



# Rapid propagation of *Celastrus paniculatus* Willd.: an endangered medicinal plant through indirect organogenesis

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Received: 13 October 2019 / Revised: 27 December 2019 / Accepted: 25 January 2020 / Published online: 12 February 2020  
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## Abstract

A rapid and efficient propagation system through callus explant derived from leaf was established in *Celastrus paniculatus* Willd., a medicinal plant of the Celastraceae family. Seed dormancy and vegetative propagation render it for developing an in vitro regeneration method. Murashige and Skoog (MS) media containing 6-benzylaminopurine (BAP), 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthalene acetic acid (NAA) and with various concentrations of BAP + NAA, BA + 2,4-D and BAP + IAA produced different natures of calli. Moreover, BAP + NAA produced friable callus, whereas BAP + 2, 4-D produced compact calli, which were transferred to the shoot initiation medium containing BAP supplemented with ascorbic acid and each of adenine sulfate, arginine and citric acid. Inclusion of meta-topolin in the media along with optimum concentration of BAP promoted shoot multiplication and elongation after 8 weeks of culture. The in vitro elongated shoots were treated with different auxins such as IAA (Indole 3 Acetic Acid), IBA (Indole-3-butyric acid) and NAA (Naphthalene acetic acid) individually for early rooting and the treated shoots were transferred to the half-strength MS medium. The regenerated plantlets were acclimatized in pots containing sterilized soil and sand and then transferred to the field conditions, 90% of the regenerants survived. Thus, this was the first report on indirect organogenesis of *C. paniculatus* Willd. using callus explant obtained from direct organogenesis leaf.

**Keywords** *Celastrus paniculatus* Willd. · Indirect organogenesis · meta-topolin · Shoot multiplication · Growth regulators

## Introduction

Medicinal plants play a significant role in world health care systems. Among these, *Celastrus paniculatus* Willd. is a crucial medicinal plant. It is a woody, climbing shrub, up to 10–18 m in height, native to all the hilly regions of India. It is known as the “elixir of life” and commonly termed as Prog or Malkangi, Jyotishmati and Bitter Sweet. In Ayurveda, the seeds of this plant are used to treat a variety of ailments (Handa, 1998; Kumar and Gupta 2002; Warriar and Nambiar 1993). In addition, the roots of this plant are used in the treatment of cancerous tumors (Parrotta 2001), skin diseases, fever, and neurological disorders (Ahmad et al. 2010). The natural propagation of this plant is poor due to the extremely low viability of the seeds and less percentage of germination (11.5%). The vegetative propagation through

the rooting of stem cuttings is not successful. Considering its medical properties and the low rate of natural propagation, the development of alternative methods of propagation for the conservation and utilization of biodiversity of *C. paniculatus* is an urgent requirement (Arya et al. 2001).

Micropropagation of this plant species through internodal explants (Rao and Purohit 2006), shoot tips and leaf discs (De Silva and Senarath 2009), nodal segments (Phulwaria et al. 2013) and shoot bud differentiation (Senapati et al. 2013) has been reported. However, the available protocols cannot be completed promptly. Owing to its medicinal and industrial application, this species has been overexploited from wild conditions by pharmaceutical companies and local tribes throughout India. Hence, it is now considered a vulnerable species (Lal and Singh 2010; Martin et al. 2006; Singh et al. 2017), especially in the Western Ghats of South India (Rajasekharan 2002).

The increased scientific and commercial attention on medicinal plants has led many wild medicinal plants at risk of extinction. The Ministry of Environment and Forest (MoEF) of the Indian Government has identified

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approximately 9500 plant species (*C. paniculatus* among one) that play an important role in the pharmaceutical industry. Jose et al. (2001) reported that the use of 7% medicinal plants can lead to decreased growth rate of wild medicinal plants. According to Sarasan et al. (2006), more than 8000 species were added to the International Union for the Conservation of Natural Resources (IUCN) and RET (Rare, Endemic, Endangered and Threatened) species during the last decade. They also noted that the number of plants recorded as “Critically Endangered” increased by over 60 percent (Subbaiyan et al. 2014).

Thus, in the present study, we attempted to develop a standard reproducible protocol for achieving a high number of shoots through indirect organogenesis. Regeneration via indirect organogenesis will also be useful in genetic transformation studies and the production of somaclonal variants in medicinal plants with high secondary metabolites (Siddique et al. 2010).

