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EFFICIENT PROTOCOL FOR MICROPROPAGATION OF HIGH PSORALEN YIELDING CHEMOTYPE OF *CULLEN CORYLIFOLIUM* (L.) MEDIK.- A TRADITIONAL HERB OF IMMENSE MEDICINAL VALUE

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ABSTRACT

Cullen corylifolium (L.) Medik. is a traditional herb of immense medicinal value. *In vitro* micropropagation of an elite chemotype of *C. corylifolium* was achieved using nodal explants employing different growth regulators (BA, Kn and 2iP). BA at 5 µM proved optimum to differentiate the maximum average number of multiple shoots. Indirect regeneration via caulogenesis from the two month old cultures of nodal explants was achieved when subcultured on B5 medium containing BA, 2iP or Kn. BA at 10 µM ~~BA~~ proved most effective with an average of 12.33 shoots per culture. The frequency of *in vitro* shoot formation enhanced markedly by the addition of adjuvants, viz. Ad.S. or CW along with optimum level of BA. Rhizogenesis of *in vitro* shoots was achieved on half strength MS basal medium and 97% plants survived after field transfer. Genetic and biochemical fidelity of the *in vitro* regenerants was established through RAPD and HPLC analysis, respectively.

KEYWORDS: ***Cullen corylifolium* (L.) Medik., Fidelity, Micropropagation, Psoralen**

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INTRODUCTION

Cullen corylifolium (L.) Medik. (Syn. *Psoralea corylifolia*), commonly known as Babchi is rich in psoralen content and several other important bio-active compounds such as daidzein, genistein, etc[1] and has been enormously honored in traditional medicinal system such as Ayurveda, Siddha and Unani. Psoralen exhibiting photosensitizing and phototoxic effects is predominantly used in photochemotherapy for curing skin disorders such as mycosis, psoriasis, vitiligo and has also been reported to inhibit different types of tumors in modern clinical applications[2-4]. Psoralen is abundantly present in fruits and seeds as well as its significant amount has also been reported in different plant parts of *C. corylifolium*, viz. leaves, roots and stems[5,6]. Despite this, biotechnologists have also made some efforts for the *in vitro* synthesis of this important furanocoumarin, psoralen[5-9]. The species belongs to family Fabaceae and is distributed almost throughout India but availability of good quality raw materials and its authentication regarding quality control parameters are the major concerns for this taxon. In our previous attempts, investigations have been carried out for quantitative analysis of psoralen as well as to assess the genetic diversity among different chemotypes of *C. corylifolium* growing in North western region of India. The different variants were found to be genetically diverse and the highest amount of psoralen was detected in the plant samples from Sirohi region, Rajasthan, India[10]. Since, chemotype, variety, genotype and cultivar influence the micropropagation[11] therefore, *in vitro* conservation of the elite germplasm of this medicinal herb is the prime necessity. Besides, the selection of elite germplasm in terms of high bioactive molecule (psoralen) is a serious constraint faced by the biotechnologists or Ayurveda/ Siddha/ Unani practitioners. Furthermore, *in vitro* plants in general are usually susceptible to genetic changes due to culture stress[12,13] therefore, the development of highly efficient plant regeneration system and assessment of clonal fidelity and psoralen content stability of the *in vitro* raised plants of *C. corylifolium* are crucial for successful commercial application of micropropagation protocol. In the present communication, an attempt has been undertaken to develop a simple, reproducible and genetically viable micropropagation protocol for the elite chemotype of *C. corylifolium* which could meet the industrial demand for the supply of raw material as well for commercial cultivation.

MATERIALS AND METHODS

(i) Plant materials for RAPD analysis

Seeds of elite chemotype of *C. corylifolium* were collected from Rajasthan (Sirohi) and were sown on different seed beds in the month of March in the Botanical garden, Department of Botany, University of Delhi. All the plants set seed in the month of November. For evaluation of *in vitro* regenerated plants, 11 lines (6 from directly regenerated and 5 from indirectly regenerated plants) of *C. corylifolium* were randomly chosen for analysis of their genetic stability.

(ii) Extraction of genomic DNA

Total genomic DNA from 2 g each leaf tissue of mature plants of *C. corylifolium* was extracted using modified CTAB (Cetyl Trimethyl Ammonium Bromide) method[14]. The quantity and quality of the RNase- treated DNA of all the plant samples of *C. corylifolium* was determined spectrophotometrically and using agarose gel (0.8%) electrophoresis.

