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An Effective Protocol for *In Vitro* Multiplication of *Celastrus paniculatus* Willd. - An Important Medicinal Plant

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Abstract

Celastrus paniculatus Willd. a threatened taxon belongs to the family Celastraceae is one of the important medicinal plants. The seeds contain alkaloids and the oil extracted from them has long been regarded as highly beneficial and medicinally effective. Due to its medicinal importance, the plant has been over exploited and effective measures are needed to be taken to conserve the precious germplasms. In present study, an effective protocol for micropropagation has been developed through nodal cultures. Nodal explants from mature plant were collected and cultured on MS medium supplemented with various concentrations of auxins (IAA,IBA,NAA,2,4-D) and cytokinins (BAP,Kin,2-ip,Zea,TDZ) either alone and in combinations under controlled photoperiod and temperature. Maximum number of 15.43±0.17 shoots was regenerated directly from the nodal explants on MS medium supplemented with BAP+NAA+GA_3. Histological studies have confirmed the direct regeneration of shoots from the cultures. Thus, obtained shoots were sub-cultured to rooting medium containing auxins for root induction. The regenerated plantlets were successfully acclimatized on mixture of organic manure, clay and sand (1:1:1) and 75% of regenerates were survived.

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Introduction

Celastrus paniculatus belongs to family Celastraceae is an important medicinal plant whose curative properties are yet to be fully exploited though the plant is used extensively in pharmaceutical industries and indigenous systems of medicine. Chemical constituents as revealed by phytochemical analysis were alkaloids like celastrin, paniculatin, celapagine, celapanigine and celapanine (CSIR, 1992). The seed oil is bitter, thermogenic, intellect promoting and is useful for treating abdominal disorders, beriberi and sores (Warrier et al., 1994). Apart from seeds, other parts like bark, leaf and roots are considered as having medicinal properties (Rastogi and Mehrotra, 1998; Govil, 1993; Parotta, 2001). Since all the parts of the plant are used in various indigenous systems of medicine, destructive harvesting has led to the threatened status of the taxon (De Silva and Senarth, 2009; Lal and Singh, 2010). The species is vulnerable in the Western Ghats of South India (Rajashekharan and Ganeshan, 2002). The consequences are possible for the verge of extinction and this provides the justification for conservation and propagation of this valuable plant. Further, increasing demand for the essential oils and chemicals by pharmaceutical

industries has necessitated large-scale production of plant. Since the percentage of seed germination is low (11.5%) and other vegetative propagation methods cannot be used for cultivation (Rekha et al., 2005), *in vitro* culture technique is emerging recently as an alternative method for conservation and propagation of threatened taxa. Through, there are reports on mass multiplication through direct organogenesis (Martin et al., 2006; de Silva and Senarth, 2009; Lal and Singh, 2010), the present study is an attempt to develop an efficient and improvised protocol for induction of multiple shoots from the nodal cultures.

Materials and methods

Explant source

Approximately 15-year-old healthy mature plant of *C .paniculatus* maintained in GKVK, University of Agricultural Sciences and plants growing in outskirts of Yelahanka, Bangalore, Karnataka, India were used as source plants to collect the explants for *in vitro* cultures (Fig. 1A and 1D).

Surface sterilization

Nodes (1cm) were used as explants. They were surface sterilized sequentially in running water for 30min with few drops of Tween-20 (Himedia, India) and 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. Explants were then surface sterilized under aseptic conditions with freshly prepared 0.1% mercuric chloride solution for 2-5min and then washed with sterile water. The materials were then treated with 0.1% streptomycin for 2-5min to avoid bacterial infections. Explants were washed thoroughly 4-5 times after each treatment in sterile double distilled water before inoculating them on various media in laminar airflow chamber.

