioned in the middle range exhibited superior potential for shoot organogenesis compared to both newer and more mature leaves (Table 3). Notably, the fifth leaf stood out as displaying the most prominent

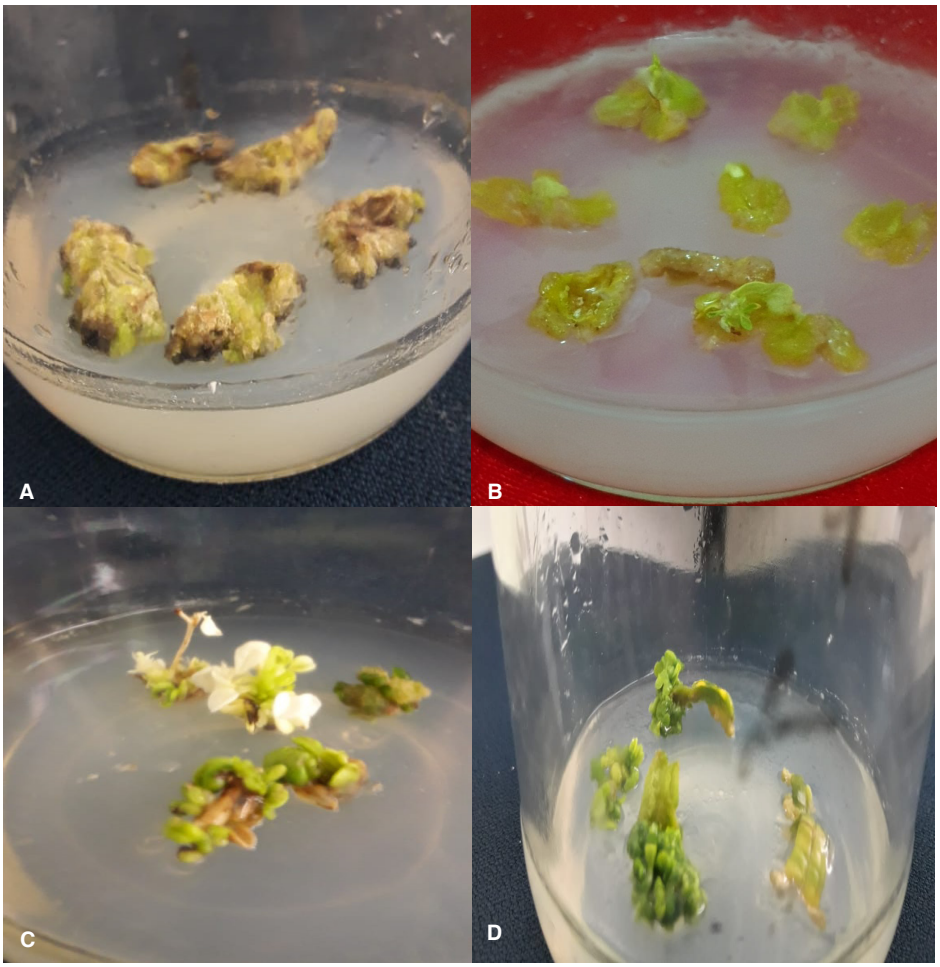

Figure 1: Shoot regeneration of *E. alba* A-B. Initiation of shoot regeneration from leaf explants of *E. alba* on MS medium supplemented with 15.0 µM NAA and 1.0 M BA C-D. Shoot regeneration from leaf explants of E. alba on MS medium. supplemented with 15.0 µM NAA and 1.0 µM BA.

shoot regeneration capacity, as indicated in the Table 3. Middle leaves' enhanced regeneration potential is attributed to their intermediary developmental stage, which involves less differentiation and enhanced metabolic activity in comparison to both newer and older leaves.[25] This physiological state translates into enhanced plant regeneration capabilities under suitable hormonal and nutritional conditions.[26] Earlier also the impact of leaf position on shoot regeneration potential have been documented in various other plant species.[27] Role of leaf position will be valuable, for regeneration of transgenic shoots post transformation as most of the time regeneration post transformation is very difficult to achieve.