## Materials and methods

### Media and culture conditions

The nutrient medium consisted of Murashige and Skoog’s (MS) media (Murashige and Skoog 1962), 3.0% sucrose (Hi-Media, Mumbai, India) supplemented with various concentrations of different plant growth regulators (PGRs); the pH was adjusted to 5.6–5.8 using 1 N HCl or NaOH. The media was solidified with 0.7% (w/v) agar and sterilized for 15 min at 121 °C. The culture was maintained at  $25 \pm 2$  °C for 16 h under a light intensity of 3000 lx supplied by cool white fluorescent lamps (Philips, India) and > 85% relative humidity.

### Callus induction

In this study, the effect of the combination of various concentrations of auxins and cytokinin’s was analyzed for callus induction through leaf explants derived from direct regeneration (Anil and Ranjitha Kumari 2019). The taxa were identified by the Botanical Survey of India, Southern Regional Centre, Coimbatore (voucher specimen number BSI/SRC/5/23/2019/Tech./111). Explants were placed on the MS medium fortified with 1-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP) and BAP in combination with NAA, 2,4-D and IAA. The cultures were incubated at 16/8 h light/dark regimes. A complete randomized design (CRD) was used with 10 replicates in all experiments.

### Shoot initiation and multiplication

Leaf-derived green and compact callus grown to 4 weeks were cultured in Murashige and Skoog’s medium supplemented with sucrose (3%) and BAP (0.5–2.0 mg/L) with additives (50 mg/L ascorbic acid, 25 mg/L adenine sulfate, 25 mg/L arginine and 25 mg/L citric acid) as described by Arya et al. (2001) and Phulwaria et al. (2013) for shoot initiation, well-established MS media supplemented with different concentrations of BAP. For further multiplication, the in vitro produced cultures were transferred to MS medium with optimal concentration of BAP (1.0 mg/L) with metoprolol (0.5–2.0 mg/L) along with additives the culture was incubated in 16/8 h light/dark regimes for 3 weeks. After 4 weeks of culture, the frequency of shoots producing callus was scored. The number of shoots per explant, mean shoot length, and mean number of leaves per shoot were recorded.

### Microscopic preparation of callus

BAP+2,4-D-derived calli, cultured for 14 and 21 days on the regeneration medium, respectively, were collected from the culture tubes and fixed according to the protocol by Jang et al. (2016) with slight modifications. The callus sample was maintained in a solution containing 10% formaldehyde buffer (0.05 M phosphate buffer solution) for 24 h at room temperature, followed by dehydration in graded alcohol (60%, 80%, 90%) for 30 min each, and absolute ethanol (2 times  $\times$  15 min each). The sections were sliced with tungsten knives on an automatic rotary microtome (Yarco YSI122) and stained with hematoxylin and eosin for 5 min before examining under a Leica DMR light microscope (Carl Zeiss, Jena, Germany).

### Rooting of shoots

Elongated healthy shoots (4.8 cm long) were transferred into root induction media comprising of MS medium supplemented with different concentrations of auxins (IAA, IBA, and NAA), each at 0.1, 0.3, 0.5, and 1.0 mg/L. Rooting percentage, number of roots per shoot, and mean root length were measured after 3 weeks.

### Hardening and acclimatization of regenerated plantlets

The rooted plantlets were taken out from the rooting medium and washed with sterilized water to remove the traces of agar–agar. Then, the well-rooted plantlets were covered with transparent polythene bags to ensure high humidity, maintained in the growth chamber and watered every alternate

day with half-strength MS salts lacking organic supplements (Ahmed et al. 2017). Acclimatized plants were transferred to pots containing normal garden soil and sand (autoclaved) in 2:1 ratio and maintained in greenhouse conditions for further growth and field experiments.

### Statistical analysis

Every treatment conducted in a CRD of ten replicates contained one explant, and every experiment was repeated three times for statistical analysis. The data were subjected to analysis of variance (ANOVA) using SPSS. The significance of differences among the means was evaluated using Duncan's multiple range test at  $p=0.05$ . The data are represented as mean  $\pm$  standard error of the mean of three experiments.

