Transverse thin cell layer (tTCL) technology: a promising tool for micropropagation of Centratherum punctatum Cass

NV. Aswathi
Central University of Kerala
Dennis Thuruthiyil Thomas (den_thuruthiyil@yahoo.com)
Central University of Kerala
Dennis Thomas
Central University of Kerala https://orcid.org/0000-0001-9283-5723

Research Article

Keywords: Transverse thin cell layer (tTCL), callus organogenesis, direct shoot regeneration· medicinal plant, Centratherum punctatum

Posted Date: May 23rd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1668660/v1

Abstract

Centratherum punctatum Cass. is an important medicinal herb used for various purposes. The present study describes an efficient protocol for the micropropagation of *C. punctatum* through transverse Thin Cell Layers (tTCLs). The effect of plant growth regulators (PGRs), thickness of tTCLs and source of tTCLs, were evaluated. The tTCLs of varying thickness (0.5-5.0 mm) were excised from 10-day-old leaf, 45-day-old node and internode and cultured on MS medium supplemented with various concentrations of PGRs (BAP, TDZ, KN; 0.1-2.0 mg/l) alone or in combination with NAA (0.1-1.0 mg/l) for shoot induction. For callus induction 2, 4-D (0.5-2.0 mg/l) was employed. The leaf and node tTCLs showed direct shoot regeneration and internodal tTCLs resulted in calli which successfully regenerated to shoots on subculture. A significant effect of thickness/width on shoot induction was observed. For leaf, 1.0 mm width and for node and internode 2.0 mm thick tTCLs showed maximum response. MS medium supplemented with 1.5 mg/l TDZ, 0.5 mg/l TDZ and 1.0 mg/l BAP in combination with 0.2 mg/l NAA were found optimum for shoot induction from tTCLs of leaf (96% response with 41.5 shoots per explant), internode (64% response with 22.9 shoots per explant) and node (87% response with 11.9 shoots per explant) respectively. Rhizogenesis was obtained when micropropagated shoots were transferred to half strength MS medium supplemented with various concentrations (0.25-2.0 mg/l) of IBA. The rooted plantlets were eventually acclimatized and transferred to soil. The procedure described here is a promising tool for micropropagation of *C. punctatum* as it produces high frequency healthy shoots from minimum explant source.

Key Message

A rapid and efficient micropropagation protocol has been standardized in *C. punctatum* through transverse thin cell layer (tTCL) explants from leaf, node and internode.

Introduction

Plants have been a valuable source of medicine from ancient times attributing to the presence of various therapeutic agents in them. The world health organization has estimated that around 60% of the world population depends on the herbal medicine for their health care needs, as they are easy to access, readily available and cost effective (World Health Organisation 2000; Mahesh and Satish 2008). The percentage of population depending on traditional medicine increased to around 80% in developing countries (World Health Organisation 2000). More than 50% of modern medicines existing in clinical use are derived from plants (David 2001).

Centratherum punctatum Cass. is an important medicinal plant of the family Asteraceae and is commonly known as Brazilian button flower (Madhumita et al. 2020). It is a perennial bushy herb, up to 60 to 80 cm tall with well branched stem. The leaves are aromatic and flowers are in terminal heads, purple in colour (Chitra and Brindha 2014). It is native to Latin America and has very high occurrence in Brazil. *C. punctatum* has very wide distribution and is found in Australia, Galappagos, Florida, Mexico,

Central America, Asia and Africa (Alves and Loeuille 2021; Flann 2009). *C. punctatum* is used against cancer, snake bite, urethritis, ulcer, inflammation, sore throat and is also used as a wound healer and pain killer (Whistler 2000). The plant is reported to have anti-microbial, anti-oxidant, anti-tumour, anti-plasmodial, HIV reverse transcriptase and wound healing properties (Pawar and Arumugam 2011; Chitra and Brindha 2015; Chiappeta et al.1983; Sivasubramanian and Brindha 2013; Sivasubramanian and Brindha 2014). Recently, many important compounds have been identified from the essential oil obtained from different plant parts of *C. punctatum* (Ogunwande et al. 2015; Satyal et al. 2018). The essential phytochemicals present in this plant include centratherin, isocentratherin and sesquiterpene lactones (Ohno et al. 1979; Bevelle 1981). Centratherin is reported to possess antimicrobial, anti-inflammatory, trypanocidal and genotoxic activities (Junior et al. 2015; Burim et al. 2001). Cytotoxic property of isocentratherin was reported by Bevelle (1981). Floral protease activity which attributes to the wound healing property of *C. punctatum* has been studied by Chitra et al. (2016).

In view of the medicinal importance of *C. punctatum* there is a need for developing suitable micropropagation protocol for this plant. In vitro micropropagation is considered as an alternate method for the efficient and rapid propagation of plants (Rout et al. 2006; Loberant and Altman 2010). Through micropropagation identical plants can be produced in abundance within limited space and time and thus, this technique is widely employed in the medicinal plant research (Lemma et al. 2020). Micropropagation aids in the continuous production of useful plant secondary metabolites, irrespective of seasonal variations (Sethy and Kullu 2022; Yeshi et al. 2022).

