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Induction of multiple shoots from the callus culture of *Celastrus paniculatus* Willd

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Abstract: *An efficient and reproducible protocol for plant regeneration with an intervening callus phase was developed for Celastrus paniculatus using leaf and nodal explants for the first time. Explants from mature plant were cultured on Murashige and Skoog's medium supplemented with various concentrations of cytokinins and auxins either alone and in various combinations. The maximum amount of callus was recorded on the MS medium supplemented with NAA+BAP/ TDZ. Callus obtained from leaf and nodal culture further sub-cultured to NAA+BAP+L-glu to produce maximum number of shoots. The regenerated shoots were transferred to medium containing GA_3 at the concentrations of 1.44µM. The shoots thus elongated are sub-cultured to rooting medium for root induction. Finally the plantlets were hardened in pots containing sterilized soil and sand mixture (3:1) and well established in field, survival rate is 70%.*

Key words: *Celastrus paniculatus, Indirect Regenerations, Micropropagation, Nodal cultures.*

INTRODUCTION

Celastrus paniculatus Willd. (Celastraceae) commonly known as Jyothismathi in Sanskrit, Malkangani in Hindi and Bitter Sweet in English, is a rare medicinal plant distributed throughout India mostly in tropical forest and subtropical Himalayas. It is a large woody, unarmed climbing shrub occurring up to an altitude of 1200m (Figs. 1A-D). Indiscriminate over exploitation coupled with insufficient attempts for replenishment of wild stock have contributed to its threatened status requiring scientific efforts for conservation and commercial cultivation [1, 2]. The species is categorized under vulnerable status in Western Ghats of South India [3].

Celastrus paniculatus is widely used to cure depression, paralysis, leprosy, fever, abdominal disorder and cancerous tumors [4,5]. It is well known for its ability to improve memory [6]. Pharmacological studies suggest that the oil obtained from the seeds possess sedative and anti-convulsant properties. Seed oil has been also found to be beneficial to psychiatric patients [7] and increased the intelligence quotient of mentally retarded children [8]. Bark is reported to be arbotifacient, depurative and brain tonic and taken internally for snake bite [9]. Root barks also show antimalarial activity [10]. The powdered root is considered useful for the treatment of cancerous tumors [11].

Chemical constituents of seeds as revealed by phytochemical analysis were Sesquiterpene alkaloids like Celapagine, Celapanigine and Celapanine[12]. Due to the over exploitation of these taxa for various medicinal uses the plant is threatened. The consequences are possible for the verge of extinction and this provides justification of conservation and propagation of this valuable plant. Seed germination is as low as 11.5% and other vegetative propagation methods also cannot be used for cultivation [13]. In this context *In-vitro* technique plays an important role. Tissue culture techniques are used globally for the conservation and utilization of genetic resources. Many studies regarding direct regeneration had been done in *C.paniculatus* [14, 15, 16,

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[17] with limited success. Indirect regeneration hasn't been done in leaf or nodal culture in this species so far and hence this study plays a major role in propagation of *C.paniculatus.* Further variations could be introduced through callus culture studies and elite variants can be selected for better yield. Hence, the present work is an attempt to develop an efficient protocol for mass multiplication through *in-vitro* regeneration from callus culture of leaf and nodal explants. That has prompted us to regenerate the shoots from callus culture by supplementing the MS medium with various growth regulators either alone or in combination.

MATERIALS AND METHODS

Explant preparation and surface sterilization

An approximately 15 year old healthy mature plant of *Celastrus paniculatus* maintained in GKVK, University of Agricultural Sciences and outskirts of Yelahanka, Bangalore, Karnataka, India were used as the source plants to collect the explants for *In-vitro* cultures. Young leaf and nodal segments were excised. Customary tissue culture techniques were followed to raise the cultures. Shoot tips (0.5 to 1cm), nodes (1cm) and leaf (1cm²) were selected for the present study. They were surface sterilized sequentially in running water for 30min with few drops of Tween-20 (Himedia, India), Bavistin (BASF, India Limited, India) for 10min to reduce the chances of fungal contamination. Explants were washed in sterile double distilled water after each treatment, finally these were surface sterilized under aseptic conditions with freshly prepared mercuric chloride (0.1%) solution for 2-5min and then washed with double distilled water. The materials are again sterilized with streptomycin (0.1%) for 2-5min to avoid bacterial infection. Explants were washed thoroughly 4-5 times in sterile double distilled water and taken into LAF for inoculation.

