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Research Article
Development of an Efficient Technology for Rapid Clonal Multiplication of *Celastrus paniculatus*–Willd, an Endangered Medicinal Plant

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Abstract

Background and Objective: *Celastrus paniculatus* Willd. (*Celastraceae*) is a large, woody climbing shrub commonly known as 'Jyotishmati', "Intellect tree" or 'Bitter sweet' is an important medicinal plant in India. Due its high pharmaceutical applications, this species has been over exploited and is now considered as endangered species. The present investigation was focused on development of an efficient shoot induction protocol for the large scale production of *Celastrus paniculatus*. **Materials and Methods:** The pathological screening of *in vitro* propagated progenies was done to ensure their disease free nature. Murashige and Skoog (MS) basal medium supplemented with varying combination of growth regulators such as Ammonium nitrate (NH_4NO_3), Benzyl Amino Purine (BAP) and Kinetin (Kin) with a concentration ranging from 0.5-3.0 mg L^{-1} for shoot induction. To detect the presence of any microbial growth, the *in vitro* derived clones with 0.5 mm in size were transferred into Potato Dextrose Agar (PDA) or Plate Count Agar (PCA) medium and the visual observation for the detection of endogenous and exogenous fungal and bacterial growth. A random sampling of 50% of *in vitro* propagated plants listed for microbial contamination. The results were recorded and expressed as Mean±SD for all the experiments. **Results:** The maximum number of shoots was induced in MS medium with 1.5 mg L^{-1} of BAP, it was 4.67±1.58 shoots. Subsequent culture on medium with 1.0 mg L^{-1} of BAP facilitated rapid multiplication and a mean of 30.52±2.64 shoots were developed. The developed shoots were healthy and a height of approximately 8 cm. **Conclusion:** The developed protocol was successfully employed for the large scale production of this endangered species. The pathological screening revealed the disease free nature of the developed shoots.

Key words: *Celastrus paniculatus*, *in vitro* propagation, shoot induction, plant growth regulators, pathological screening

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Plant Biotechnology has immense role in the field of micropropagation, conservation and improvement for the production of various secondary metabolites with therapeutic applications[1]. Previous studies reported that *in vitro* propagation as an efficient technique in plant biotechnology for the rapid and efficient propagation of plants from a minimum plant material[2]. Plant hormones such as auxins and cytokinins have a vital role in the developmental pathway of plant cells and tissues in culture medium[3].

Celastrus paniculatus-Willd belonging to the family Celastraceae is an endangered medicinal plant, especially distributed in tropical and subtropical regions of India. It contains a large number of bioactive constituents such as malkanguniol, malkangunin, celapanine, celapanigine, celapanin, celapagin, celastrine, paniculatine, celastrol, pristimerin, zeylasteron, zeylasteral[4] etc. It has a beneficial role on learning and memory process in mentally retarded children[5] and also used for the treatment of various diseases includes beri-beri, malaria, anxiety, asthma and leucoderma[6]. Many pharmacological studies have proven the antioxidant, anti-inflammatory and anticancerous activity of this plant[7]. Because of its high pharmaceutical application, lack of proper vegetative propagation method, poor seed viability and low germination percentage[8], this plant species is now considered as endangered species. Propagation through tissue culture is a viable alternative in this species because it could also be used as a complimentary strategy for conservation and utilization of genetic resources.

A few studies on callus induction, shoot induction and multiplication[9], bud differentiation and micropropgation through adventitious root formation[10] have been reported for this plant species. This study evaluated the effect of different concentration of NH_4NO_3, BAP and Kin on *in vitro* shoot induction responses in *Celastrus paniculatus* and the pathological screening of *in vitro* raised clones with PDA medium. The available reports on the micropropagation of *C. paniculatus* could not reproduced. So the objective of the study is the large scale production of this endangered species *Celastrus paniculatus* -Willd by applying MS medium fortified with different concentrations of selected plant growth regulators.