The thin cell layer (TCL) system involves excising tiny explants either longitudinally (lTCL) or transversely (tTCL) from different plant organs or tissues like stem, leaf, inflorescence, cotyledon, hypocotyle, epicotyl, internode, shoot tip, petiole and node. The TCL technology was originated about forty-eight years ago with organogenesis from tobacco pedicel (Tran Thanh Van 1973). Since then, this technique has been successfully applied in the micropropagation of various taxa including vegetable crops, legumes, ornaments and medicinal plants (Abdolinejad et al. 2020; Hieu et al. 2018; Nhut et al. 2003a; 2003b; Teixeira da Silva and Nhut 2003; Sudhakaran et al. 2006; Taylor and Van Staden 2006; Leguillon et al. 2003; Ghnaya et al. 2008; Swarna and Ravindharan 2013). The technique of TCL culture has the advantage over traditional tissue culture methods in terms of overall plantlet production, minimal use of explant tissue and sensitivity and responsiveness to the substances added to the culture medium due to the close contact with the growth medium and faster diffusion of nutrients and PGRs as the explant size is smaller (Lakshmanan et al. 1995; Teixeira da Silva and Tanaka 2011). Therefore, the objective of our study was to develop an efficient and reliable protocol for micropropagation of *C. punctatum* by using tTCL explants and the successful establishment of micropropagated plants in field conditions. To the best of our knowledge this is the first report of in vitro propagation of *C. punctatum* via tTCL culture.

Materials And Methods

Establishment of primary culture

Healthy *C. punctatum* plants were collected from Periye, Kasaragod district, Kerala, India. The plant material was identified by Dr. A.K. Pradeep, Assistant Professor, Department of Botany, University of Calicut, Kerala, India. The voucher specimens (CU 7087) was deposited in the Calicut University Herbarium (CALI). The nodal segments were excised, surface sterilised and cultured on MS (Murashige and Skoog 1962) medium supplemented with 1.0 mg/l 6- benzyl aminopurine (BAP). All the explants for the present study were obtained from these in vitro maintained shoots.

Thin cell layer culture of leaves

Ten-day-old leaves were excised from in vitro grown shoots maintained in MS medium supplemented with 1.0 mg/l BAP and segmented transversely into tTCL of varying width (0.5-5.0 mm) to evaluate the effect of explant size on regeneration. All excisions were carried out with the help of a sterilized forceps and scalpel. The tTCLs were transferred to MS medium supplemented with different concentrations of BAP, TDZ, KN (0.1-2.0 mg/l) alone or in combination with NAA (0.1-1.0 mg/l). The excised tTCLs of leaves were placed on media in such a way that the abaxial surface is in contact with the medium.

Thin cell layer culture of nodes and internodes

The nodal segments were excised from in vitro grown shoots and were transversely sliced into segments of 0.5-5.0 mm thickness. Utmost care was taken to avoid internodal region while excising the nodal tTCLs. Similarly, internodal segments were sliced into tTCLs of varying thickness (0.5-5.0 mm). Nodal tTCLs were inoculated on MS medium supplemented with various concentrations of BAP, TDZ, KN (0.1-2.0 mg/l) alone or in combination with NAA (0.1-1.0 mg/l). Internodal tTCLs failed to produce any direct shoots on above media and therefore, they were transferred to 2, 4-D (0.5-2.0 mg/l) for callus induction. The explants were placed on media with the basal surface in touch with the media.

Rooting and acclimatization

Shoots below 0.5 cm length were cultured on MS medium supplemented with 1.0 mg/l BAP for elongation. For rooting, tTCL derived shoots having a length of 1.5-2.0 cm were individually excised and cultured on half strength MS medium supplemented with different concentrations of indole-3-butyric acid (IBA; 0.25-2.0 mg/l). The rooted shoots were acclimatized in paper cups (10.0 cm × 7.6 cm, length × diameter) containing sterile sand and soil (1:1) and were covered with perforated polythene bags. The plants were supplied with half strength MS liquid medium for 7 d, thereafter watered every alternate day. After 2 months, the fully acclimatized plants were transferred to garden pots and subsequently to the field.

Culture conditions and Data analysis

The media were solidified with agar (0.8% w/v) and the pH was adjusted to 5.8 by 0.1 N sodium hydroxide (NaOH) and sterilized by autoclaving at 120° C and 0.1 kPa pressure for 20 min. Cultures were incubated under a photoperiod of 16 h with light intensity of 36 $\mu molm^{-2}s^{-1}$ provided by white

fluorescent lamps, with 22 ± 2° C and 65% relative humidity. For each experiment three replicates each with 25 explants was conducted and the data was recorded. The values were analysed by one-way analysis of variance (ANOVA) test and difference among means were compared using Duncan's Multiple Range Test (DMRT; Duncan 1955) at 5% level of significance using SPSS software version 24.

Results And Discussion

The tTCL explants from leaf, node and internode were evaluated for induction of shoots or callus on MS medium containing various concentrations of BAP, TDZ, KN (0.1-2.0 mg/l), 2, 4-D (0.5-2.0 mg/l) alone or in combination with NAA (0.1-1.0 mg/l). The leaf and node tTCLs responded by direct shoot regeneration and internodal tTCLs gave rise to calli which successfully regenerated to shoots after subculture.

Effect of tTCL thickness

To test the influence of explant thickness/width on plant regeneration or callus induction, the tTCLs were excised at varying thickness/width (0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mm). The results showed that width (in case of leaf tTCLs) or thickness (in case of nodal and internodal tTCLs) have significant effect on direct regeneration, callus induction and shoot organogenesis from callus. In leaf, tTCL of width 1.0 mm exhibited the highest direct shoot induction (96%) on MS medium supplemented with 1.5 mg/L TDZ. Most of the 0.5 mm tTCLs turned necrotic with very low percentage of response (13%). Nodal tTCLs of 2.0 mm thickness gave optimum direct shoot induction (87%) on MS medium supplemented with1.0 mg/l BAP in combination with 0.2 mg/l NAA. Both above and below to this thickness the response showed a corresponding decrease (Fig. 1). Hence, for further experiments with leaf tTCLs a width of 1.0 mm and for nodal tTCLs 2.0 mm thickness was employed.