## Results and discussion

### Callus induction from leaf explants

Callus induction was observed in all the tested media combinations depending on the concentration of the growth hormones (Table 1). Interestingly, the calli in BAP, after 7–10 days post-incubation and the explants in control medium, within 10 days, were dead. Wounding promotes callus formation, but its color and texture are varied, necrotic white compact, white compact, cream friable, necrotic friable, hard compact and green compact callus (Fig. 1 and Table 1). The optimal growth regulator in combinations for callus induction were as follows: 0.5 mg/L BAP with 0.5 mg/L NAA showed 91.66% callus response and 0.5 mg/L BAP with 0.3 mg/L 2,4-D showed 89.33% callus response, while callus formation decreased with increasing concentration of the hormones. The mean callus diameter was significantly higher in BAP fortified with NAA as compared to other combinations. However, the fastest callus formation was achieved in 0.5 mg/L BAP combined with 0.3 mg/L 2,4-D. The callus induction was also recorded in auxins (NAA and 2,4-D) individually; however, the nature of calli differed according to the combinations as described above. Nevertheless, auxins alone enriched medium induced higher efficiency of callus as compared to BAP fortified with IAA. This finding indicated a satisfactory callus formation and growth in 1:1 ratio of cytokinin: auxin or the auxin concentration was slightly less than that of the cytokinin. In contrast to our results, Anusha et al. (2016) reported 88.10% callus response in MS medium fortified with 1.5 mg/L 2,4-D alone with in vivo plant material as an explant. Previous studies reported similar findings in *Brassica* species (Ahmad and Spoor 1999) and *Sesamum indicum* L. (Asad et al. 2019; Bakar et al. 2014). Available literature reveals that the use of leaf explants generated via direct regeneration of *C.*

**Table 1** Effect of cytokinin's and auxins on callus formation and nature of calli from leaf explants of *Celasturus paniculatus*

Hormone	Callus formation (%)	Nature of callus
NAA		
0.1	65.33 $\pm$ 1.20 <sup>f</sup>	Pink green
0.3	72.33 $\pm$ 2.18 <sup>e</sup>	Cream friable
0.5	67.33 $\pm$ 1.22 <sup>f</sup>	Friable
0.7	59.33 $\pm$ 0.98 <sup>h</sup>	Cream white
2,4-D		
0.1	55.00 $\pm$ 0.88 <sup>i</sup>	Hard compact
0.3	62.33 $\pm$ 1.12 <sup>gh</sup>	Light green compact
0.5	66.33 $\pm$ 1.32 <sup>fg</sup>	White green compact
0.7	61.00 $\pm$ 1.20 <sup>h</sup>	White compact
BA+NAA		
0.5+0.1	80.66 $\pm$ 0.78 <sup>d</sup>	Cream friable
0.5+0.3	86.33 $\pm$ 0.96 <sup>bc</sup>	Friable compact
0.5+0.5	91.66 $\pm$ 0.69 <sup>a</sup>	Friable callus
0.5+0.7	83.33 $\pm$ 1.34 <sup>cd</sup>	Necrotic friable
BA+2,4-D		
0.5+0.1	84.33 $\pm$ 1.20 <sup>cd</sup>	Necrotic white compact
0.5+0.3	89.33 $\pm$ 1.11 <sup>ab</sup>	Hard compact
0.5+0.5	85.00 $\pm$ 1.43 <sup>c</sup>	White green compact
0.5+0.7	80.66 $\pm$ 0.99 <sup>d</sup>	White green compact
BA+IAA		
0.5+0.1	62.00 $\pm$ 0.88 <sup>gh</sup>	White compact
0.5+0.3	65.66 $\pm$ 1.34 <sup>fg</sup>	Hard compact
0.5+0.5	59.66 $\pm$ 1.53 <sup>h</sup>	Light green compact
0.5+0.7	52.33 $\pm$ 0.89 <sup>ij</sup>	Hard compact

Mean in each column followed by the same superscript letters are not significantly different according to DMRT AT  $P < 0.05$