Nutrient Media and culture conditions

Murashige and Skoog's[18], Phillips and Collins[19] and MMS medium supplemented with sucrose (3%) and Agar agar were used for cultures initiation. Agar agar (Himedia, India) was added to culture media as gelling agent at a concentration of 0.8% (w/v). The P^H of the medium adjusted to 5.8±0.02 and the medium was then autoclaved for 15min at 121°C. Cultures were maintained at 25±2°C under 16h day photoperiod with a light intensity of 40-25µmol $m^{-2}s^{-1}$photon flux density, provided by cool white fluorescent lamps (Phillips, India).

Callus multiplication

To study the effect of plant growth regulators, nodal and leaf cultures were cultured on MS medium containing various concentrations of BAP, Kin and TDZ alone or in combinations. Cultures were repetitively transferred to MS media containing IAA+BAP+L-glu of an optimized concentration for shoot regeneration.

Rooting of Micropropagated shoots

To induce rooting, shoots were excised and transferred to MS medium containing different concentrations of IBA as described by Bilochi[20]and rooting by dip treatment of auxin recommended by Harry and Thorpe[21].*In vitro* developed Shoots (5-8cm long) were excised and basal ends were treated with different concentrations of IBA for 3-5min. The rooting response differed according to different concentrations and combinations of auxins used [22]. Thus treated shoots were transferred to bottles containing MS basal medium for further growth.

Hardening of *In-vitro* produced plantlets

After the root initiation, none of the plantlets directly transferred from rooting medium to the potting mixture and grown under natural conditions survived. However, the survival percentage rose to 70% after the plantlets were hardened under aseptic conditions before being transferred to the polybags. Plantlets were transferred to polybags containing a mixture of sterilized soil and sand (3:1). Such plantlets were kept in the greenhouse for 7-8 weeks, shifted to field for the evaluation of percentage survival of plants.

Growth Studies

Leaf (1cm²) and Nodal (1cm) explants were inoculated on various concentrations of auxins and cytokinins and their combinations to study the rate

of growth of cultures under controlled conditions. Initial fresh weight of the explants was recorded before inoculating on the growth medium and dry weight was recorded for the same explant after drying in the oven at 60 °C for 24h. Subsequent fresh and dry weights of the cultures were recorded at interval of 5 days up to 30 days. Initial weight of node and leaf are 0.096±0.01and 0.051±0.03 respectively. Initially dry weight of node and leaf are 0.016±0.01 and 0.004±0.00 respectively. Thus obtained data was tabulated for further analysis (Table 1).

Experimental Design and Statistical Analysis

All the experiments were conducted with a minimum of 10 replicates per treatment. Each explant was taken as a replication and all the experiments were repeated 3-times. The results were expressed as the Mean±SE for all experiments as given by Snecdecor[23]. The effect of different treatments was quantified and the level of significance was determined by analysis of variance (ANOVA) and least significant difference (LSD) at $\alpha = 0.05$ probability level.

RESULTS AND DISCUSSION

In the present study cultures were established from leaf and nodal explants of *C.paniculatus* derived from an approximately 15year old plant. Cultures responsiveness was found to be better for the explants collected during February- April. However, those collected during other periods were found to be less responsive and exhibited more contamination. The seasonal effect of culture establishment has also been reported in *Azadirachta indica*[24], *Salvadora persica*[25] and *Sauropus androgynous*[26]. Direct regeneration of shoot was also found in the present study from different combinations of auxin and cytokinin but the present study was to develop successful protocol for mass propagation through indirect regeneration. High percentage of green nodulated callus was observed in the explants cultured on MS medium containing NAA+BAP/TDZ compared to L_2, and MMS media. The results confirm the morphogenetic potential of *Celastrus* explants to form callus. In general, callus induction was not much influenced by the growth regulator types or combination tested [27, 28]. Cultures from Calli are potentially useful as a method for commercial propagation due to its ability of achieving high multiplication rate [29]. Colors of callus varied from white to green (Figs. 2A-B). Calli obtained from leaf were green hard nodulated compared to other explants. Leaves give the best response for callus initiation in *Munronia pinnata*[30] and *Cajanus cajan*[31]. To the best of our knowledge there is no literature available on the use of leaf explants of *C. paniculatus* for plant regeneration except cotyledonary leaf explants [32] and Inflorescence explants by Gowdru[33]. Among the cytokinins and other concentrations tested NAA (0.53µM) + BAP (8.87µM) were found to be most favorable for callus induction. (Table 2).

Table 1
Effect of NAA+BAP on the growth of Callus proliferation from nodal and leaf cultures of *Celastrus paniculatus*.