Callus induction from internodal tTCLs was optimum (93%) when 3.0 mm thick tTCLs were cultured on MS medium supplemented with 1.5 mg/l 2, 4-D. Although maximum callus induction was observed at the above-mentioned thickness, tTCLs of all the other thickness also showed good callus induction (Fig. 2). However, maximum shoot regeneration was obtained from calli derived from 2.0 mm thick tTCL (64% response with 22.9 shoots per explant; Fig. 1) on MS medium supplemented with 0.5 mg/l TDZ. From our observations, it is clear that size of the tTCLs play a crucial role in direct and indirect organogenesis in *C. punctatum.* In *Sorghum bicolor* size of the TCLs play an important role and the highest response was observed when 3.0 mm thick TCLs were used (Gendy et al. 1996). In *Lilium longiflorum* TCLs, of the various thickness (1.0–5.0 mm) used 3.0 and 4.0 mm showed 100% shoot induction (Nhut et al. 2001a). In *Allium ampeloprasum*, 5.0 mm thick TCLs exhibited maximum response of 40 shoots per explant (Silvertand et al. 1992).

Effect of PGRs

Direct shoot regeneration from TCLs of leaves

In vitro leaves were transversely segmented into 1.0 mm wide TCLs and cultured (Fig. 3A, B). In all concentrations and combinations of PGRs (BAP, KN, TDZ, NAA) the leaf tTCLs responded except for basal MS medium where the explants showed browning and necrosis after 5–7 d of culture (Table 1). The responding explants remained healthy and green, showed signs of direct shoot regeneration after 5 d of culture. Tiny shoot buds emerged from the surface of responding explants after 7 d (Fig. 3C). The number of shoots increased gradually after 14 d (Fig. 3D). Several elongated shoots were visible after 30 d (Fig. 3E). The results showed that the concentration of PGRs in culture media significantly affect the percentage response and shoot number per leaf tTCL explants. Among the different concentration of BAP tested 1.5 mg/l BAP gave maximum response (42% regeneration with 6.6 shoots per explant). When various concentration of KN was added to the media, 1.0 mg/l resulted in optimum percentage response (28% with 3.1 shoots per explant) but the shoot number was higher in 1.5 mg/l KN (5.3 shoots per explant with 18% response). The highest response was observed on MS medium supplemented with 1.5 mg/l TDZ where 96% cultures responded with a mean number of 41.5 shoots per explant after 45 d of culture (Fig. 3F). Both above and below this concentration shoot induction rate and number decreased (Table 1). The addition of NAA in combination with TDZ did not improve the shoot regeneration rate. The leaf tTCLs when subjected to various concentrations of 2, 4-D either resulted in necrosis or non- regenerative compact callus.

Table 1
Effect of MS medium supplemented with different concentrations of PGRs on shoot induction from tTCL explants of leaves 45 d after culture.

PGRs (mg/l)				Percent response*	Mean no. of shoots*	Mean shoot length (cm)*
BAP	KN	TDZ	NAA			
0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.1	-	-	-	8.0 ± 1.2i	1.2 ± 0.07d	5.6 ± 0.6b
0.2	-	-	-	11 ± 1.8hi	1.1 ± 0.05d	6.2 ± 0.4a
0.5	-	-	-	19 ± 1.9h	2.5 ± 0.08d	6.0 ± 0.5a
1.0	-	-	-	31 ± 3.4f	3.8 ± 0.11d	6.2 ± 0.4a
1.5	-	-	-	42 ± 3.9e	6.6 ± 0.5d	5.4 ± 0.6b
2.0	-	-	-	27 ± 2.4g	2.3 ± 0.1d	5.7 ± 0.4b
-	0.1	-	-	6.0 ± 1.1i	1.2 ± 0.06d	5.2 ± 0.5b
-	0.2	-	-	9.0 ± 1.3hi	1.8 ± 0.08d	5.5 ± 0.6b
-	0.5	-	-	15 ± 1.9h	2.4 ± 0.07d	5.8 ± 0.5b
-	1.0	-	-	28 ± 2.7g	3.1 ± 0.14d	6.1 ± 0.4a
-	1.5	-	-	18 ± 2.1h	5.3 ± 0.2d	6.3 ± 0.6a
-	2.0	-	-	12 ± 1.6hi	1.9 ± 0.06d	6.0 ± 0.5a
-	-	0.1	-	17 ± 1.9h	1.4 ± 0.04d	6.1 ± 0.6a
-	-	0.2	-	38 ± 4.1f	3.2 ± 0.13d	5.9 ± 0.5b
-	-	0.5	-	57 ± 4.9d	9.5 ± 0.7d	5.8 ± 0.4b
-	-	1.0	-	71 ± 5.2b	16.7 ± 1.3c	5.7 ± 0.4b
-	-	1.5	-	96 ± 6.1a	41.5 ± 2.1a	5.6 ± 0.5b
-	-	2.0	-	67 ± 4.6c	22.3 ± 1.8b	5.3 ± 0.6b
1.5	-	-	0.1	45 ± 3.2e	6.4 ± 0.3d	5.3 ± 0.5b
1.5	-	-	0.2	36 ± 2.8f	5.8 ± 0.3d	5.1 ± 0.5b
1.5	-	-	0.5	31 ± 2.7f	3.1 ± 0.1d	4.8 ± 0.4c
1.5	-	-	1.0	19 ± 2.1h	1.2 ± 0.08d	4.9 ± 0.5c
-	1.0	-	0.1	17 ± 1.9h	3.8 ± 0.2d	4.7 ± 0.6c