Media and culture conditions

Murashige and Skoog's (MS, 1962), Phillips and Collins (L2, 1979) and MMS containing salts of MS medium and vitamins of L2 medium supplemented with various concentrations and combinations of both auxins and cytokinins were used to raise the cultures. Sucrose (3%) and agar-agar (0.8%) were added as carbon source and gelling agent respectively. The pH of the medium was adjusted to 5.8±0.2 and media was then autoclaved for 15min at 121°C. Cultures were maintained at 22±2°C under 16h day-¹ photoperiod with a light intensity of 40-50µmol^{-1} photon flux density, provided by white fluorescent lamps (Phillips, India).

Rooting and acclimatization

The excised shoots were sub-cultured to MS media supplemented with NAA, IBA and IAA at various concentrations for root induction. After the development of roots, none of the plantlets survived when they were directly transferred from rooting medium to the potting mixture and grown under natural conditions. However, the survival percentage rose to 75% after the plantlets were hardened under aseptic conditions. *In vitro* raised plantlets were transferred to pots containing soilrite before shifting them to polybags containing mixture of organic manure, soil and sand (1:1:1). Such plantlets were kept in the greenhouse for 7-8weeks for hardening before transferring them to field for the evaluation of percentage of survival of plants.

Histology

Segments of morphogenic callus were fixed in FAA (Formalin: Acetic Acid: Alcohol) for 48h. Customary paraffin technique was followed for histological studies. Microtome sections of about 15-18µm thickness were taken and stained with Haidenhain's haemotoxylin and counter stained with Orange-G/Eosin. Photomicrographs of sections were taken with Nikon binocular microscope using Cannon camera.

Data analysis

All the experiments conducted were repeated thrice in 5-replicates. The mean was calculated and represented as Mean±SE. The data was subjected to one way ANOVA for analysis. Significant 'F' ratios between group mean were further subjected to DMRT using SPSS. Probability values<0.05 were considered significant (Snedecor, 1946).

Results and discussion

Initially MS, L2 and MMS media were used to raise the cultures. However, MS medium was found more suitable in supporting the growth of cultures. Murashige and Skoog's medium is highly nutritious containing high concentrations of ammonium salts used widely to raise the cultures of various plants including *C. paniculatus*

(George and Sherrington, 1984; Nair and Seeni, 2000). Hence, the data was accordingly recorded for the cultures grown on MS medium (Table 1). Explants were failed to respond on basal medium. Explants were then inoculated on MS supplemented with various concentrations of auxins and cytokinins either alone or in combinations. Explants on auxin-supplemented media had not shown any response even after four weeks of culture. However, presence of cytokinins in the medium promoted either the growth of the axillary meristem into single shoot or induction of multiple shoots depending on the type and concentration of the growth regulators. Proliferation of seven to eight shoots was recorded from the nodal cultures grown on MS+BAP (6.65µM) in the present study. However, Rao and Purohit (2006) have reported the regeneration of 3-4 shoots from the nodal cultures grown on MS+BAP (4.44µM). However, 8-10 shoots are recorded in the present study from the nodal cultures grown on MS+BA (2.12µM) (Fig. 2C). Similar work was reported from the cultures grown on BA supplemented medium (Martin et al., 2006).

Table1. Effect of different concentrations of growth regulators on multiple shoot regeneration from nodal culture of *Celastrus paniculatus* on MS medium.

MS media with combinations of hormones (µM)	Shoot Numbers (Mean ± SE)
NAA(0.53 µM)+BAP(4.43)+GA_3(0.28 µM)	11.0 ± 0.0^{b}
NAA(1.07 µM)+BAP(4.43)+GA_3(0.57 µM)	15.43 ± 0.17^{a}
NAA(0.53 µM)+BAP(6.65)+GA_3(0.28 µM)	8.66 ± 0.14^{c}
NAA(1.07 µM)+BAP(6.65)+GA_3(0.57 µM)	6.33 ± 0.10^{d}
NAA(0.53 µM)+BAP(8.87)+GA_3(0.28 µM)	5.32 ± 0.10^{e}
NAA(1.07 µM)+BAP(8.87)+GA_3(0.57 µM)	4.66 ± 0.14^{f}
NAA(0.53 µM)+BAP(11.1)+GA_3(0.28 µM)	3.0 ± 0.00^{gh}
NAA(1.07 µM)+BAP(11.1)+GA_3(0.57 µM)	3.66 ± 0.14^{g}
NAA(0.53 µM)+BAP(13.3)+GA_3(0.28 µM)	2.66 ± 0.20^{i}
NAA(1.07µM)+BAP(13.3)+GA_3(0.57 µM)	2.33 ± 0.23^{ij}
NAA(1.07µM)+BA(0.42)+GA_3(0.57 µM)	3.0±0.00
NAA(0.53 µM)+BA(2.12)+GA_3(0.28µM)	8.66±0.14