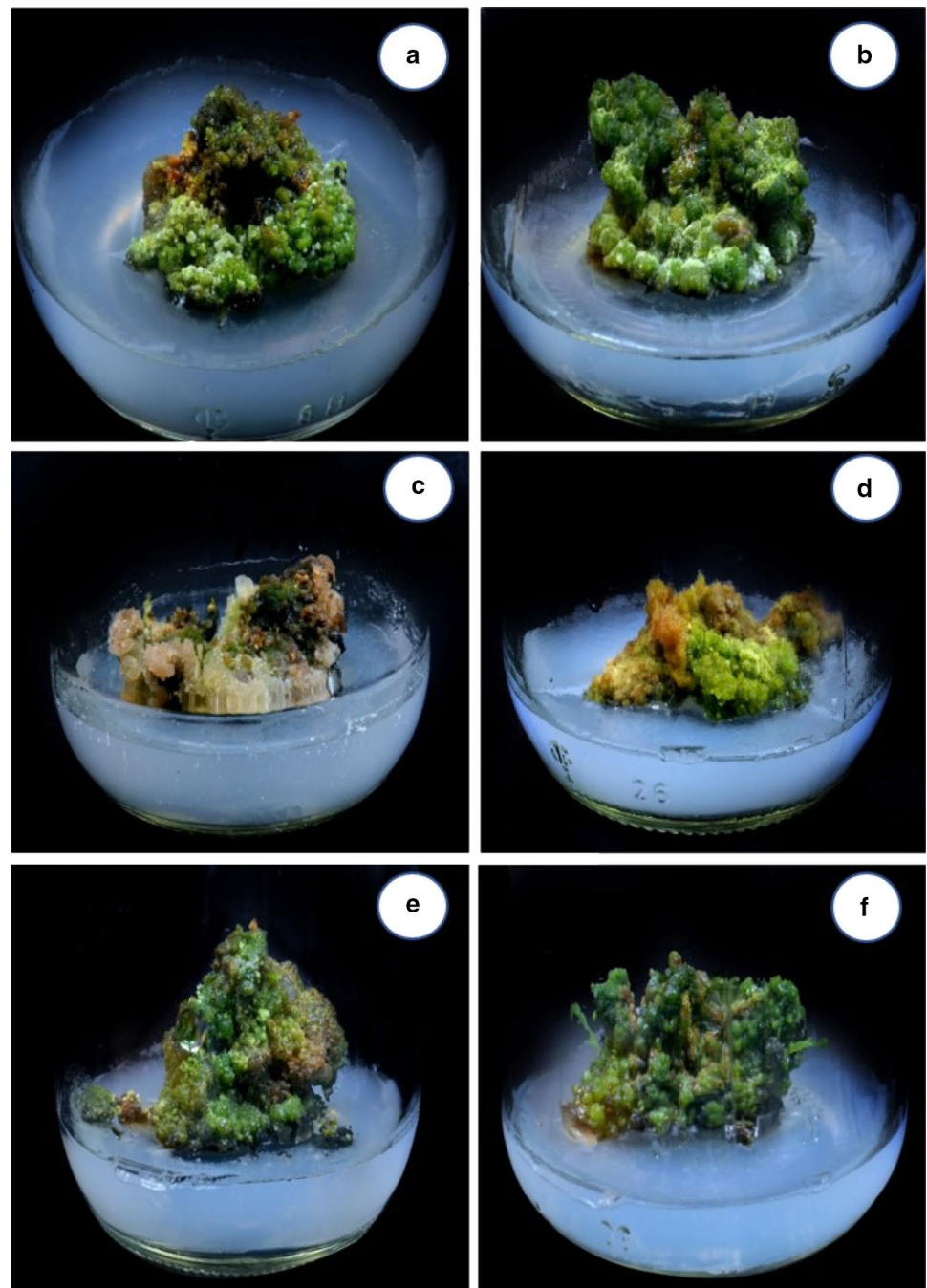
*paniculatus* Willd. has not been reported. The callus was observed every 7 days and sub cultured at regular intervals for 21–25 days.

### Shoot organogenesis from callus and regeneration

The effectiveness of BAP on shoot organogenesis is well known for several plant species (Ahmad et al. 2010; Kumar et al. 2010; Maity et al. 2005; Singh and Tiwari 2012), including *C. paniculatus* (Phulwaria et al. 2013). In this report, green compact and friable callus were transferred to shoot induction medium containing different concentrations (0.5, 1.0, 1.5, and 2.0 mg/L) of BAP along with additives, such as 50 mg/L ascorbic acid and 25 mg/L each of adenine sulfate, arginine, and citric acid (Phulwaria et al. 2013).

The calli become more greenish and started to initiate shoots after 1 week of inoculation, exhibiting 79.33% shooting with 2.64 shoots per callus at 1.0 mg/L BAP (Fig. 2). However, friable callus did not show differentiation even after transfer to different concentrations of auxins and