* Values represent mean ± SE. Means followed by same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test

PGRs (mg/l)				Percent response*	Mean no. of shoots*	Mean shoot length (cm)*
BAP	KN	TDZ	NAA			
-	1.0	-	0.2	13 ± 1.7hi	2.4 ± 0.1d	4.3 ± 0.4c
-	1.0	-	0.5	10 ± 1.5hi	1.2 ± 0.1d	4.5 ± 0.5c
-	1.0	-	1.0	9.0 ± 0.9hi	1.1 ± 0.2d	4.2 ± 0.6c
-	-	1.5	0.1	76 ± 5.8b	12.3 ± 1.2c	5.1 ± 0.6b
-	-	1.5	0.2	59 ± 4.9d	26.7 ± 1.9b	5.2 ± 0.6b
-	-	1.5	0.5	24 ± 3.1g	17.1 ± 1.4c	5.1 ± 0.4b
-	-	1.5	1.0	12 ± 1.6hi	8.3 ± 0.6d	5.3 ± 0.4b

* Values represent mean ± SE. Means followed by same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test

Leaf is an excellent material for tTCL culture as evidenced by various reports. In *Scutellaria ocmulgee* tTCLs culture, the optimum shoot regeneration was obtained from leaf derived tTCLs as compared to shoot derived tTCLs (Vaidya et al. 2016). In our study, leaf tTCL culture responded by direct shoot regeneration. Similarly, in *Passiflora edulis*, direct shoot regeneration from leaf tTCLs has been successfully achieved by Hieu et al. (2018). Direct organogenesis from leaf lamina and petiole derived TCL was obtained in commercially important *Saintpaulia ionantha* (Ohki 1994). Shoot induction was achieved from leaf and petiole TCL segments of *Tanacetum cinenariifolium* by Hedayat et al. (2009). In *Bacopa monnieri* leaf TCLs were cultured on MS medium supplemented with 2.27 mg/l BAP produced shoots (Croom et al. 2016). Baskaran et al. (2018) reported that *Uperoleia altissima* leaf lTCLs when cultured on MS medium supplemented with 0.45 mg/l BAP and 2.4 mg/l metatopolin followed by transfer to a fresh medium resulted in 100% response with 17.4 shoots per explant whereas explants in PGR free medium did not form any shoots. There are reports on superiority of leaf TCLs over TCLs derived from other explants. According to Welander (1988) leaf segments are superior in shoot regeneration than stem segments in apple TCL culture. Similarly, Dobránszki and Teixeira da Silva (2011) reported low shoot regeneration capacity of shoot/stem TCL as compared to leaf TCL in Royal Gala cultivar of apple.

In agreement with our findings, there are several reports of shoot regeneration from leaf tTCLs in presence of TDZ. According to the study conducted by Tubić et al. (2016), tTCLs derived from the base of leaf sheaths cultured on MS medium supplemented with 2.4 mg/l TDZ resulted in 100% shoot induction with 20.0 shoots per explant. However, shoot formation was significantly lower in KN, BAP and metatopolin. Nhut et al. (2005) achieved high frequency shoot regeneration in *Begonia tuberous* TCLs on half strength MS medium supplemented with 0.2 mg/l TDZ alone. Similarly, *Lillium longiflorum* TCLs gave shoots and protocorm-like bodies in TDZ containing medium (Nhut et al. 2001b).

Direct shoot regeneration from nodal tTCLs

The nodal tTCLs of 2.0 mm thickness were excised from in vitro derived nodes (Fig. 4A) and cultured on MS medium with or without PGRs showed shoot regeneration. In node derived tTCLs BAP was more favorable for direct shoot induction than TDZ and KN. TDZ at 1.5 mg/l resulted in 48% response with an average number of 1.2 shoots per explant. The optimum concentration of KN for shoot regeneration from nodal tTCL explant was 1.0 mg/l (35% response with 1.9 shoots per explant). Among the various concentrations of BAP tested, the optimum response was observed on 1.0 mg/l. Here, 65% explants responded with an average number of 6.8 shoots per explant (Table 2). This concentration of BAP was selected for further experiments with NAA (0.1-1.0 mg/l). Highest frequency of shoot regeneration (87%) and number of shoots (18.9 shoots per explant) was obtained on MS medium supplemented with 1.0 mg/l BAP in combination with 0.2 mg/l NAA (Table 2). On MS medium supplemented with various concentrations of 2, 4-D nodal tTCLs either turned necrotic or resulted in poor callus induction. These calli on subculture did not show any sign of regeneration (data not shown). In responding cultures, the nodal tTCLs showed emergence of tiny shoot bud 7 d after culture (Fig. 4B). The shoot bud enlarged in size after 14 d and the explants exhibited a hollow region at the center (Fig. 4C) and more shoots were produced within 20 d of culture (Fig. 4D). Within 30 d of culture the mean shoot number reached 7.8 per explant (Fig. 4E) and after 45 d it has increased to 18.9 (Fig. 4F). This result indicates the superiority of synergetic action of cytokinin - auxin combination in shoot induction from nodal tTCL explants. Our results are in agreement with several other reports on positive effect of cytokinin - auxin interaction. Successful shoot regeneration has been established by using BAP-NAA combination in *Dendrobium candidum* nodal tTCLs (Zhao et al. 2007). Similar results of synergetic action of BAP-NAA induced enhanced shoot regeneration have been reported by Swarna and Ravindran (2013) in nodal tTCLs of *Talinum triangulare*. In *Justicia gendarussa* a combination of BAP and NAA induced enhanced shoot regeneration (Thomas and Yoichiro 2010). Synergistic action of BAP-NAA combination induced shoot regeneration has been reported in other systems such as *Sesamum indicum* (Chattopadhyaya et al. 2010), *Brassica napus* (Ghnaya et al. 2008) and *Cajanus cajan* (Franklin et al. 2000). Successful shoot regeneration from tTCLs of nodal region was reported in *Eclipta alba* cultured on MS medium supplemented with BAP in combination with NAA (Singh et al. 2012).