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.

Arya et al. in 2001 have obtained 3-4 shoots from the nodal cultures of *C. paniculatus* on MS supplemented with various concentrations of ascorbic acid in addition to adenine sulphate, arginine and citric acid. When Kin was supplemented to MS, a single shoot has grown from the axillary meristem (Fig.1E) which is in agreement with the report of Yadav et al. (2011), where a growth of a single shoot was observed from the nodal cultures of *C. paniculatus* on Kin supplemented medium. BAP found to be more efficient than Kin in promoting the morphogenetic potential of the explants. Similar observations have been made in other species including *C. paniculatus* (Kumar and Singh, 2009; De Silva and Senarth, 2009; Tejavathi and Padma, 2011). However, combinations of two cytokinins such as BAP and TDZ resulted in the growth of callus from both node and leaf explants (node and leaf explants (Tejavathi and Niranjan, 2016). BAP (6.65µM) +TDZ (0.45µM) has induced green nodulated callus after four weeks of culture (Fig.1F). Critical balance between the concentrations of auxins and cytokinins in the cultures determines the specific morphogenetic potential of the explants (Skoog and Miller, 1957). MS media supplemented with combination of auxin and cytokinins in lower concentration initiated multiple shoots. The highest mean number of shoots (15.43±0.17) observed from nodal segments on MS medium supplemented with NAA (0.53µM) + BAP (8.87µM) +GA_3 (0.28µM) (Fig 2A-2B). As the concentration of BAP increases, the morphogenetic potential also reduced with less number of shoot proliferation. Whereas, Kin along with NAA had induced the growth of the axillary bud into a single shoot. Organogenesis depends upon hormonal balance and other factors such as plant tissue, environmental conditions, and compositions of the medium, polarity and growth substances (George and Sherrington, 1984).

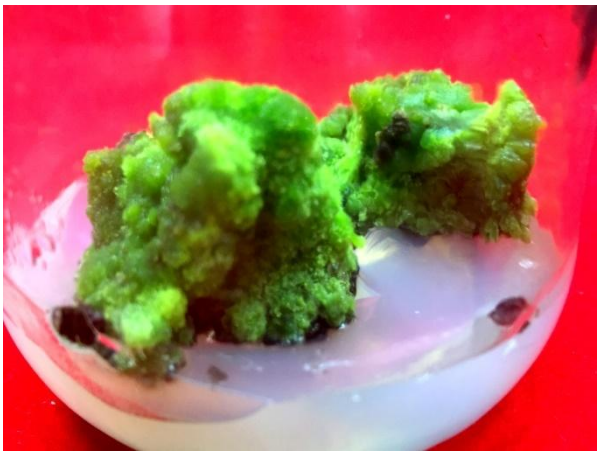
A - Woody liana

B - Young flowers

C - Inflorescence

D - Tricarpellary fruits

E- Nodal explant showing single shoots on MS+NAA (0.53µM) +Kin (4.64µM).



F - Nodal explant showing green callus on BAP (6.65µM) +TDZ (0.45µM).