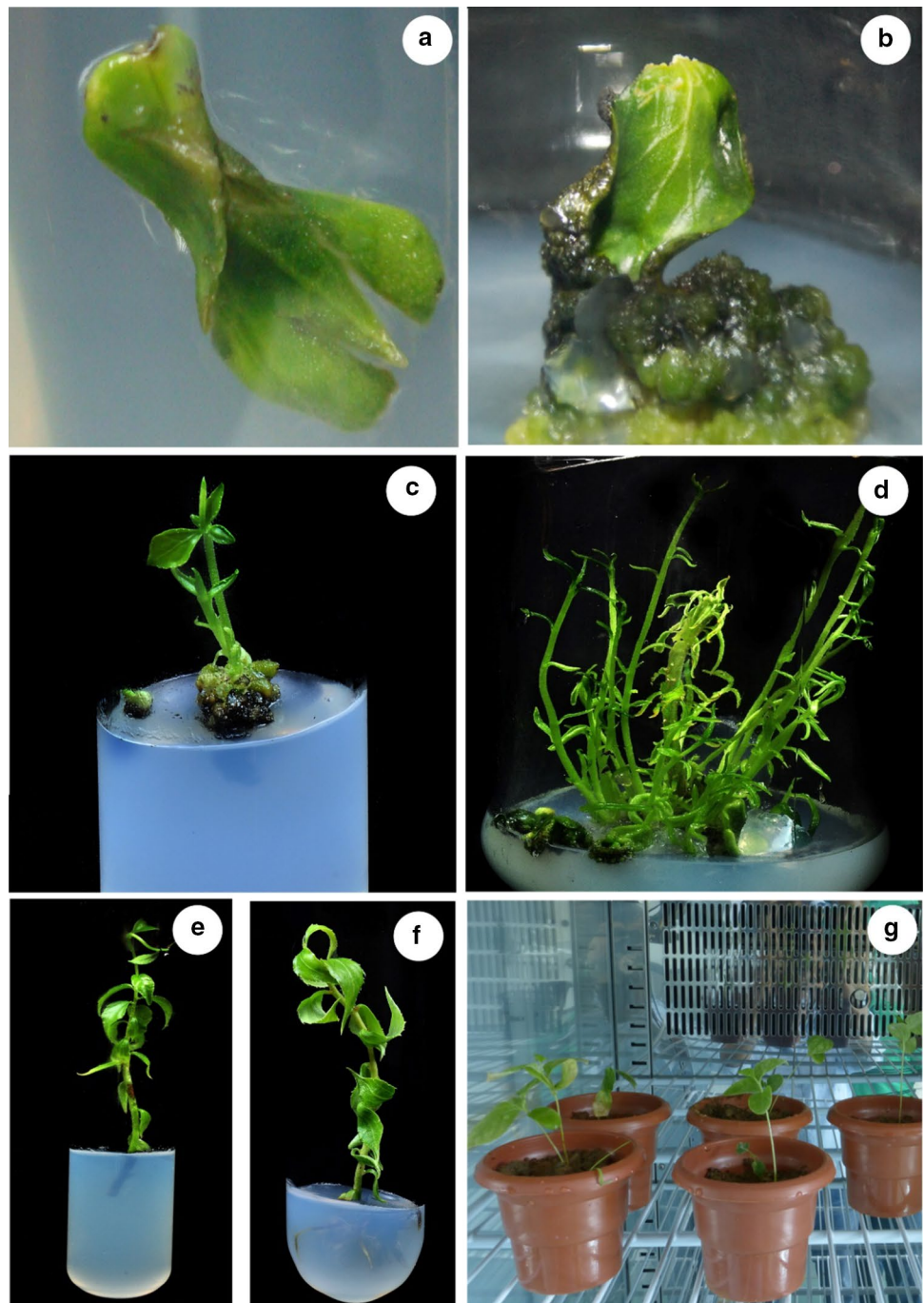
**Fig. 1** Effect of Plant Growth Regulators in forming callus from *Celastrus paniculatus* leaf explants. **a** Necrotic white compact, **b** white compact, **c** cream friable, **d** necrotic friable, **e** hard compact, **f** green compact



cytokinins. In order to increase the shoot organogenesis, the in vitro explants were transferred to the MS medium fortified with 1.0 mg/L BAP and meta-topolin (0.5, 1.0, 1.5, and 2.0 mg/L) supplemented with additives as mentioned above. Although BAP is one of the most effective cytokinin used (Valero-Aracama et al. 2010) for shoot organogenesis, the slow release of benzyladenine from its derivatives might reduce the root number at the acclimatization stage, which has been shown in other plant species (Werbrouck et al. 1995). Therefore, hydroxylated benzyl adenine analog and active natural growth substance, such as meta-topolin,

a cytokinin, were used for the first time in *C. paniculatus* species. Adenine sulfate stimulates cell growth and enhances shoot formation (Raha and Roy 2001), whereas arginine helps in increasing the number of shoots per plant and citric acid plays a role in increasing shoot length (Sanjaya et al. 2005). This could be attributed to adenine sulfate that shows a synergistic effect with other cytokinin's BAP and meta-topolin. mT is reported to be a potential alternative to BAP (Bairu et al. 2007). In comparison, mT occurs naturally with an aromatic structure similar to BAP and has not been associated with hyperhydricity (Bairu et al. 2007).