(iii) RAPD analysis

Twenty 10-mer primers (VAA-01 to VAA-20, Sigma Aldrich, USA) were screened individually for their repeatable amplification with the pooled DNA sample of the aforesaid eleven lines and 1 line of mother stock plant of *C. corylifolium*. The primers were selected on their ability to produce the optimum number of banding patterns across the eleven lines of the regenerants as well as the elite mother plant. PCR amplification solutions (25 µl) included approximately 50 ng of genomic DNA, 3 mM $MgCl_2$, 0.1 mM each of

dNTPs, 1U of Taq polymerase (Invitrogen Corporation, USA) and 2.5 µl of 10 X PCR reaction buffer [500 mM KCl, 200 Mm Tris-HCl (pH 8.4)] and 1 µl of 0.8 µM of RAPD primer (Sigma Aldrich, USA). DNA amplifications were carried out in a 2720 Thermal Cycler (Applied Biosystems). The reaction conditions started with pre-PCR at 94^0C for 1 min, 36^0C for 30 sec, 72^0C for 1 min, followed by 45 cycles of the following: denaturation at 94^0C for 5 sec, annealing at 36^0C for 15 sec and elongation at 72^0C for 1 min and a final extension at 72^0C for 7 min. The amplification products were resolved on 1.2% (w/v) agarose gels containing ethidium bromide and gels were digitally photographed under UV gel documentation system (Alpha Imager HP; Alpha Innotech).

(iv) Plant materials for in vitro regeneration

Healthy twigs (15- 20 cm long) excised from field grown mature plants of *C. corylifolium* were immediately dipped in freshly prepared 1% (w/v) citric acid (SRL, Mumbai, India) solution to minimize browning. Surface sterilization was done by treating the nodal explants with 5% (v/v) Teepol (Rickett and Colman India Ltd.) and 0.5% (w/v) bavistin solution (a systemic fungicide; BASF India Ltd., Mumbai), followed by 70% (v/v) ethanol and freshly prepared 0.1% $HgCl_2$ (SRL, Mumbai) and 100 mg/l streptomycin solution for 5 min with continuous shaking and subsequent washing for four to five times with autoclaved double distilled water in the laminar hood After sterilization, cut ends, which turned brown were trimmed prior to implantation on semisolid media.

(v) Culture media

The B5[15] basal medium was used either alone or supplemented with N^6-benzyladenine (BA), N^6-(2-isopentenyl) adenine (2iP), 6-furfuryl aminopurine (Kn) and adenine sulfate (AdS). For rhizogenesis, B5 and MS[16] basal medium alone or alongwith α-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) were employed. All plant regulators were procured from Sigma Aldrich (USA). Salts and other chemicals were obtained from Qualigens, SRL, Mumbai. In addition, adjuvants like coconut water (CW) either alone or along with the optimum concentration of BA was also added to B5 medium to promote caulogenesis. As the carbon source, 3% (w/v) sucrose (DCM, Daurala, India) was added to the media and the pH was adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl. Approximately, 20 ml media was dispensed in each "150 x 25 mm" test tubes (Borosil, India), plugged with non-absorbent cotton wrapped in two-layered muslin cloth and sterilized by autoclaving at 1.06 kg cm^{-2} at 121^oC for 15 min.

(vi) Raising and incubation of cultures

The nodal explants were cultured in test tubes containing semisolid medium in a vertical orientation. Only one explant per test tube was inoculated. Cultures were incubated at 25 ± 2^0C temperature under continuous cool day light (28-35 µmol m^{-2} s^{-1}) emitted by fluorescent incandescent tubes (40 W, Philips, Kolkata) with 55±5% relative humidity.

Recording of data

The average number of shoots per explant, the average shoot length, the average number of roots per shoot and the average root length has been represented as mean values along with standard error (mean ± SE). The mean values were calculated on the basis of a minimum of 24 replicates in each experiment and repeated at least for once or twice. The evaluation of psoralen content from the seed samples of *C. corylifolium* was performed with four replicates. The data expressed as mean ± SE have been statistically analyzed using ANOVA (Analysis of Variance) through SPSS (Statistical Package for Social Sciences). The differences between means were tested for significance by Duncan's multiple range test (DMRT) at p=0.05. For genetic fidelity studies, amplified DNA bands were recorded with the selected RAPD primers and only clear and scorable bands at a particular position were considered. Each band was treated as a marker and scoring of bands was done on the basis of their presence ('1') or absence ('0') in the gel. NTSYSpc (Numerical Taxonomy and Multivariate Analysis System) Version 2.1 software[17] was used to perform the distance matrix and cluster analysis of the complete data set of the marker employed. Genetic association amongst the different

individuals was measured by the Jaccard's similarity coefficient[18] with the SIMQUAL (Similarity for the qualitative data program in NTSYS) module of NTSYS-pc software.