MATERIALS AND METHODS

The experiment was done at the laboratory of Department of Biotechnology, University of Calicut on 2016 June-December. The chemicals used for the experiments were procured from Himedia, Mumbai; Merck, Mumbai; SRL, Mumbai; Sigma-Aldrich, Germany and Qualigens, Mumbai. The chemicals used were of analytical grade.

Plant material: An approximately 10 year old plant of *Celastrus paniculatus* was collected from the Botanical garden, University of Calicut was selected for the present study. The collected plant was identified and authenticated from Department of Botany, University of Calicut. A voucher specimen with Accession No 6890 has been deposited in the hebarium of the same Department. The nodal cuttings, shoot tips and midrib of the leaves with approximately 3cm in length were used as explant.

Chemicals, Culture vessels and instruments: All the chemicals and media components were purchased from Hi-Media Pvt.Ltd, Mumbai, India. The *in vitro* culture studies were performed in culture tubes (25×100 mm, 25×200 mm), conical flasks (250 mL) of glass (Borosil) and culture bottles (53×125 mm; 20 mm thick). Cotton plug made up of non-absorbent cotton wrapped with cotton guaze were used as closures for culture tubes and conical flasks, while polypropylene autoclavable lids (14 mm height; 48 mm inner diameter; 20 mm thick) were used for jam bottles. The glassware after thorough wash with Extran (a neutral liquid detergent; Merck India Ltd; Mumbai) solution were washed well in running tap water followed by rinses with distilled water. The culture vessels were dried at 100°C in a hot air oven (Kemi make). Double distillation unit and Millipore water purification system (ELIX-3) was used for collecting water for media and stock preparation. The electric balance (Sartorious and Denver) was used for accurate weighing of chemicals, sucrose, agar and growth regulators for preparation of stock solution and tissue culture medium.

The magnetic stirrer (Remi make) was used for the proper mixing of chemicals, sucrose etc. for stock and media preparation. The pH meter (Cyberscan 510, Eutech, Singapore) was used for adjusting the pH of the media. Microwave oven (Sharp) was used for melting agar by boiling the media. Autoclave (Nat steel and Inlab) was used for the sterilization of media, forceps, blade holders, petriplate, cotton and all other items required for performing aseptic culture. To reduce the chance of contamination, transfer of the explants were done in laminar air flow chamber. A glass bead sterilizer (Steripot) was used for heating forceps and blade holders during aseptic transfer. The Olympus trinocular compound research microscope (CX41) with microphotographic attachment (digital camera E330) was used for stomatal studies. Olympus digital camera (CAMEDIA C5060) was used for taking the photographs of *in vitro* cultures and plates.

Surface sterilization: The endophytic microflora is a major cause of contamination in tissue culture technique, so in order to prevent the chance of contamination surface sterilization was done. The explants were washed in running tap water for 30 min, then treated with 0.1% (w/v) mercuric chloride ($HgCl_2$) and Tween 20 (2 drops per 100 mL) for 5 min and washed with distilled water 4 or 5 times. Explants were then taken into the laminar air flow chamber and treated with 0.1% (w/v) $HgCl_2$ for 2 min and washed with sterile double distilled water 4 or 5 times. The cut ends of the explants were trimmed off and the culture were aseptically transferred in to MS medium supplemented with 3% sucrose, 0.7% agar.

Media preparation: The full strength MS medium[11] was modified with different plant growth regulators such as NH_4NO_3, BAP and Kin were used in order to study the shoot induction responses. The stock solutions of MS medium such as macronutrients, micronutrients, iron chelators and vitamins were prepared separately and kept under 4°C. For *in vitro* studies, the pH of the medium was adjusted to 5.8 prior to the addition of agar. The culture medium was autoclaved at 120°C for 20 min. The cultures were maintained at 24±2°C under 12 h photoperiod (provided by fluorescent lamb). Each treatment was carried out with 12 culture bottles with single explant per bottle and each experiment repeated as twice. The response in various media was expressed in terms of mean number of shoots, roots, leaves per explants and length of shoots after 20, 40 and 60 days of regular intervals.