Table 2
Effect of PGRs on direct shoot regeneration from tTCL explants taken from nodal region 45 d after culture.

PGRs (mg/l)				Percent response*	Mean no. of shoots*	Mean shoot length (cm)*
TDZ	KN	BAP	NAA			
0.0	0.0	0.0	0.0	29 ± 1.9ef	1.1 ± 0.3g	5.7 ± 0.5b
-	-	0.1	-	31 ± 1.7ef	1.1 ± 0.4g	5.6 ± 0.5b
-	-	0.2	-	36 ± 1.8e	2.2 ± 0.2f	5.8 ± 0.4b
-	-	0.5	-	47 ± 1.6d	4.3 ± 0.5e	6.2 ± 0.6a
-	-	1.0	-	65 ± 3.1b	6.8 ± 0.7d	6.1 ± 0.6a
-	-	1.5	-	52 ± 2.7c	5.5 ± 0.4e	5.8 ± 0.4b
-	-	2.0	-	35 ± 1.4e	2.6 ± 0.3f	5.6 ± 0.4b
0.1	-	-	-	14 ± 0.7g	1.2 ± 0.2g	6.1 ± 0.5a
0.2	-	-	-	27 ± 0.9f	2.1 ± 0.1f	5.9 ± 0.4b
0.5	-	-	-	32 ± 1.1ef	2.5 ± 0.3f	6.2 ± 0.6a
1.0	-	-	-	44 ± 2.1d	1.7 ± 0.1g	6.1 ± 0.7a
1.5	-	-	-	48 ± 1.8d	1.2 ± 0.4g	6.2 ± 0.6a
2.0	-	-	-	37 ± 1.3e	1.3 ± 0.2g	5.9 ± 0.5b
-	0.1	-	-	9 ± 0.02g	1.3 ± 0.1g	5.6 ± 0.6b
-	0.2	-	-	15 ± 0.8g	1.8 ± 0.4g	5.8 ± 0.6b
-	0.5	-	-	23 ± 0.9f	2.2 ± 0.3f	6.1 ± 0.4a
-	1.0	-	-	35 ± 1.1e	1.9 ± 0.4g	5.9 ± 0.5b
-	1.5	-	-	31 ± 1.7e	1.6 ± 0.4g	5.4 ± 0.5b
-	2.0	-	-	19 ± 0.7g	1.4 ± 0.2g	5.1 ± 0.6b
-	-	1.0	0.1	69 ± 2.4b	11.3 ± 0.6a	5.8 ± 0.7b
-	-	1.0	0.2	87 ± 3.5a	18.9 ± 0.7a	5.9 ± 0.5b
-	-	1.0	0.5	71 ± 2.1b	8.1 ± 0.2b	5.8 ± 0.6b
-	-	1.0	1.0	52 ± 3.4c	4.8 ± 0.3c	5.4 ± 0.4b
-	1.0	-	0.1	22 ± 1.9f	1.2 ± 0.1g	5.1 ± 0.7b

* Values represent mean ± SE. Means followed by same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test

PGRs (mg/l)				Percent response*	Mean no. of shoots*	Mean shoot length (cm)*
TDZ	KN	BAP	NAA			
-	1.0	-	0.2	18 ± 1.8g	1.1 ± 0.2g	4.6 ± 0.3c
-	1.0	-	0.5	16 ± 1.5g	1.1 ± 0.1g	4.3 ± 0.5c
-	1.0	-	1.0	9.0 ± 1.9g	1.0 ± 0.3g	4.2 ± 0.4c
1.5	-	-	0.1	43 ± 2.9d	1.1 ± 0.2g	4.9 ± 0.5c
1.5	-	-	0.2	37 ± 0.9e	0.9 ± 0.1g	5.1 ± 0.4b
1.5	-	-	0.5	27 ± 1.2f	1.1 ± 0.1g	5.6 ± 0.4b
1.5	-	-	1.0	14 ± 0.7g	1.2 ± 0.2g	5.3 ± 0.6b

* Values represent mean ± SE. Means followed by same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test

Indirect shoot regeneration from internodal tTCLs

Internodal region collected from 45-d-old in vitro plants were excised into tTCLs (Fig. 5A) and cultured on MS medium supplemented with different concentrations (0.1-2.0 mg/l) of BAP, TDZ and KN. No sign of direct shoot regeneration was observed in all treatments and the explants turned brown and necrotic or developed nonregenerative whitish powdery calli (data not shown). Hence, the internode derived tTCLs were cultured on MS medium fortified with various concentrations (0.5-2.0 mg/l) of 2, 4-D. Calli started originating from the cut portions of the explants 7 d after culture (Fig. 5B) and later spread to the entire surface of the explant. On all concentrations of 2, 4-D used, luxuriantly growing greenish friable calli were induced. Of the various concentrations of 2, 4-D tried, 1.5 mg/l was optimum. On this medium, 75% of internode derived tTCLs produced calli with a mean fresh weight of 358 mg/tube (Table 3). However, media without PGRs did not result in callus induction. For callus induction from various explants 2, 4-D is routinely employed in several systems like *Zinnia elegans* (Samantaray and Singh 2021) and *Solanum tuberosum* (Shirin et al. 2007). During callogenesis, 2, 4-D can revert differentiated cells of the explant to dedifferentiated state and cause them to divide (George et al. 2008). Ashton and Crafts (1981) reported that 2, 4-D causes cell enlargement by rising the activity of enzymes responsible for cell wall loosening and cell wall material synthesis.