RESULTS

1. Differentiation of shoots from in vivo nodal explants

Nodal explants excised from 3 to 4 month-old-field grown plants of Sirohi region were cultured on B5 medium supplemented with different concentrations (0, 1, 5, 10, 15, 20 and 30 µM) of BA. Interestingly, multiple shoots were obtained on all the concentrations of BA tried, but differences were observed in terms of percentage of explants forming multiple shoots as well as average number of shoots (Data not shown). Maximum response was achieved on 5 µM BA, where 85.7% explants developed multiple shoots with an average of 3.57±0.3[a] shoots per explant and average length being 1.64±0.2[a] cm (Fig. 1).

2. Differentiation of shoots from explants of in vitro raised micro shoots

In order to evaluate the regeneration potential of *in vitro* raised shoots on 5 µM BA, the nodal explants were excised from such shoots and further sub-cultured on B5 medium fortified with different concentrations of cytokinins, viz. BA, Kn, 2iP and the regeneration were achieved via direct as well as indirect organogenesis (Table 1, 2; Fig. 1). Nodal explants differentiated direct shoots within 30 days of culture. If the cultures were retained for more than 30 d on same medium, good amount of brown and nodular calluses were formed and the counting of axillary shoots became difficult due to differentiation of shoots from callus covering the entire explants. Such calli were further subcultured on same but fresh medium to obtain indirect organogenesis (Table 1, 2; Fig. 1).

3. Direct organogenesis

Nodal explants excised from *in vitro* raised shoots induced direct multiple shoots on B5 medium when fortified with 0.1, 1, 5, 10, 20 and 30 µM of different cytokinins (BA, Kn and 2iP). Incidentally, the morphogenic response enhanced significantly and in case of nodal explants, 5 µM BA elicited an optimum response in cent percent cultures with an average of 5.72±0.17[a] shoots after 30 d. 2iP proved best for elongation of shoots (Table 1; Fig. 1). The maximum shoot length (7.25±0.09[a] cm) was achieved on 1 µM 2iP (Table 1; Fig. 1).

4. Indirect organogenesis via callus phase

Indirect regeneration was achieved through callus phase derived from the calluses excised from the two months old cultures of nodal explants which were subcultured on B5 medium containing BA, 2iP and Kn. Amongst all the different cytokinins such as BA, 2iP and Kn, BA was most effective for caulogenesis of nodal explants (Table 1; Fig. 1). A maximum average of 12.34±0.65[a] shoots per culture was developed in 100% nodal explants on ~~a~~ 10 µM BA (Table 1). With a view to further enhance the morphogenic response in this high yielding chemotype of Sirohi, the B5 medium was also adjuvanted with Kn, CW and Ad.S. either alone or along with the optimum concentration of BA (10 µM BA). Surprisingly, adjuvants (CW or Ad.S) when used alone, did not enhance the morphogenic response in the nodal explants. Though lower concentrations of Kn were not promotory but its high concentration proved to be effective when tried alone. Therefore, the response was also evaluated on B5 medium fortified with different combinations of Kn and BA. It was observed that Kn when used along with the optimum concentration of BA, the average shoot number went up from around 12.34±0.65[g] to 20.15+0.25[c] at 0.1 µM Kn + 10 µM BA and 21.25±0.25[b] at 1 µM Kn + 10 µM BA in 100% cultures (Table 2). Likewise, the other adjuvants when used along with optimum level of BA markedly enhanced the response in nodal explants and showed cent percent multiple shoot differentiation on all the combinations (Table 2; Fig. 1). The nodal explants induced an optimum average of 17.13±0.17[d] and 24.75±0.94[a] shoots at 0.1 µM Ad.S.+ 10 µM BA and 15% CW + 10 µM BA combinations, respectively (Table 2; Fig 1).

5. In vitro flowering

Incidentally, *in vitro* flowering was also observed in about 12.5% and 16.7% cultures on 5 µM BA and 10 µM BA within 6 weeks of culture (Fig. 2 a).

6. Rooting of excised shoots

For rhizogenesis, nearly 2 cm long *in vitro* shoots were excised separately from direct and indirect shoot cultures and transferred to full as well as half strength of MS or B5 basal medium with or without auxins (0.1, 1, 5 and 10 µM of IBA, NAA and IAA). Half strength MS medium proved effective for direct rhizogenesis where approximately 91.66% shoots induced an average of more than 3.33 roots within 15 d (No significant difference was observed with reference to direct or indirect shoots therefore, representative data is presented here). On all auxins supplemented media, roots were induced through intervening callus (Table 3; Fig. 2 b).

7. In vitro hardening and acclimatization to field

The *in vitro* developed plantlets regenerated directly or indirectly were transplanted to jars containing sterilized soilrite mixture for acclimatization separately and further transferred to the field gradually. The rate of plantlets survival was ~87% in either case (Fig. 2 c). Almost all the micropropagated plants flowered well after four months of field transfer (Fig. 2 c).