Shoot induction: Here the shoot induction was carried out in MS medium supplemented with varying concentrations of NH_4NO_3, BAP and Kin (0.5-3.0 mg L^{-1}) alone to study their effect on shoot induction responses in *Celastrus paniculatus*. The nodal segments, shoot tips and midrib were used as explants and the cultures were incubated in 12 h photoperiod. The medium favoured the shoot induction. The observations in terms of number of shoots per explant, shoot length and number of leaves per shoot were done at regular intervals of 20, 40 and 60 days.

Stomatal study: The lower surface of leaf was pealed and the green chlorophyll was removed by gentle scratching with scalpel. The thin epidermal layer was then stained using saffranin for about 10-15 min and mounted on a microscopic slide using glycerin and viewed under microscope. Type, nature and status of stomata were observed.

Pathological screening of *in vitro* raised clones: Pathological screenings were done to ensure the disease free nature of the *in vitro* raised clones. To detect the presence of any microbial growth, the *in vitro* derived clones with 0.5 mm in size were transferred into Potato Dextrose Agar (PDA) or Plate Count Agar (PCA) medium. These plates were incubated at dark and observed for 2 week at a regular interval of 24 h. The visual observation for the fungal growth or any disease symptoms were recorded throughout the growing season.

Observation and data analysis: The experimental designs were completely randomized. Twelve replicates were used for each treatment. The results were recorded and expressed as the Mean±SD for all the experiments[12]. The cultures were observed at an alternate days for factors like contamination and growth response. Data on growth response were recorded after every 20, 40 and 60 days in all trials. The observations were compared and the difference in their growth pattern was studied. Percentage of culture in terms of number of shoot, root and leaves and length of shoot and root was taken as parameters for analyzing the growth.

RESULTS

Shoot induction: The shoot induction response showed that among the applied growth regulators BAP exhibited superiority over NH_4NO_3 and Kin. The nodal explants cultured on MS medium without any growth regulators facilitated only slight emergence of 1 or 2 shoots. MS medium modified with different growth regulators such as NH_4NO_3, BAP and Kin with a concentration of 0.5-3.0 mg L^{-1} favoured the shoot induction responses from the nodal explants. The length and number of induced shoots relied on the levels of NH_4NO_3, BAP and Kin. Shoot inductions were obtained within 12 days of culture incubation and data on growth responses were recorded every after 20, 40 and 60 days. The results are detailed below separately.

Shoot induction responses after 20 days: The nodal explants of *Celastrus paniculatus* showed specific response in all applied concentrations of NH_4NO_3, BAP and Kin (0.5-3.0 mgL^{-1}). Of the different levels of NH_4NO_3 concentrations added to MS medium maximum number of shoots were obtained in 1.0 mgL^{-1} and the number of leaves were maximum in 2.0 mg L^{-1} NH_4NO_3 and minimum in 3.0 mgL^{-1} NH_4NO_3. The higher levels above the optimal concentration lead to the decline of number of shoots and leaves formed. Lengths of shoots were higher in 2.0 mg L^{-1} and the higher and lower levels from the optimum concentrations showed gradual decrease in the length of

shoots. Various levels of BAP supplement to the MS medium, maximum number and length of shoots were obtained at same concentration i.e., 3.0 mg L^{-1} for both shoot number and shoot length. The maximum number of leaves per explants was obtained in MS medium enriched with 2.0 mg L^{-1} of BAP.

When comparing the effect of applied growth regulators (NH_4NO_3, BAP and Kin with a concentration of 0.5-3.0 mg L^{-1}) after 20 days of culture period, it was concluded that , the maximum number of shoot response was in medium with 1.0 mg L^{-1} of NH_4NO_3 . Maximum shoot length was obtained in 3.0 mg L^{-1} of Kin. In case of number of leaves the maximum responses were obtained in medium with 2.0 mg L^{-1} of BAP. The leaves were with pale green in color and not fully developed. The results are shown in Table 1 and Fig. 1.