Apart from Plant Growth Regulators (PGRs), numerous other compounds are acknowledged for their impact on growth and morphogenesis, especially under *in vitro* conditions.[28] Among these compounds, antibiotics hold particular significance, finding utility in various applications within plant tissue culture. They are employed for purposes such as selecting transformed tissue and eradicating residual *Agrobacterium* from leaf tissue.[29] Considering this, an experiment was conducted to evaluate the influence of various antibiotics, specifically cefotaxime, carbenicillin and cephalexin, on the capacity for shoot regeneration in *E. alba* (Table 4). This study involved the investigation of a diverse set of concentrations spanning from 0 to 500 mg/L. In our investigation, the addition of cefotaxime to the growth medium increased shoots regeneration, elevating the frequency from 46.2% to 53.4% of explants. Conversely, carbenicillin exhibited a hindrance to shoot organogenesis, while cephalexin entirely precluded shoot organogenesis (Table 4). The effect on antibiotics including that of cefotaxime and carbenicillin has been documented in previous studies in many plants.[30] The modulatory influence of these antibiotics on growth processes is intertwined with their ability to interfere with the metabolism of Plant Growth Regulators (PGRs). Further, it is reported that their breakdown

products in the culture medium can differently influence plant cell growth.[30] For example, cefotaxime has been shown to inhibit ethylene production, whereas carbenicillin has the ability to break down into active auxins like phenylacetic acid.[31] Hence, the observed effects of these antibiotics on shoot regeneration could be linked to changes in the natural levels of particular Plant Growth Regulators (PGRs) and will be helpful in achieving regeneration after transformation.

The method of regeneration along with that the choice of the explant source can lead to the introduction of genetic variations in the plants.[28] The application of markers such as RAPD and ISSR has proven effective in identifying genetic similarities or differences within micropropagated material derived from various plants. Molecular markers, like RAPD and ISSR, have proven to be valuable in generating DNA profiles and serving as valuable method for evaluating the stability of *in vitro* cultured plants at genetic level.[14] The *in vitro* propagated plants from the current study exhibit a remarkable level of genetic uniformity, as evidenced by the analysis shown in Figure 2A and 2B. Of 40 primers employed

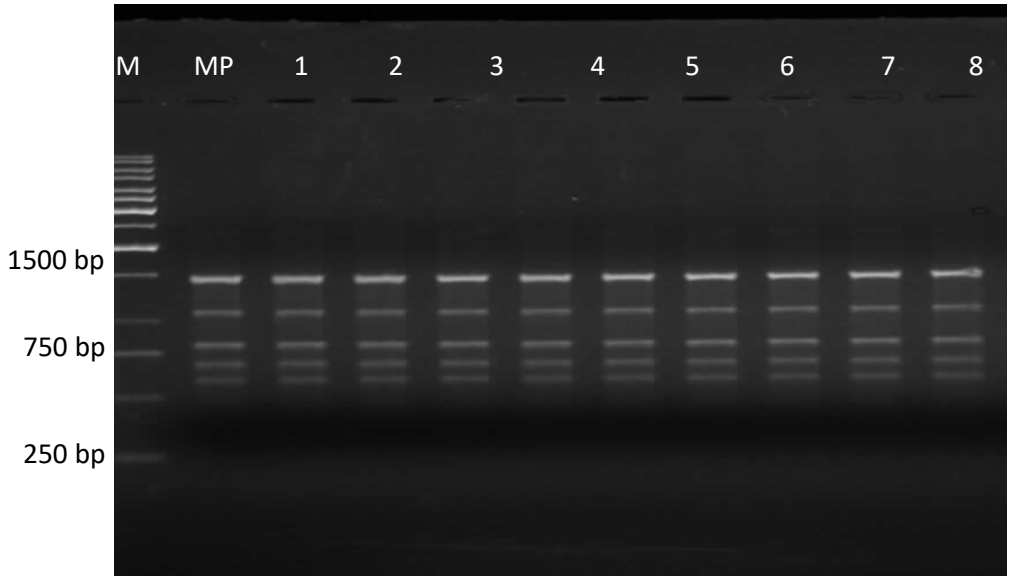

Figure 2 A: ISSR profile (primer 7) of *in vitro* raised plantlets and mother plant of *E. alba* plants, Lane-MP: Mother Plant; Lane 1-9: *in vitro* raised plants Lane M: 1 kb molecular weight markers.