8. Genetic fidelity among regenerants and elite mother chemotype

Of the twenty random primers, *i.e.* VAA-01 to VAA- 20, nine primers produced optimum monomorphic banding patterns in the regenerated plants as well as their donor mother plant. A total of 478 amplification products were detected from the aforesaid eleven regenerants along with the mother plant with an average of 53 fragments per primer (Table 4). Each primer produced a unique set of amplification products, ranging in size from 100 bp (VAA-02, VAA-11, VAA-16 primers) to 1000 bp (VAA-03, VAA-05 primer) (Table 4). VAA-03 was more practicable than other primers in this experiment due to more monomorphic bands. The regenerated plants shared the same banding patterns as those of the donor plants, implying that they possibly were genetically identical to each other (Fig. 3). Jaccard's coefficient of similarity varied from 0.97 to 1.00, among the regenerants and the elite mother plant, indicative of a high level of similarity among the twelve plants investigated (Table 5). Cluster analysis through UPGMA analysis revealed 97% similarity between the elite mother plant and the eleven micropropagated lines.

9. Estimation of psoralen content from regenerants

HPLC analysis of the psoralen content from the seeds of elite mother stock, eleven elite *ex vitro* regenerated mature plants individually as well as of the pooled seed samples revealed more or less similar quantity of psoralen. The seeds collected from the *ex vitro* grown mature elite mother stock yielded 4773.17 ± 0.48^{f} µg/g fresh weight of psoralen in comparison to 4786.50 ± 0.43^{a} µg/g fresh weight in the case of the pooled seed samples of the eleven regenerants. However, 4783.00 ± 1.29^{b}, 4785.00 ± 1.08^{a}, 4779.50 ± 0.65^{e}, 4782.00 ± 0.91^{e}, 4781.50 ± 0.65^{e}, 4783.75 ± 1.10^{b} µg/g fresh weight of psoralen was detected in the randomly selected six directly micropropagated plants, whereas in the five indirectly regenerated plants, selected randomly 4783.00 ± 1.08^{d}, 4784.50 ± 0.65^{b}, 4782.50 ± 1.19^{e}, 4783.50 ± 1.19^{b}, 4782.00 ± 1.08^{e} µg/g fresh weight of psoralen was estimated (Table 6).

DISCUSSION

During present investigations an efficient regeneration protocol has been developed for the high psoralen yielding *chemotype*, *i.e.* Sirohi (Figs 1, 2). Though some published work is on record regarding simple regeneration of *C. corylifolium* from a variety of explants but none of the earlier workers[5,7,8,19-26] reported the selection and micropropagation of high psoralen yielding chemotype, its regeneration and establishment of fidelity at DNA level as well as stable quantity of metabolites of the

regenerants. In the present study, it has also been shown that the nodal explants of mature *C. corylifolium* yielded quite low morphogenic response in terms of average shoot number being optimum on 5 µM BA. When such shoots were further subcultured, multifold enhancement was obtained in the morphogenic potential of nodal explants and the best morphogenic potential was seen on 5 µM BA for direct organogenesis and 10 µM BA for indirect organogenesis (Table 1; Fig. 1). However, other cytokinins, like Kn or 2iP when used alone were not much effective (Table 1, 2; Fig. 1). The efficiency of BA in *in vitro* regeneration studies has been reported in *Arnica montana*, *Hollarhena antidysenterica* and *Simmondsia chinensis*[27-29]. The promotory effect of BA over other cytokinins could be due to its easy permeability, increased affinity for active cell uptake, less resistance to the enzyme cytokinin oxidase, or receptor abundance in its perception apparatus which interacts with the coupling elements in the signal transduction chain[30]. Even in the present investigations, the higher shoot elongation on Kn containing medium is also in accordance that Kn containing medium release relatively lesser amount of ethylene than medium incorporated with BA[31]. Based on the above observations, it can be assumed that BA is necessary for shoot proliferation and Kn plays a vital role on account of preventing ethylene production. Thus, the synergistic effect of combination of cytokinins (BA + Kn) was optimal for enhanced shoot regeneration from nodal explants of *C. corylifolium* owing to inhibition of ethylene as reported earlier[32-34].