Table 3
Effect of 2, 4-D on callus induction from tTCLs of internode after 45 d of culture.

2, 4-D (mg/l)	Percent response*	Fresh weight (mg/tube)*	Dry weight (mg/tube)*
0.0	0.0	0.0	0.0
0.5	39 ± 1.67c	229 ± 6.2b	18 ± 3.2c
1.0	56 ± 3.22b	319 ± 8.2a	32 ± 8.2b
1.5	75 ± 4.61a	358 ± 6.3a	37 ± 7.5b
2.0	48 ± 2.89c	144 ± 4.2c	11 ± 5.3c

* Values represent mean ± SE. Means followed by same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test

The friable organogenic calli were subcultured on MS medium supplemented with various concentrations of BAP, KN and TDZ (0.1-2.0 mg/l) alone or in combination with NAA (0.1-1.0 mg/l) for shoot induction. On MS basal medium there was no shoot organogenesis. MS medium supplemented with BAP alone or in combination with NAA did not yield any shoot induction (data not shown). However, on all other media combinations shoot organogenesis from calli was observed. The percentage of shoot induction and mean number of shoots varied with the type and concentration of PGRs used. In KN alone or in combination with NAA the response was poor. Here, KN alone was better than with NAA combination. Optimum shoot induction response was obtained on 1.0 mg/l KN where 19% cultures responded with an average number of 4.6 shoots per explant (Table 4). The highest response was obtained on MS medium supplemented with 0.5 mg/l TDZ. On this medium, 64% cultured calli responded with an average number of 22.9 shoots per explant (Table 4). However, TDZ in combination with NAA was not promising. The initial sign of shoots originated from green friable calli as small outgrowths. Later, these outgrowths transformed in to shoot buds (Fig. 5C- E). Large number of elongated shoots were visible from calli after 45 d of culture (Fig. 5F). TDZ is a potential cytokinin extensively used for shoot induction from various explants (Huetteman and Preece 1993). TDZ stimulated callus organogenesis has been reported in several systems such as *Jatropha curcas* (Kumar et al. 2010), *Tylophora indica* (Thomas and Philip 2005), *Caryopteris terniflora* (Wu et al. 2021) and *Plumbago zeylanica* (Sharma and Agarwal 2018). In this study we obtained callus induction and shoot organogenesis from internodal derived tTCLs of *C. punctatum*. A similar study in *Talinum triangulare* where callus induction and shoot regeneration has been achieved from internodal tTCLs by Swarna and Ravindran (2013). Shoot organogenesis from internode tTCLs derived calli of *Tanacetum cinenariifolium* was obtained on MS medium supplemented with various concentrations of BAP and IBA (Mao et al. 2013). Chattopadhyaya et al. (2010) reported successful shoot regeneration from internode tTCLs of *Sesamum indicum* on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA.

Table 4
Effect of different PGRs on shoot induction from in vitro tTCL derived callus from internodes 45 d after culture.

PGRs (mg/l)			Percent response*	Mean no. of shoots*	Mean shoot length (cm)*
TDZ	KN	NAA			
0.0	0.0	0.0	0.0	0.0	0.0
0.1	-	-	8.0 ± 0.1f	6.1 ± 1.2c	6.2 ± 0.5a
0.2	-	-	31 ± 1.2d	12.4 ± 2.4b	5.9 ± 0.6b
0.5	-	-	64 ± 4.5a	22.9 ± 4.3a	6.1 ± 0.6a
1.0	-	-	46 ± 3.9c	14.3 ± 2.5b	6.3 ± 0.6a
1.5	-	-	58 ± 4.3b	11.2 ± 1.2b	6.2 ± 0.4a
2.0	-	-	37 ± 2.9d	9.3 ± 1.3b	6.1 ± 0.5a
-	0.1	-	8.0 ± 0.2f	1.2 ± 0.8d	5.3 ± 0.b
-	0.2	-	11 ± 0.3ef	1.5 ± 1.1d	5.8 ± 0.5b
-	0.5	-	13 ± 0.2ef	2.3 ± 1.3d	5.9 ± 0.4b
-	1.0	-	19 ± 0.9e	4.6 ± 2.3c	5.7 ± 0.5b
-	1.5	-	12 ± 0.5ef	3.8 ± 2.4d	6.3 ± 0.6a
-	2.0	-	10 ± 0.3ef	2.9 ± 1.4d	5.6 ± 0.7b
0.5	-	0.1	46 ± 1.8c	18.3 ± 1.5b	5.9 ± 0.4b
0.5	-	0.2	42 ± 2.3c	9.9 ± 0.6b	6.1 ± 0.5a
0.5	-	0.5	38 ± 2.1d	4.1 ± 0.7c	5.9 ± 0.6b
0.5	-	1.0	14 ± 1.8ef	2.8 ± 0.4d	5.4 ± 0.5b
-	1.0	0.1	17 ± 1.1e	3.1 ± 0.5d	5.8 ± 0.4b
-	1.0	0.2	14 ± 0.8e	2.4 ± 0.6d	5.6 ± 0.5b
-	1.0	0.5	12 ± 0.6de	1.9 ± 0.5d	5.4 ± 0.6b
-	1.0	1.0	11 ± 0.9de	1.3 ± 0.4d	5.2 ± 0.3b