Shoot induction response after 40 days: After 40 days of culture period, there is a significant increase in the growth response over 20 days. In the case of MS medium with NH_4NO_3, the best response in terms of numbers of shoots was obtained in 1.0 mg L^{-1} of NH_4NO_3 . The length of shoot was highest in medium with 1.5 mg L^{-1} of NH_4NO_3 .When considering the number of leaves the most responsible concentration was MS supplemented with 2.0 mg L^{-1} of NH_4NO_3. In the case of BAP, the number of shoots and number of leaves were highest in MS medium supplemented with 1.0 mg L^{-1} of BAP. Whereas in the case of length of the shoots the best response was obtained in MS medium supplemented with 2.5 mg L^{-1} of BAP. In the case of MS medium with Kin the highest number and length of the shoots were in 2.0 mg L^{-1} of Kin. The maximum numbers of leaves were obtained in 2.5 mg L^{-1} of BAP. The results are tabulated in Table 2.

So from the above results it was concluded that after 40 days of culture period, the highest number of shoots were obtained in medium with 1.0 mg L^{-1} of BAP. In the case of length of the shoot MS medium with 1.5 mg L^{-1} NH_4NO_3 and in the case of number of leaves the maximum response were obtained in MS medium with 2.0 mg L^{-1} of NH_4NO_3.

Shoot induction response after 60 days: After 60 days of culture period the growth response was reduced and vitrification was observed in culture bottles. The rate of vitrification was higher in MS medium supplemented with Kin. The higher growth responses in terms of number of shoots were noted in MS medium with 1.5 mg L^{-1} of BAP. The more lengthy shoots were produced in MS medium with 1.0 mg L^{-1} of BAP. The number of leaves produced was higher in MS medium supplemented with 3.0 mg L^{-1} of Kin. The results were shown in Table 3.

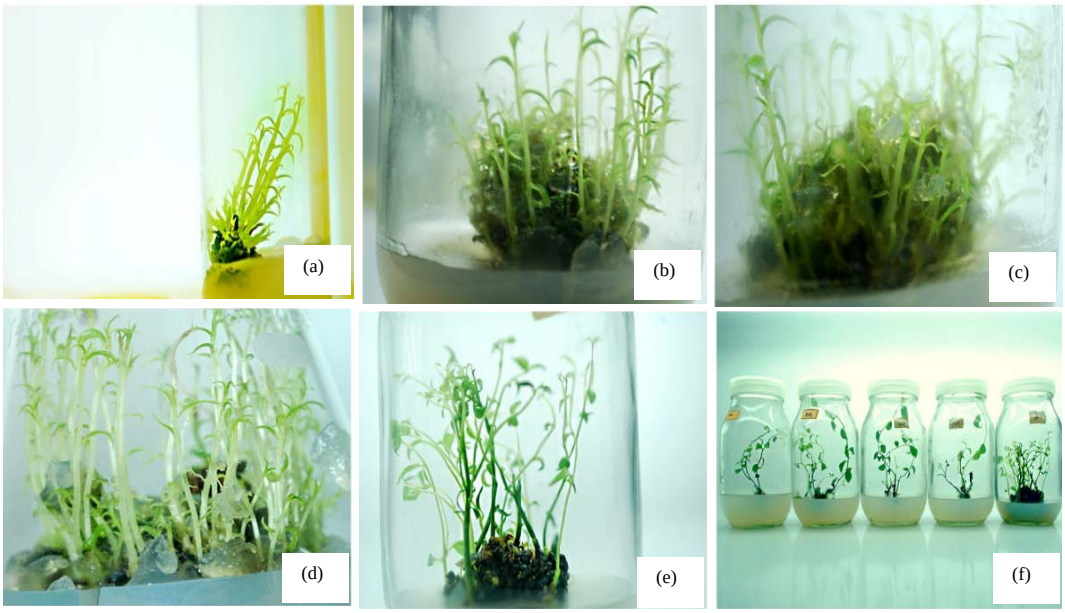

Fig. 1(a-f): Different stages of *in vitro* response in culture derived internode segments and midrib of *Celastrus paniculatus* on MS medium supplemented with varying concentrations of phytohormones, (a) Shoot induction response after 15 days on Ms medium supplemented with 2.0 mg L^{-1} of NH_4NO_3, (b-d) Shoot multiplication response on MS medium with BAP (1.0 mg L^{-1}) and (e-f) Growth response just before hardening