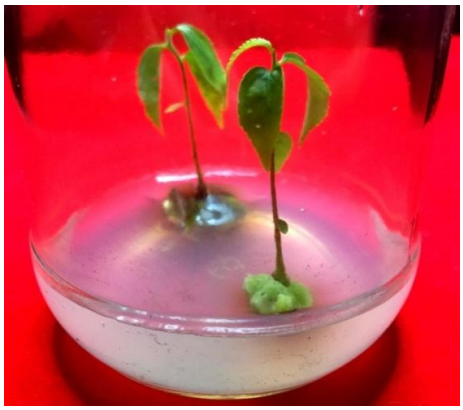
Fig. 1: Morphology and morphogenetic potential of *C. paniculatus*.

Thus, obtained regenerated shoots were excised from the cultures and transferred to various strengths of MS supplemented with different concentrations auxins.

Induction of healthy roots was obtained on half strength MS salts containing 1% Sucrose with 0.6% agar as a gelling medium supplemented with IBA (4.90µM) and NAA (1.07µM). NAA proved to be best with 80% rooting and very less callusing at the base followed by IBA and IAA (Fig 2D).

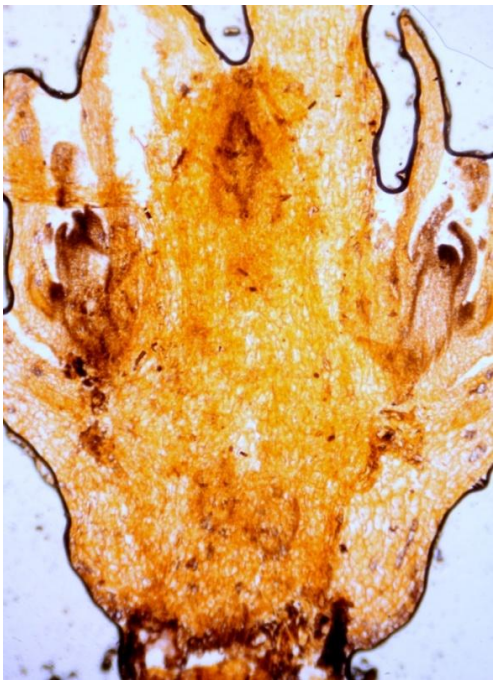
A, B- Multiple shoots on MS+ NAA (0.53µM) +BAP (8.83µM) +GA_3 (3.42µM)

C- Multiple shoots on MS+ NAA (0.53µM) +BA (2.12µM) +GA_3 (3.42µM)



D- Callusing at base of the regenerated shoot when treated with IAA

E- Hardening of tissue cultured plants in pots.



F - Microtome section of nodal cultures showing direct regeneration of multiple shoots. Arrows indicate the proliferation of axillary buds.

Fig. 2: Micropropagation of *C. paniculatus* through nodal cultures.

Peeters et al. (1991) found that NAA was taken six times faster than IAA in inducing roots from the basal parts of shoots. Consequently, the efficacy of rooting in the presence of NAA may be due its faster uptake. This may be due to variation in the route of auxin uptake (De-Klerk et al., 1997). The rooting response differed according to different concentrations and combinations of auxins used (Gaspar and Coumans, 1987). Similarly, Singh and Lal (2007) also achieved root induction on MS medium fortified with 0.5 or 1.0mg l^{-1} of NAA in *Leucaena leucocephala*. The rooted plantlets immediately after removed from agar medium were subjected to *in vitro* hardening in culture bottles containing autoclaved soilrite, which is moistened with liquid ¼ MS salts. Bottles were subsequently transferred into greenhouse condition and plants are gradually transplanted into pots containing mixture of organic manure, soil and sand (1:1:1) and were allowed to grow in nursery shade conditions (Fig. 2E).

Histology

The microtome sections of the cultures have confirmed the direct origin of multiple shoots from the cultures (Fig. 2F). Even the axillary buds of proliferated shoots were seen growing along with apical bud conforming the overcoming of apical dominance in culture conditions (van Staden et al., 2008). A delicate balance between the cytokinins, auxins and gibberllins (Woolley and Wareing, 1972) controls lateral bud development.

Conclusion

It can be concluded by above data that the described method can be successfully employed for large-scale production, multiplication, and establishment of *Celastrus paniculatus* in *ex situ* conditions.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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