The frequency of *in vitro* shoot formation has also been seen to be increased markedly by the addition of additives (Ad. S.) and unidentified supplements, viz. CW along with optimum level of BA. Adenine is itself a product of cytokinin metabolism and it can serve as a precursor for zeatin synthesis[35-36]. CW is a natural complex supplement that comprises mineral nutrients and several organic compounds with high level of zeatin and known to play a vital role in plant growth and development[37-38]. Another feature of the current investigation is the early induction of *in vitro* flowering in *C. corylifolium* (Fig. 2) which offers an attractive alternative to obtain flower throughout the year. Even the field transferred plants also successfully flowered after four months of field transfer and the ripened seeds were harvested and further used for estimation of psoralen content. *In vitro* flowering was also reported earlier in Gentian[39] and *Vernonia anthelmintica*[40] under the influence of BA, Kn, Ad.S. or 2,4-D supplemented medium. Genetic uniformity of *ex vitro* plants of *C. corylifolium* as revealed by analysis through RAPD confirmed that all the micropropagated plants had a high (97%) affinity amongst them and their donor mother plants. This small genetic variation (3%) in DNA may be attributed to naturally occurring variation or due to the accumulation of mutation by various factors such as *in vitro* process and its duration, auxin to cytokinin ratio (hormonal balance), *in vitro* stress induced by added biochemicals, or other nutritional conditions, all of which are known to induce somaclonal variations[37,41-42]. Yet another significant feature of this study is the establishment of consistency in secondary metabolite content which is of immense practical utility and commercial implications. However, it has been reported that the seeds of callus derived plants yielded less psoralen in comparison to those of naturally-grown plants of *C. corylifolium*[8]. Similar to our work, almost equal amount of essential oils from *in vitro* derived plantlets and *in vivo* mother stock plants of *Mentha spicata*[43] and *Pogostemon cablin* (Blanco) Benth[44] was reported.

Table 1

Effect of different growth regulators augmented to B5 medium on direct and indirect morphogenic potential of nodal explant of C. corylifolium after 30 d of culture.

Growth Regulators (µM)	Percentage of Explants developing Shoots				Average** number of shoots per explant		Average** shoot length (cm)	
	Direct		Indirect		Direct	Indirect	Direct	Indirect
	Single	Multiple	Single	Multiple				
BA								
0	91.66^{a}	8.33^{k}	91.66^{a}	8.33^{m}	1.17±0.17^{n}	1.18±0.12^{q}	2.49±0.18^{e}	1.05±0.35^{j}
0.1	66.66^{d}	33.33^{h}	45.83^{e}	54.16^{h}	1.74±0.09^{h}	2.04±0.26^{l}	1.37±0.32^{k}	0.91±0.16^{k}
1	25^{i}	75^{d}	12.5^{j}	87.5^{b}	3.87±0.12^{c}	5.93±0.73^{g}	4.22±0.46^{d}	0.92±0.19^{k}
5	0^{m}	100^{a}	0^{l}	100^{a}	5.72±0.17^{a}	9.64±1.00^{d}	0.88±0.16^{l}	1.03±0.19^{j}
10	8.33^{l}	91.66^{b}	0^{l}	100^{a}	5.62±0.22^{b}	12.34±0.65^{a}	0.53±0.35^{m}	0.74±0.15^{m}
20	8.33^{l}	91.66^{b}	12.5^{j}	83.33^{c}	3.42±0.24^{d}	10.33±1.3^{b}	0.43±0.02^{n}	0.65±0.19^{n}
30	16.66^{k}	83.33^{c}	4.17^{k}	83.33^{c}	2.13±0.16^{f}	9.87±2.14^{c}	0.33±0.05^{o}	0.36±0.05^{o}
Kn								
0.1	91.66^{a}	8.33^{k}	91.66^{a}	8.33^{m}	1.16±0.16^{n}	1.25±0.46^{p}	1.55±0.48^{j}	2.57±0.45^{f}
1	87.5^{b}	12.5^{j}	87.5^{b}	12.5^{l}	1.23±0.13^{m}	1.52±0.25^{n}	2.13±0.45^{h}	2.17±0.41^{h}
5	79.16^{c}	20.83^{i}	79.16^{c}	20.83^{k}	1.43±0.24^{j}	1.85±0.64^{k}	2.36±0.43^{g}	2.15±0.42^{h}
10	33.33^{h}	62.5^{f}	33.33^{g}	66.66^{f}	2.03±0.24^{g}	2.81±0.21^{h}	2.37±0.41^{g}	2.15±0.33^{h}
20	33.33^{h}	66.66^{e}	29.16^{h}	70.83^{e}	2.04±0.20^{g}	7.34±0.25^{e}	1.37±0.20^{k}	1.56±0.23^{i}
30	20.83^{j}	75^{d}	20.83^{i}	75^{d}	2.18±0.22^{e}	6.06±3.75^{f}	0.89±0.15^{l}	0.82±0.16^{l}
2iP								
0.1	87.5^{b}	12.5^{c}	87.5^{b}	12.5^{l}	1.13±0.06^{o}	1.25±0.16^{p}	4.57±0.80^{c}	3.56±0.70^{e}
1	87.5^{b}	12.5^{c}	87.5^{b}	12.5^{l}	1.25±0.07^{l}	1.39±0.44^{o}	7.25±0.09^{a}	5.27±0.80^{a}
5	87.5^{b}	12.5^{c}	70.83^{d}	29.16^{j}	1.36±0.04^{k}	1.54±0.29^{m}	6.35±0.87^{b}	4.67±0.84^{c}
10	54.16^{e}	45.83^{b}	37.5^{f}	62.5^{g}	1.56±0.13^{i}	1.93±0.22^{j}	3.62±0.59^{d}	4.72±0.76^{b}
20	37.5^{g}	62.5^{f}	45.83^{e}	45.83^{i}	1.72±0.12^{h}	1.72±0.21^{l}	2.62±0.5^{f}	3.74±0.66^{d}
30	45.83^{f}	45.83^{g}	91.66^{a}	8.33^{l}	1.52±0.16^{j}	1.18±0.12^{q}	2.07±0.26^{i}	2.43±0.52^{g}