Table 1: Effect of NH_4NO_3, BAP and Kin on shoot induction responses of *in vitro* cultures of *Celastrus paniculata* after 20 days of incubation

Media combination (mg L^{-1})	No. of shoot	Length of shoot (cm)	No. of leaves
Control	1.19±0.32	3.42±2.12	2.22±0.62
MS+1.0 NH_4NO_3	2.93±0.86	6.78±1.57	3.04±0.88
MS+1.5 NH_4NO_3	1.89±0.70	5.73±1.73	2.96±0.70
MS+2.0 NH_4NO_3	1.76±0.65	6.88±1.04	3.11±0.96
MS+2.5 NH_4NO_3	1.74±0.83	6.66±1.49	2.67±0.63
MS+3.0 NH_4NO_3	1.65±0.60	6.32±1.90	2.55±0.60
MS+1.0 BAP	1.36±0.45	7.36±1.32	3.85±2.68
MS+1.5 BAP	1.47±0.48	6.66±1.29	3.62±0.98
MS+2.0 BAP	1.35±0.56	6.25±0.88	4.74±0.69
MS+2.5 BAP	1.52±0.78	7.04±2.43	3.86±0 98
MS+3.0 BAP	1.64±0.32	8.15±2.57	3.59±0.32
MS+1.0 Kin	1.89±0.45	10.12±1.09	2.36±0.48
MS+1.5 Kin	2.01±0.79	9.48±1.17	2.17±0.35
MS+2.0 Kin	1.92±0.64	8.54±1.32	2.38±0.47
MS+2.5 Kin	2.43±1.62	9.39±1.78	1.68±0.48
MS+3.0 Kin	2.10±0.72	10.28±1.79	2.26±0.78

Values are an average of 12 replicates and expressed as the Mean±SD for all the experiments

Table 2: Effect of NH_4NO_3, BAP and Kin on shoot induction of *in vitro* cultures of *Celastrus paniculatus* after 40 days of incubation

Media combination (mg L^{-1})	No. of shoot	Length of shoot (cm)	No. of leaves
Control	1.28±1.68	4.32±1.23	1.36±0.24
MS+1.0 NH_4NO_3	3.36±1.12	8.69±1.46	3.36±0.16
MS+1.5 NH_4NO_3	2.82±0.88	9.56±1.28	3.62±0.47
MS+2.0 NH_4NO_3	2.92±1.54	8.83±1.68	4.27±1.22
MS+2.5 NH_4NO_3	3.23±1.69	8.51±1.79	3.96±0.36
MS+3.0 NH_4NO_3	3.10±0.42	7.67±1.63	3.47±0.43
MS+1.0 BAP	4.83±1.46	8.86±1.68	3.67±0.98
MS+1.5 BAP	4.67±1.16	8.52±1.40	3.59±0.71
MS+2.0 BAP	3.26±0.44	7.50±0.56	3.27±0.80
MS+2.5 BAP	4.78±1.06	9.38±2.58	3.45±0.75
MS+3.0 BAP	4.60±1.47	9.16±1.16	3.23±0.59
MS+1.0 Kin	1.20±0.32	7.04±3.22	1.79±0.36
MS+1.5 Kin	1.45±0.68	7.83±1.68	1.46±0.20
MS+2.0 Kin	1.80±0.77	8.15±2.57	2.18±0.15
MS+2.5 Kin	1.65±0.70	7.00±1.38	2.68±0.63
MS+3.0 Kin	1.30±0.48	5.91±1.46	2.29±0.29

Values are an average of 12 replicates and expressed as the Mean±SD for all the experiments

Table 3: Effect of NH_4NO_3, BAP and Kin on shoot induction of *in vitro* cultures of *Celastrus paniculata* after 60 days of incubation