* Values represent mean ± SE. Means followed by same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test

Rooting and acclimatization

Shoots derived from all the different tTCLs were used for root induction. Shoots below 0.5 cm length were excised and cultured on elongation medium i.e., MS medium supplemented with 1.0 mg/l BAP (Fig. 6A). Shoots having a size of 1.5- 2.0 cm were excised and cultured on ½ MS medium supplemented with various concentrations of IBA (0.25-2.0 mg/l) for rooting. There was no significant difference in rooting of shoots derived from different tTCLs. The highest frequency of rooting was observed on ½ MS medium supplemented with 0.5 mg/l IBA. Here, 95% shoots rooted with a mean number of 15.6 roots per shoot (Table 5; Fig. 6B). Induction of rooting by IBA has been well documented in other systems such as *Withania somnifera* (Vdawale et al. 2004), *Gymnostachyum febrifugum* (Silpa and Thomas 2021), *Nicotiana tabacum* (Shoyeb et al. 2020) and *Hemidesmus indicus* (Sreekumar et al. 2000). Well developed in vitro derived rooted plants were transferred to paper cups containing sand and soil (1:1) and acclimatized (Fig. 6C).

Table 5
Effect of different concentrations of IBA on root induction from tTCL derived in vitro grown shoots. Medium: ½ MS, culture period 45 d.

Concentration of IBA (mg/l)	Percent response*	Mean no. of roots*	Mean root length (cm)*
0.0	9.0 ± 1.2d	2.5 ± 0.3c	3.2 ± 0.2c
0.25	45.1 ± 2.9b	8.2 ± 0.9b	2.5 ± 0.6d
0.5	95.0 ± 4.5a	15.6 ± 2.0a	7.2 ± 0.9a
1.0	60.5 ± 3.6b	9.2 ± 2.3b	6.3 ± 1.2b
1.5	23.0 ± 2.1c	3.6 ± 1.1c	2.1 ± 1.0d
2.0	20.6 ± 1.9c	3.1 ± 1.4c	1.5 ± 0.7e

* Values represent mean ± SE. Means followed by same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test

Conclusions

In conclusion tTCLs are an excellent explant for micropropagation of *C. punctatm.* Leaf and nodal explants are ideal for direct shoot induction whereas internodal explants responded by indirect organogenesis. The shoot regeneration depends on source of tTCLs, their thickness/width as well as on type of PGRs in media. For *C. punctatm* leaf derived tTCL was observed to be superior than node and internode derived tTCLs. The favourable thickness/ width of tTCL varied according to explant source. For leaf 1.0 mm wide and node 2.0 mm thick tTCLs resulted in highest shoot regeneration. For internodal tTCLs, maximum callus induction was achieved in 3.0 mm thickness, but highest shoot regeneration was obtained from 2.0 mm tTCL derived callus. The micropropagation technique using tTCLs is advantageous in terms of minimum usage of explant tissue as well as efficient shoot regeneration. This protocol can be effectively implemented for the mass propagation of *C. punctatm*.

Abbreviations

TCL	Thin cell layer
tTCL	Transverse thin cell layer
lTCL	Longitudinal thin cell layer
PGR	Plant growth regulator
BAP	6- benzyl aminopurine
TDZ	Thidiazuron
KN	Kinetin
NAA	∞- Naphthaleneacetic acid
2, 4-D	2, 4-Diclorophenoxyacetic acid
IBA	Indole 3- butyric acid
MS	Murashige and Skoog

Declarations

Acknowledgements

Aswathi N V thankfully acknowledges the financial support from CSIR, Govt. of India in the form of JRF and SRF [Award no. 09/1108(0019)/2017-EMR-I].

Author contributions

ANV collected materials and performed the experiments, evaluated data, wrote the manuscript. TDT designed the experiments, contributed to writing and corrected manuscript.

Conflict of interest : The authors declare that they have no conflict of interest.

Ethical approval : This research paper does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate : Informed consent was obtained from all individual participants included in the study.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

References

Abdolinejad R, Shekafandeh A, Jowkar A, Gharaghani A, Alemzadeh A (2020) Indirect regeneration of *Ficus carica* by the TCL technique and genetic fidelity evaluation of the regenerated plants using flow cytometry and ISSR. Plant Cell Tissue Organ Cult 143:131-144

Alves FV, Loeuille BF (2021) Geographic distribution patterns of species of the subtribe Lychnophorinae (Asteraceae: Vernonieae). Rodriguésia 72: e02072019. https://doi.org/10.1590/2175-7860202172072

Ashton FM, Crafts AS (1981) Mode of action of herbicides, John Wiley and Sons, Inc, USA. Pp. 65-77

Baskaran P, Kumari A, Van Staden J (2018) In vitro propagation via organogenesis and synthetic seeds of *Urginea altissima* (Lf) Baker: a threatened medicinal plant. 3 Biotech 8. https://dx.doi.org/10.1007%2Fs13205-017-1028-7

Bevelle CA, Handy GA, Segal RA, Cordell GA, Farnsworth NR (1981) Isocentratherin, a cytotoxic germacranolide from *Centratherum punctatum* (Compositae). Phytochemistry 20:1605-1607

Burim RV, Canalle R, Lopes JL, Vichnewski W, Takahashi CS (2001) Genotoxic action of the sesquiterpene lactone centratherin on mammalian cells in vitro and in vivo. Teratog Carcinog Mutagen 21:383-393

Chattopadhyaya B, Banerjee J, Basu A, Sen SK, Maiti MK (2010) Shoot induction and regeneration using internodal transverse thin cell layer culture in *Sesamum indicum* L. Plant Biotechnol Rep 4:173-178