SL.No	*Time of Interval (days)*	*Node*		*Leaf*	
		Fresh Weight (gm) (Mean±SE)	*Dry Weight(gm) (Mean±SE)*	*Fresh Weight(gm) (Mean±SE)*	*Dry Weight(gm) (Mean±SE)*
1	0 day	0.096±0.01^{g}	0.016±0.01^{g}	0.051±0.03^{fg}	0.004±0.00^{fg}
2	5	0.132±0.00^{f}	0.024±0.00^{f}	0.071±0.00^{f}	0.006±0.01^{f}
3	10	0.185±0.04^{e}	0.033±0.00^{de}	0.244±0.02^{e}	0.046±0.00^{e}
4	15	0.234±0.03^{cd}	0.041±0.04^{d}	0.320±0.03^{d}	0.078±0.03^{cd}
5	20	0.280±0.07^{c}	0.056±0.01^{c}	0.579±0.00^{c}	0.085±0.02^{c}
6	25	0.307±0.01^{ab}	0.071±0.00^{b}	0.597±0.00^{ab}	0.098±0.02^{ab}
7	30	0.333±0.00^{a}	0.083±0.02^{a}	0.614±0.01^{a}	0.109±0.02^{a}

Data represented by Mean±SE of three independent experiments. Mean followed by the different letter within columns are significantly different ($p<0.05$) using Duncan's multiple range test.

Table 2
Effect of different concentrations of NAA+BAP+L-glu on multiple shoot regeneration from Callus culture of *Celastrus paniculatus* on MS medium.

NAA(μM)	*BAP(μM)*	*L-glu(μM)*	*Shoot Numbers (Mean±SE)*	
			Callus-Nodal Explant	*Callus-Leaf Explant*
0.53	4.43	0.68	8.66±0.25^{b}	7.33±0.05^{d}
1.07	4.43	3.42	12.33±0.05^{a}	13.0±0.00^{a}
0.53	6.65	0.68	8.33±0.05^{bc}	12.33±0.05^{b}
1.07	6.65	3.42	5.66±0.25^{d}	12.0±0.00^{bc}
0.53	8.87	0.68	5.0±0.00d^{e}	6.33±0.05^{e}
1.07	8.87	3.42	4.0±0.00^{f}	4.33±0.05^{f}
0.53	11.1	0.68	3.0±0.35^{g}	4.0±0.00^{gh}
1.07	11.1	3.42	3.0±0.00^{gh}	3.33±0.05^{i}
0.53	13.3	0.68	2.66±0.25^{i}	4.0±0.04^{g}
1.07	13.3	3.42	2.0±0.25^{ij}	2.33±0.05^{j}

Data represented by Mean±SE of three independent experiments. Mean followed by the different letter within columns are significantly different ($p<0.05$) using Duncan's multiple range test.