**Fig. 2** Effect of plant growth hormones on indirect regeneration of *Celastrus paniculatus* Willd. **a** Leaf explant, **b** Callus initiation, **c** shoot initiation from Callus region, **d** multiple shoots, **e** shoot elongation, **f** rooting, **g** hardening of plants



Doubling the shoot number, 83.33% (Table 2) shoot response was observed after 25–30 days of inoculation in the presence of 1.0 mg/L of BAP and meta-topolin. Currently, only a few reports are available on the micropropagation in *C. paniculatus* through callus (Sharada et al. 2003), nodal shoot segments (Arya et al. 2001) and nodal explants (Martin et al. 2006; Senapati et al. 2013). Endophytic microflora is a major cause of contamination in wild type explants. Hence, in the present study, we used embryo derived leaves as starting material. The leaf explants

derived from in vitro culture were cultured to get callus and the resulting callus tissue was regenerated into whole plantlets. This method not only avoids the endophytic microflora contamination but also tedious process of identifying and collecting leaves from wild type plants. The mean number of shoots obtained in the present study was lower i.e. 7.12 in compared to De Silva and Senarath (2009) and Lal and Singh (2010) reports in *C. paniculatus* was 8.60 and 8.9 respectively. This is because of the nature of explants used by the authors, De Silva and Senarath (2009) and Lal and

**Table 2** Effect of continuous treatment of cytokinin and additives on shoot multiplication from callus explants of *Celastrus paniculatus* Willd

BAP (mg/L)	Meta topolin (mg/L)	Percentage of shooting	Shoot number (mean ± SE)	Shoot length (mean ± SE)
0.0	–	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
0.5	–	40.66 ± 0.88 <sup>f</sup>	1.23 ± 0.02 <sup>g</sup>	0.56 ± 0.01 <sup>g</sup>
1.0	–	79.33 ± 1.20 <sup>b</sup>	2.64 ± 0.19 <sup>e</sup>	1.75 ± 0.06 <sup>d</sup>
1.5	–	62.66 ± 0.88 <sup>d</sup>	1.84 ± 0.10 <sup>f</sup>	1.12 ± 0.07 <sup>f</sup>
2.0	–	44.66 ± 0.88 <sup>e</sup>	1.25 ± 0.05 <sup>g</sup>	0.45 ± 0.05 <sup>g</sup>
1.0	0.0	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
1.0	0.5	64.66 ± 0.88 <sup>d</sup>	4.58 ± 0.14 <sup>c</sup>	2.78 ± 0.06 <sup>c</sup>
1.0	1.0	83.33 ± 1.20 <sup>a</sup>	7.12 ± 0.05 <sup>a</sup>	4.84 ± 0.09 <sup>a</sup>
1.0	1.5	72.33 ± 1.45 <sup>c</sup>	5.23 ± 0.11 <sup>b</sup>	3.02 ± 0.07 <sup>b</sup>
1.0	2.0	65.66 ± 0.88 <sup>d</sup>	3.76 ± 0.10 <sup>d</sup>	1.54 ± 0.05 <sup>e</sup>

Mean in each column followed by the same superscript letters are not significantly different according to DMRT AT P < 0.05

Singh (2010) used nodal explants from the mature plants, whereas, in our study we have used embryo derived leaf explants. The major advantage of our protocol is that, we have used the tissue culture raised explants which will not only avoid endophytic contamination but also the availability of explants is throughout the year contrast of mature plant derived explants. The obtained results are contradictory to the results obtained by Sharada et al. (2003), as they found the maximum number of shoots in MS medium supplemented with BAP. This may be because the donor mother plants grew in two different regions, leading to the possible involvement of genes in modulating hormone levels (Tantikanjana et al. 2001).