Media combination (mg L^{-1})	No. of shoot	Length of shoot (cm)	No. of leaves
Control	1.14±1.13	4.31±1.52	3.12±1.24
MS+1.0 NH_4NO_3	3.28±1.12	8.23±1.21	3.62±0.98
MS+1.5 NH_4NO_3	2.75±0.47	10.13±1.32	3.59±0.75
MS+2.0 NH_4NO_3	3.46±1.65	9.34±1.67	4.05±0.95
MS+2.5 NH_4NO_3	3.97±1.87	10.22±1.46	5.69±1.51
MS+3.0 NH_4NO_3	4.14±1.48	8.39±1.41	2.20±0.72
MS+1.0 BAP	3.18±1.15	10.88±1.08	2.61±0.24
MS+1.5 BAP	4.67±1.58	9.31±1.56	1.85±0.28
MS+2.0 BAP	3.83±0.32	10.74±1.31	1.62±0.13
MS+2.5 BAP	4.16±1.23	10.76±0.56	1.86±0.53
MS+3.0 BAP	4.35±0.67	9.43±1.31	1.41±0.29
MS+1.0 Kin	1.30±0.72	8.03±1.63	6.25±1.78
MS+1.5 Kin	1.40±0.48	8.05±1.58	5.85±1.85
MS+2.0 Kin	1.45±0.59	8.88±1.26	4.75±1.11
MS+3.0 Kin	1.50±0.65	9.38±1.83	6.80±2.47

Values are an average of 12 replicates and expressed as the Mean±SD for all the experiments

Stomatal study: The leaves were amphistomatic and numerous in numbers. They were with kidney shaped guard cells and subsidiary cells were aligned completely with guard cells. The stomas were with specific sub stomatal cavity.

Pathological screening: The small cuttings of *in vitro* raised clones of approximately 0.5 and 1.0 mm in size of the shoots were transferred to PDA and PCA medium to examine the microbial infection. No microbial growth was observed on the medium after 3-4 days of incubation ensure the disease free nature of in vitro raised progenies.

DISCUSSION

The medium devoid of growth regulators failed to induce *in vitro* shoot induction responses. Similar results were obtained in *Crataeva nurvala*[13], *Nyctanthus arbor-tristis*[14], *Peganum harmala*[15] and *Celastrus paniculatus* [16] on MS basal medium. Few workers reported the successful micropropagation protocol of *Celastrus paniculatus*[17-19]. The present study is an effort for the large scale production of this highly valuable endangered medicinal plant through *in vitro* propagation using different plant growth regulators and explants. For this MS medium supplemented with varying concentrations of growth regulators such as NH_4NO_3, BAP, Kin were used alone but previous micropropagation studies on BAP in combination with Kin resulted in a number of shoots and percentage response that was higher when compared to BAP alone[20]. The combined effect of BAP and Kinetin on efficient shoot induction has been well proved in asteracae member such as *Capsicum chinense* and *Bambusa balcoo*[21]. It is demonstrated that high frequency of plantlets regeneration is possible from inter node segments and midribs of the leaves were used as explants. The growth and morphogenetic responses of tissues are strongly influenced by the type and the concentration of the mineral nutrients applied.

The *in vitro* studies revealed that the propagation using tissue culture technique for multiplication of a number of medicinal plants is highly effective for their large scale production[22]. The study revealed the effect of different concentrations of the selected growth regulators on *in vitro* shoot induction responses in terms of number of shoot, length of shoot and number of leaves. Ammonium nitrate is identified as an important factor affecting *in vitro* storage organ formation. It affects numerous *in vitro* responses such as such as shoot induction, shoot regeneration[23], root induction from shoot cultures[24] and regulation of growth and biomass production[25]. The study revealed that the optimum response in terms of number of shoot is recorded in MS medium fortified with 3.5 mg L^{-1} of NH_4NO_3 after 60 days of culture incubation. Previous studies documented that minor quantities of complex organic nutrients like amino acids, peptides, fatty acids, carbohydrates, vitamins influence the high rate of proliferation and regeneration of *in vitro* medicinal crops[26].