Figure 1: Morphology of *C.Paniculatus.*

(A) Woody liana
(B) Inflorescence
(C) Tricarpellary fruit
(D) Seeds

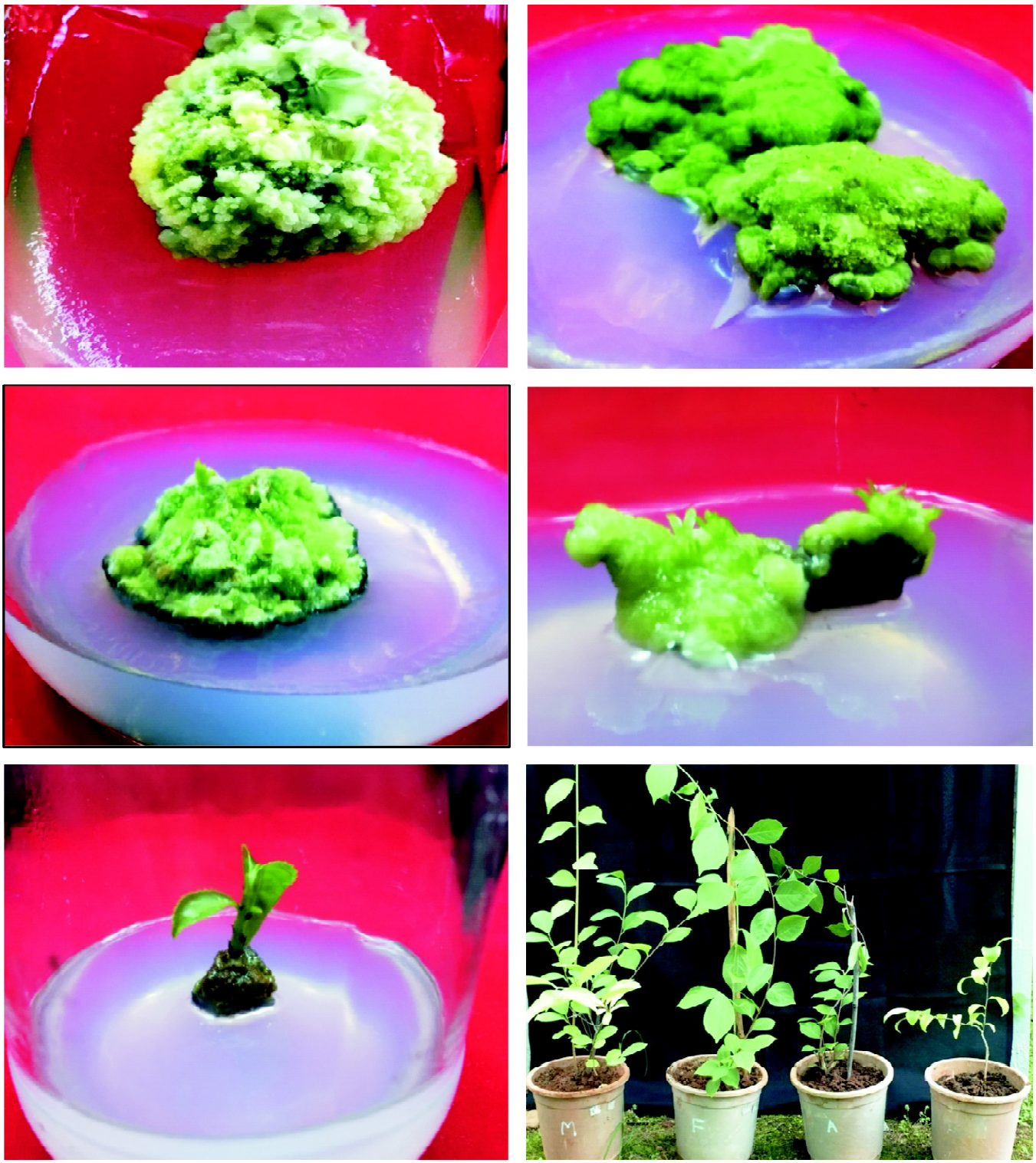

Figure 2: Micropropagation of *C.paniculatus*.

(A) Nodal Explant Showing White Callus on MS+NAA (0.53µM) +Kin (4.64µM),
(B) Leaf explants Showing Nodulated Green Callus on MS+NAA (0.53µM) + BAP/ TDZ (8.87µM)
(C,D) Indirect Regeneration through Callus obtained from Leaf on MS+NAA (0.53µM) +BAP (4.43µM) + L-glutamine (3.42µM)
(E) Single Shoots sub-cultured in rooting medium
(F) Hardening of tissue cultured plants in pots.

Sub-Culture for Shoot Initiation from Callus

Thus obtained green nodulated callus were repetitively sub-cultured to the fresh shoot developing MS medium NAA (1.07µM) +BAP (4.43µM) +L-glutamine (3.42µM). The highest number of shoots 12-14 per explants was produced during these passages (Fig. 2C-D). Glutamine plays a major role in friable-callus initiation and somatic-embryoid formation increased linearly with addition to L-glutamine in medium [34, 35]. Repeated transfer of the original explants was suggested as an efficient technique for rejuvenation and reinvigoration of *in-vitro* culture [36], which was further supported by subsequent reports on different plants [37,38,39].

Rooting and acclimatization of plantlets

Compared with NAA and IAA, IBA was found to be better auxin for root induction. Here root dip treatment was used to induce rooting. It showed healthier and longer roots compared to other methods with 85% of root induction. Similar work has been done using IBA for root induction by Turker, Casako and Singh lal[40,41,42]in other taxa. Plants were transferred to greenhouse for secondary hardening and such plantlets are transferred to polybags containing sterilized soil and sand (3:1) (Fig. 2F). Using the described protocol, 70% of plants have been produced by Indirect Organogenesis.

CONCLUSION

The improved micro propagation (Indirect regeneration) through leaf is first of its type in *C.paniculatus,* the protocol discussed here could be exploited for large scale multiplication of this taxa, a threatened medicinally important plant. Continuous supply of plants by this method will reduce the pressure on the species natural population and thus help conserve it.

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