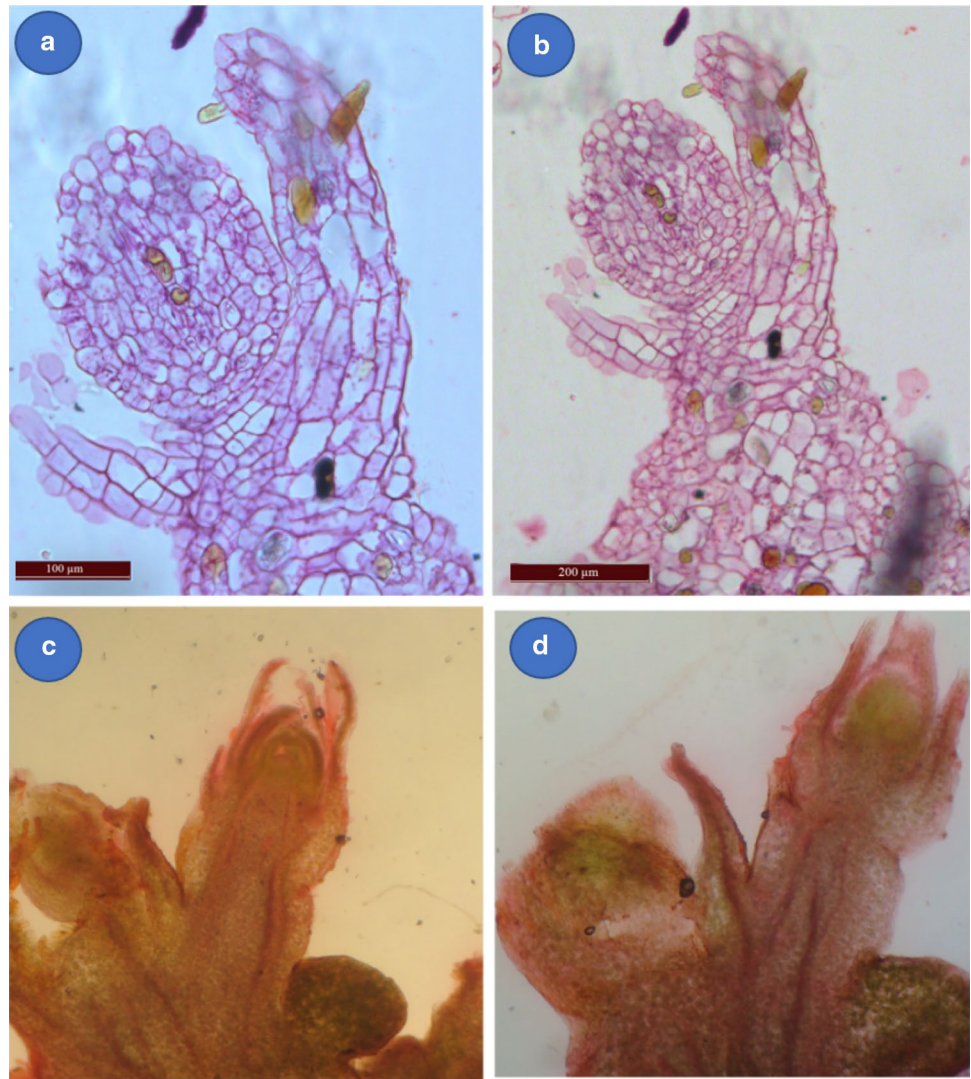
The stimulatory role of BAP was similar to that reported previously in other species (Chaturvedi and Sharma 1989; Pathak et al. 2017; Singh et al. 2019). The role of meta-topolin (Wojtania 2010) and its advantage over BAP were reported in other crops such as banana (Bairu et al. 2008) and *Beta vulgaris* (Kubalaková and Strnad 1992). The primordial shoots generated from callus derived from leaf explants exhibited meristems (Dakshayini et al. 2016). The anatomical analysis of the explants (Fig. 3) revealed that callus was mainly induced by the leaf primordia, where new meristematic regions were formed. The organized cell division from such zones turned to microshoots. These findings were in agreement with those in recent studies on *Sorghum bicolor* and chrysanthemum (Dreger et al. 2019; Verma and Prasad 2019).

### Rooting, hardening, and acclimatization

Root formation was negligible in the shoot multiplication medium. Nadgauda et al. (1978) utilized turmeric tissue culture and demonstrated that with increasing levels of BAP, root formation was negligible. Therefore, developed