Many workers reported that BAP is more effective than Kin for the induction of adventitious shoot buds from internode exlplants in *Celastrus paniculatus*[27-29]. Similar results were reported in species such as *Rotula aquatica*[30], *Cinnamomum camphora*[31], *Mamordica charantina*[32]. But in the case of shoot elongation, it has been reported that Kin was more effective over BAP[33]. The combined effect of cytokinins has significant role on adventitious shoot bud regeneration[34]. In contradiction to this the present study revealed that after 60 days of culture period the optimum response in terms of number and length of shoots were in MS medium fortified with 1.5 mg L^{-1} BAP but in terms of number of leaves the optimum response were obtained in MS medium supplemented with 3.0 mg L^{-1} of Kin. Increase in the level of BAP decreased the number as well as the length of the shoot. The higher concentration of BAP not only reduces the number of shoots but also resulted the stunted growth of shoots with characteristic symptoms of browning and shoots tip decays[35]. The BAP was also an efficient growth regulator for shoot multiplication in species such as *Chlorophytum borivilianum*[36] and *Cyphomandra betacea*[37]. Upon increasing the concentration of growth regulators, a reduction in the number of shoots per culture was observed. Similar trend was observed in *Solanum nigrum* using BAP at higher concentrations[38].

The development of callus from explants cultured on medium supplemented with BAP or Kin alone in the present study has been accomplished in *Chonemorpha grandiflora*[39] and *Baliospermum montanum*[40]. This may be because of the accomplishment of a balance between the level of endogenous auxin and exogenous cytokinins in the media, which ultimately leads to mitosis and subsequent callus formation. Vitrification or hyperhydrocity of the *in vitro* raised clones were observed in culture bottles after 60 days of incubation. Continuous subculturing in the same medium results the vitrification of the cultures, increased callus formation and decreased culture vigor followed by death. It could be overcome by transferring to lower concentrations of BAP. In addition to the shoot induction response, cytokinin such as BAP and Kin also known to accelerate and increases the rate of germination[41]. Half strength MS medium supplemented with1.0 mg L^{-1} BAP in a combination of 0.5 mg L^{-1} NAA resulted the highest efficiency of *in vitro* propagation of *Celosia argentea*[42-44]. The *in vitro* inflorescence *in Celosia argentea* var. *cristata* from nodal explants was

achieved on MS medium with 0.5 mg L^{-1} BA[45]. The application of 15% of coconut water to the culture medium containing auxin and cytokinin can induce plant cells to divide and grow rapidly. It also enhanced *in vitro* development of medicinal plants such as shoot growth and maximum shoot length (8.0 cm)[46].

MS medium with different levels of NH_4NO_3, BAP and Kin other than the optimal level decreased the number of shoots. Increase in the level of BAP decreased the number as well as height of the shoot. Cultures retained for more than 6 weeks showed necrosis of shoot tips that may have been caused by nutritional deficiencies and this can be overcome by reducing the subculturing cycle duration to 4 weeks[47]. Pathogen free nature of *in vitro* raised plants was confirmed by *in vitro* pathological screening. Earlier reports mentioned about the *in vitro* triple indexing protocol for detecting *Pseudomonas solanacearum* in the ginger explants used for tissue culture[48] and microrhizome technology for high quality, pathogen/pesticide free rhizomes production in ginger[49].

CONCLUSION

The developed shoot induction protocol could be successfully applied for the large scale production of *Celastrus paniculatus*, an endangered Indian medicinal plant, within a short period of time. The pathological screening of the tissue culture raised plantlets by using PDA medium ensure their disease free nature, which has potential commercial application in the field of pharmacology and phytomedicine.

SIGNIFICANCE STATEMENT

The significance of this study is to develop a new combination of selected plant growth regulators such as, Ammonium nitrate (NH_4NO_3), Benzyl Amino Purine (BAP) and Kinetin (Kin) with a concentration ranging from 0.5-3.0 mg L^{-1} for shoot induction responses. The growth responses were recorded after 20, 40 and 60 days of incubation. In order to ensure the disease free nature of the *in vitro* raised clones, pathological screening were done by using potato dextrose agar and plate count agar.

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