Mean of 24 explants ± SE. Values in a column followed by the same superscript are not significantly different as determined by SPSS at p=0.05.

Table 2

Effect of Kn, Ad.S. and CW added to 10 µM BA containing B5 medium on morphogenic response of nodal explants of C. corylifolium after 60 d.

Adjuvants	Explants developing shoots (%)		Average*number of shoots per explant	Average*shoot length (cm)
	Single	Multiple		
Kn + BA				
0+10 (control)	0^{b}	100^{a}	12.34±0.65^{g}	0.53±0.35^{h}
0.1+10	0^{b}	100^{a}	20.15±0.36^{c}	1.36±0.65^{a}
1+10	0^{b}	100^{a}	21.25±0.25^{b}	1.08±0.81^{b}
5+10	0^{b}	100^{a}	14.25±0.31^{e}	0.85±0.36^{d}
10+10	0^{b}	100^{a}	9.53±0.10^{h}	0.95±0.36^{c}
Ad.S. + BA				
0.1+10	0^{b}	100^{a}	17.13±0.17^{d}	1.03±0.65^{b}
1+10	0^{b}	100^{a}	14.94±0.27^{e}	0.73±0.31^{f}
5+10	0^{b}	100^{a}	9.04±0.35^{h}	0.78±0.25^{e}
10+10	0^{b}	100^{a}	9.05±0.36^{h}	0.73±0.31^{f}
CW + BA				
10+0	0^{b}	100^{a}	12.73±0.34^{g}	0.78±0.14^{e}
10+5	0^{b}	100^{a}	13.16±0.32^{f}	0.66±0.10^{g}
10+10	0^{b}	100^{a}	20.13±0.34^{c}	0.99±0.17^{c}
10+15	0^{b}	100^{a}	24.75±0.94^{a}	0.85±0.11^{d}
10+20	25^{a}	75^{b}	4.57±0.32^{i}	0.64±0.17^{g}

Mean of 24 explants ± SE. Values in a column followed by the same superscript are not significantly different as determined by SPSS at p=0.05.

Table 3

Rooting response of in vitro raised shoots of C. corylifolium on half strength MS medium supplemented with different auxins after 30 d.

Auxins (µM)	Shoots developing roots (%)		Average* number of roots per shoot	Average* root length (cm)
	With callus	Without callus		
Control	0[g]	91.66[a]	3.33±0.49[a]	3.91±0.52[a]
IAA				
0.1	16.66[e]	50[c]	2.30±0.95[b]	3.11±0.81[bc]
1	33.33[d]	50[c]	2.50±0.91[b]	3.21±0.75[bc]
5	58.33[b]	33.33[d]	2.71±0.97[ab]	3.96±0.86[a]
10	58.33[b]	33.33[d]	3.02±1.10[a]	3.54±1.10[ab]
IBA				
0.1	8.33[f]	66.66[b]	1.83±0.44[bc]	3.50±0.71[ab]
1	41.66[c]	33.33[d]	1.91±0.41[bc]	3.75±0.78[a]
5	41.66[c]	8.33[f]	3.75±1.22[a]	3.00±0.93[c]
10	58.33[b]	8.33[f]	2.67±0.88[ab]	3.66±0.73[ab]
NAA				
0.1	8.33[f]	50[c]	1.83±0.58[bc]	3.41±0.87[b]
1	33.33[d]	33.33[d]	1.92±0.32[bc]	2.50±0.92[d]
5	58.33[b]	33.33[d]	2.93±1.90[ab]	2.32±0.81[d]
10	66.66[a]	16.66[e]	2.74±0.82[ab]	2.01±0.71[e]

Mean of 24 explants ± SE. Values in a column followed by the same superscript are not significantly different as determined by SPSS at p=0.05

Table 4

List of RAPD primers, their sequences, number of scorable bands and their range of amplified fragments generated in C. corylifolium (L.) Medik. donor plant and regenerants.