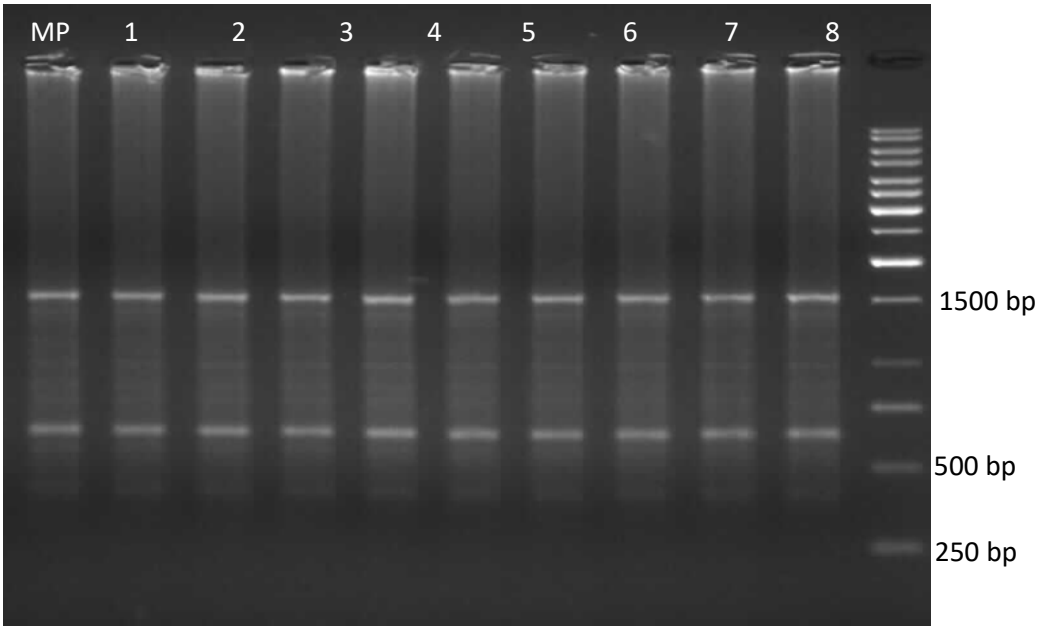

Figure 2B: RAPD profile (primer 3) of *in vitro* raised plantlets and mother plant of *E. alba* plants; Lane-MP Mother Plant; Lane 1-9: *in vitro* raised plants; Lane M: 1 kb molecular weight markers.

28 primers showed in DNA fragment amplification (Tables 5 and 6). The ISSR primers were used to score 55 of the 123 markers collected, while the RAPD primers were used to score the remaining 68 markers (Tables 5 and 6). The markers ranged in between 250-2,000 bp, similar banding profile with both the markers had established the clonal attribute of the selected plants. This similarity can be linked to the genome's resistance to various aseptic interventions as well as the diverse cultural factors faced throughout the various stages of regeneration.

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CONFLICT OF INTEREST

The authors declare that there is no competing interests.

ABBREVIATIONS

2,4-D: 2,4-Dichlorophenoxy acetic acid; **BA:** 6-Benzyl adenine; **CTAB:** Cetyl trimethyl ammonium bromide; **IAA:** Indole-3-acetic acid; **ISSR:** Inter-simple sequence repeats; **NAA:** α-Naphthalene acetic acid; **RAPD:** Random amplified polymorphic DNA.

SUMMARY

Genetic transformation facilitates means to validate gene function and trait specific improvement in highly valuable medicinal plants without affecting their desirable genetic makeup. Increasing interest in *E. alba* as a medicinal and oil yielding plant has opened avenues to develop efficient genetic transformation system in order to transfer novel traits into the plant. Therefore, present presents an efficient shoot regeneration method *E. alba*, which can be effectively used for generating transgenic *E. alba* plants. The role of different auxins and cytokinins in the shoot regeneration of *E. alba* plants was investigated. Combination of auxins and cytokinins lead to enhanced shoot regeneration capacity as compared to alone. Leaf position from top to bottom of the shoot also influenced shoot regeneration potential of *E. alba* and antibiotics especially cefotaxim lead to enhance shoot regeneration capacity. RAPD and ISSR markers, confirms the clonal nature of regenerated plants with mother plant. This protocol has the potential to facilitate work on genetic modification of *E. alba* for incorporating genes of important traits.

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