shoots were transferred to auxin (IAA, IBA and NAA) containing medium for root formation. The mean number of roots, mean root length and rooting percentage were significantly different among the tested treatments (Table 3; Fig. 3f). Roots were initiated after 15 days in the half-strength MS medium with IBA (0.5 mg/L), while all the other tested treatments required more than 20 days with less mean shoot length. The mean root number (9.2 ± 0.35), mean root length (5.7 ± 0.47), and rooting percentage (89.6%) were significantly higher in this medium, referring it to be optimal for rooting of in vitro propagated shoots of *C. paniculatus*. Notably, increasing the concentrations of auxins beyond 1.0 mg/L reduced the number of roots per shoot. The rooted plantlets were transferred into plastic pots for acclimatization, and about 90% of the rooted plantlets survived in the pot one month after the transfer. These survived plants did not show any variation and were morphologically uniform. Taken together, our findings suggested that a lower concentration of auxins was effective in initiating root formation (Table 3). In another study by Nathar and Yattoo (2014), lower concentrations of auxins could initiate root induction. Also, optimum rooting response with the aid of IBA, IAA, and NAA has been reported in *C. paniculatus* (Anil and Ranjitha Kumari 2019; Sharada et al. 2003) and other medicinal plants such as *Artocarpus lakoocha* (Singh et al. 2019) and *Tinospora cordifolia* (Mridula et al. 2017). Conversely, Senapati et al. (2013) reported maximal rooting percentage (73.3%) with IAA using nodal explants in *C. paniculatus*. However, another study by Anil and Ranjitha Kumari (2019) demonstrated that in the direct regeneration, the shoots transferred to the rooting medium failed to show root induction until pulse treatment with auxins was administered. Similar findings were also observed in *Embelia ribes* (Dhavala and Rathore 2010).

**Fig. 3** Histological examination of *Celastrus paniculatus*. **a, b** Shoot bud with pair of primordial leaves. **c, d** Shoot and shoot bud initiation



**Table 3** Effect of auxins on root induction from shoots of *Celastrus paniculatus* Willd

Plant growth hormone	Concentration (mg/L)	Percentage of rooting	Root number (mean ± SE)	Root length (Mean ± SE)
IBA	0.1	50.33 ± 0.15 <sup>f</sup>	4.7 ± 0.44 <sup>c</sup>	3.2 ± 0.33 <sup>d</sup>
	0.3	64.00 ± 0.86 <sup>d</sup>	6.2 ± 0.39 <sup>b</sup>	4.0 ± 0.51 <sup>c</sup>
	0.5	89.66 ± 0.24 <sup>a</sup>	9.2 ± 0.35 <sup>a</sup>	5.7 ± 0.47 <sup>a</sup>
	1.0	70.00 ± 0.15 <sup>c</sup>	6.8 ± 0.65 <sup>b</sup>	4.2 ± 0.76 <sup>c</sup>
NAA	0.1	54.00 ± 0.72 <sup>ef</sup>	3.8 ± 0.53 <sup>d</sup>	3.1 ± 0.13 <sup>d</sup>
	0.3	60.66 ± 0.55 <sup>d</sup>	4.6 ± 0.29 <sup>c</sup>	3.7 ± 0.36 <sup>d</sup>
	0.5	81.33 ± 0.70 <sup>b</sup>	6.4 ± 0.37 <sup>b</sup>	4.6 ± 0.23 <sup>b</sup>
	1.0	62.33 ± 0.23 <sup>d</sup>	4.0 ± 0.54 <sup>d</sup>	4.2 ± 0.49 <sup>c</sup>
IAA	0.1	49.00 ± 0.54 <sup>g</sup>	3.3 ± 0.37 <sup>d</sup>	3.1 ± 0.50 <sup>d</sup>
	0.3	59.33 ± 0.61 <sup>e</sup>	4.7 ± 0.51 <sup>c</sup>	3.9 ± 0.42 <sup>bc</sup>
	0.5	70.66 ± 0.78 <sup>c</sup>	5.9 ± 0.38 <sup>b</sup>	4.0 ± 0.24 <sup>c</sup>
	1.0	60.33 ± 0.37 <sup>d</sup>	3.5 ± 0.83 <sup>d</sup>	2.9 ± 0.36 <sup>d</sup>

Mean in each column followed by the same superscript letters are not significantly different according to DMRT at  $P < 0.05$

## Conclusion

The present study demonstrated an efficient, rapid and simple protocol for callus induction and micropropagation of *C. paniculatus*. This methodology concludes that BAP, in combination with natural cytokinin meta-topolin, exhibits a synergic effect by increasing shoot multiplication. Hence, the present protocol could be recommended for large-scale production of *C. paniculatus*, which is already endangered because of ruinous overharvesting for pharmaceuticals with little or no regard for the future requirements. In addition, the optimized regeneration protocol would be applied to enhance the secondary metabolites via various methodologies such as *Agrobacterium*-mediated genetic transformation and adventitious root culture.

**Acknowledgements** We thank CSIR for providing the Senior Research Fellowship to Anil Kumar Moola vide sanction letter number 09/475(0201)/2018–EMR–I.

## Compliance with ethical standards

**Conflict of interest** Authors declared that there is no conflict of interest.

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