Sl. No.	Primer	Sequence (5'- 3')	Total No. of bands amplified	No. of monomorphic bands	No. of polymorphic bands	Size of bands (bp)
1.	**VAA-01**	CAGGCCCTTC	36	3	0	350-750
2.	**VAA-02**	TGCCGAGCTG	48	4	0	100-600
3.	**VAA-03**	AGTCAGCCAC	60	5	0	150-1000
4.	**VAA-05**	AGGGGTCTTG	47	3	1	350-1000
5.	**VAA-11**	CAATCGCCGT	60	5	0	100-600
6.	**VAA-10**	GTGATCGCAG	59	4	1	200-700
7.	**VAA-16**	AGCCAGCGAA	60	5	0	100-800
8.	**VAA-18**	AGGTGACCGT	60	5	0	250-900
9.	**VAA-20**	GTTGCGATCC	48	4	0	200-800
Total			**478**	**38**	**2**	

Table 5

Similarity matrix computed with Jaccard's coefficient for different regenerants of C. corylifolium ~~(L.) Medik.~~ (M: Elite mother chemotype; 1-6: direct regenerants; 7-11: indirect regenerants).

	M	1	2	3	4	5	6	7	8	9	10	11
M												
1	0.975											
2	1.000	0.975										
3	1.000	0.975	1.000									
4	0.975	0.950	0.975	0.975								
5	1.000	0.975	1.000	1.000	0.975							
6	1.000	0.975	1.000	1.000	0.975	1.000						
7	1.000	0.975	1.000	1.000	0.975	1.000	1.000					
8	1.000	0.975	1.000	1.000	0.975	1.000	1.000	1.000				
9	1.000	0.975	1.000	1.000	0.975	1.000	1.000	1.000	1.000			
10	1.000	0.975	1.000	1.000	0.975	1.000	1.000	1.000	1.000	1.000		
11	1.000	0.975	1.000	1.000	0.975	1.000	1.000	1.000	1.000	1.000	1.000	

****** Mean of 24 shoots ± SE. Values in a column followed by the same superscript are not significantly different as determined by SAS at p 0.05.***

Table 6

HPLC analysis of psoralen content of seed samples of the mature plants of elite chemotype and those of the regenerants (raised directly and indirectly) of the elite chemotype of C. corylifolium

Sl. No.	Seed Sample	*Amount of psoralen (µg/g fresh wt)
1.	**Elite mother genotype**	4773.17 ± 0.48^{f}
2.	**Pooled sample of regenerants (direct and indirect regenerants)**	4786.50 ± 0.43^{a}
3.	**Direct regenerants** Regenerant 1	4783.00 ± 1.29^{b}
4.	Regenerant 2	4785.00 ± 1.08^{a}
5.	Regenerant 3	4779.50 ± 0.65^{e}
6.	Regenerant 4	4782.00 ± 0.91^{e}
7.	Regenerant 5	4781.50 ± 0.65^{e}
8.	Regenerant 6	4783.75 ± 1.10^{b}
9.	**Indirect regenerants** Regenerant 1	4783.00 ± 1.08^{d}
10.	Regenerant 2	4784.50 ± 0.65^{b}
11.	Regenerant 3	4782.50 ± 1.19^{e}
12.	Regenerant 4	4783.50 ± 1.19^{b}
13.	Regenerant 5	4782.00 ± 1.08^{e}

****Results are the mean of four replicates (±SE). Values in a column followed by the same superscript are not significantly different as determined by SPSS at p=0.05.***

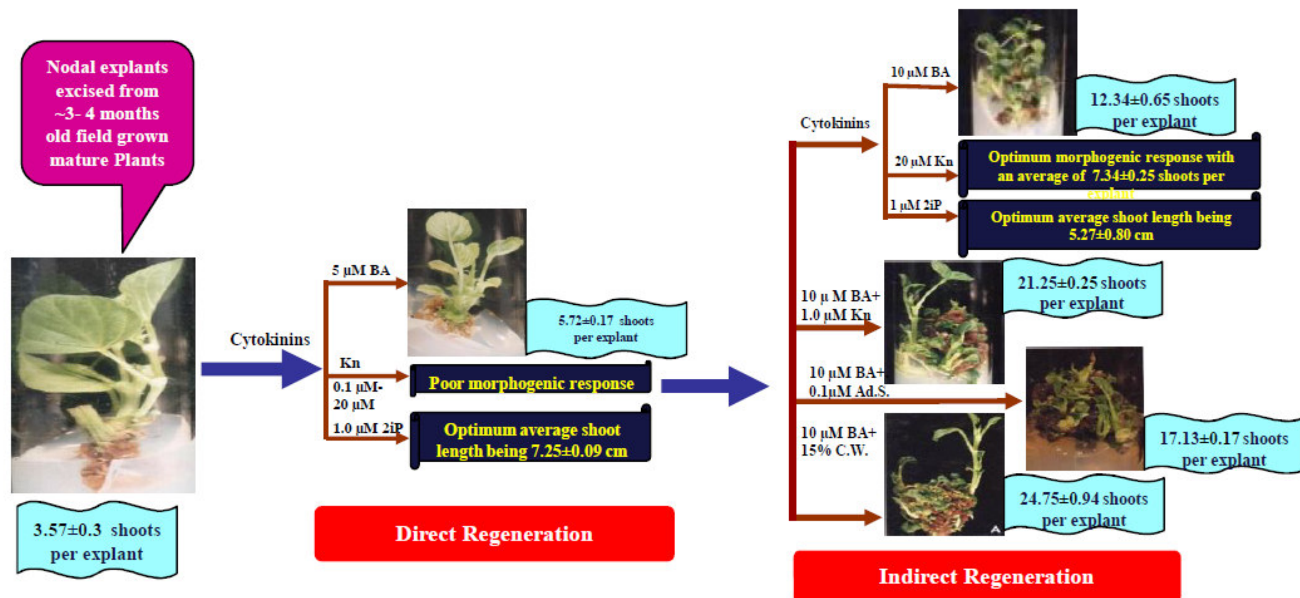

Figure 1
Flow chart showing direct and indirect in vitro regeneration from nodal explants of C. corylifolium along with their optimum morphogenic responses.

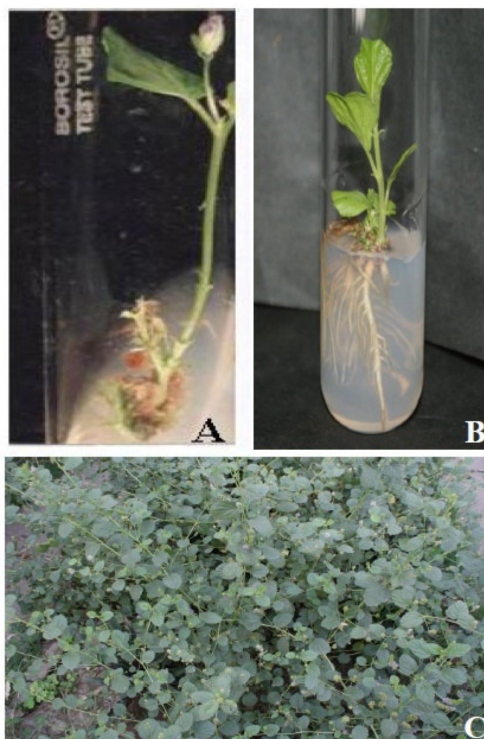

Figure 2
Excised shoot with improved shoot length and in vitro flowering on MS basal alone after 4 weeks of transfer (A) Rooting of in vitro raised shoot on half strength MS basal after two weeks (B). Tissue culture raised six month old plants growing vigorously in garden with bluish-purple flowers (C).

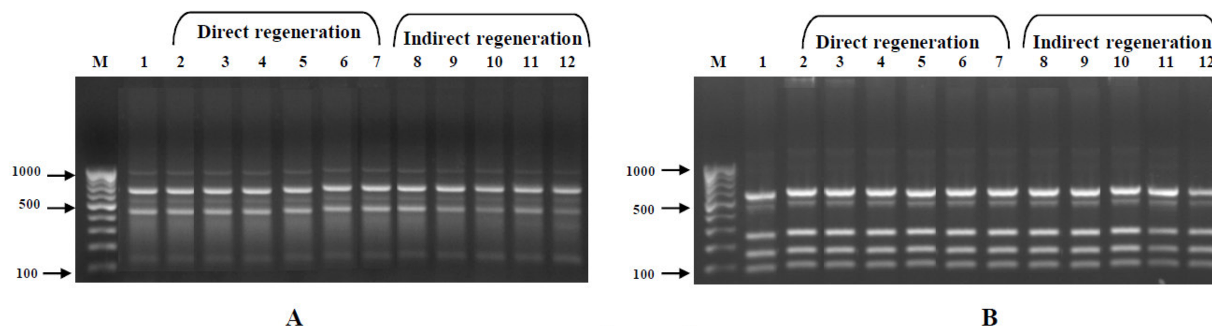

Figure 3

RAPD profile of the primers VAA-03 (A) and VAA-11 (B) of the eleven regenerants and elite chemotype of C. corylifolium (Lane M: 100 bp DNA size Marker, Lane 1: Elite mother chemotype; 2-7: direct regenerants; 8-12: indirect regenerants.

CONCLUSION

Authentication of species identity and prediction of the quantity of bioactive principles are the two major attributes of quality control which are required for the trade of herbal plants used for pharmaceutical purposes. The results presented here demonstrates a highly efficient, safe and genetically as well as biochemically viable lab to land technology for producing true- to- type plants of *C. corylifolium* which would not only provide great emphasis on enhanced cultivation of the desired germplasm but also facilitate the scaling up of the psoralen at commercial level even with genetic stability.

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