Chiappeta AD, de Mello JF, Maciel GM (1983) Higher plants with biological activity plants of Pernambuco. Rev Inst Antibiot 21:43-50

Chitra B, Brindha P (2014) Studies on preliminary phytochemical screening of different extract of *Centratherum punctatum* Cass.- a traditional wound healer. Int J Pharm Sci Res 6:19-22

Chitra B, Brindha P (2015) Antimicrobial activity of ethanol and aqueous extract of *Centratherum punctatum* Cass. World J Pharm Pharm Sci 4:1126-1131

Chitra B, Brindha P, Vijayakumar AB (2016) Protease activity of floral extracts of *Centratherum punctatum* Cass. a wound healing herb. World J Pharm Res 5:1079-1083

Croom LA, Jackson CL, Vaidya BN, Parajuli P, Joshee N (2016) Thin Cell Layer (TCL) culture system for herbal biomass production and genetic transformation of *Bacopa monnieri* L. Wettst. Am J Plant Sci 7:1232-1245

David JP (2001) Phytochemistry and medicinal plants. Phytochemistry 56:237-243

Dobránszki J, Teixeira da Silva JA (2011) Adventitious shoot regeneration from leaf thin cell layers in apple. Sci Hortic 127:460-463

Duncan DB (1955) Multiple range and multiple F test. Biometrics 11:1-42

Flann C (2009) Global Compositae checklist. http://compositae.landcareresearch.co.nz/ Default.aspx

Franklin G, Jeyachandran R, Ignacimuthu S (2000) Factors affecting regeneration of pigeon pea (*Cajanus cajan* L. Millsp) from mature embryonal axes. Plant Growth Regul 30:31-36.

Gendy C, Sene M, Van Le B, Vidal J, Thanh KV (1996) Somatic embryogenesis and plant regeneration in *Sorghum bicolor* (L.) Moench. Plant Cell Rep 15:900-904

George EF, Hall MA, De Klerk GJ (2008) Plant propagation by tissue culture. 3rd Edn, Springer, Dordrecht, Netherlands. Pp. 501-502

Ghnaya AB, Charles G, Branchard M (2008) Rapid shoot regeneration from thin cell layer explants excised from petioles and hypocotyls in four cultivars of *Brassica napus* L. Plant Cell Tissue Organ Cult 92:25-30

Hedayat H, Abdi Gh, Khosh-Khui M (2009) Regeneration via direct organogenesis from leaf and petiole segments of pyrethrum *Tanacetum cinerariifolium* (Trevir.) Schultz-Bip. American- Eurasian J Agric Environ Sci 6:81-87

Hieu T, Linh NT, Tung HT, Bao HG, Nguyen CD, Nhut DT (2018) Stimulation of shoot regeneration through leaf thin cell layer culture of *Passiflora edulis* Sims. Vietnam J Biotech 16:669-677

Huetteman CA, Preece JE (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tissue Organ Cult 33:105-119

Junior FM, Covington CL, de Albuquerque AC, Lobo JF, Borges RM, de Amorim MB, Polavarapu PL (2015) Absolute configuration of (−) - Centratherin, a sesquiterpenoid lactone, defined by means of chiroptical spectroscopy. J Nat Prod 25:2617-2623

Kumar N, Anand KV, Reddy MP (2010) Shoot regeneration from cotyledonary leaf explants of *Jatropha curcas*: a biodiesel plant. Acta Physiol Plant 32:917-924

Lakshmanan P, Loh CS, Goh CJ (1995) An in vitro method for rapid regeneration of a monopodial orchid hybrid *Aranda deborah* using thin section culture. Plant Cell Rep 14:510-514

Leguillon S, Charles G, Branchard M (2003) Plant Regeneration from thin cell layers in *Spinacia oleracea*. Plant Cell Tissue Organ Cult 74:257-265

Lemma DT, Banjaw DT, Megersa HG (2020) Micropropagation of medicinal plants. Int J Plant Breed Crop Sci 7:796-802

Loberant B, Altman A (2010) Micropropagation of plants. In: Flickinger MC (ed) Encyclopedia of industrial biotechnology. Wiley, New York. Pp. 1-7. https://doi.org/10.1002/9780470054581.eib442

Madhumitha KM, Anbumalarmathi J, Sharmili SA, Nandhini G, Priya GS (2020) A comparative study of in vivo plant and in vitro callus extracts of *Centratherum punctatum* Cass. Annu Res Rev Biol 18:1-13

Mahesh B, Satish S (2008) Antimicrobial activity of some important medicinal plants against plant and human pathogens. World J Agri Sci 4:839-843

Mao J, Zhou Y, Guo CX, Xu HL, Wang CY (2013) An *Agrobacterium*-mediated transformation system from thin cell layer explants of pyrethrum (*Tanacetum cinenariifolium*). In VI international symposium on the taxonomy of cultivated plants 1035:163-170

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473-497

Nhut DT, Hai NT, Huyen PX, Huong DT, Hang NTD, Teixeira da Silva JA (2005) Thidiazuron induces high frequency shoot bud formation from *Begonia* petiole transverse thin cell layer culture. Propag Ornam Plants 5:149-155

Nhut DT, Teixeira da Silva JA, Van Le B, Tran Thanh Van K (2003a) Thin cell layer studies of vegetable, leguminous and medicinal plants. In: Nhut DT, Van Le B, Tran Thanh Van K, Thorpe T (eds) Thin cell layer culture system: regeneration and transformation applications. Kluwer Academic Publishers, Dordrecht, The Netherlands. Pp. 387-425

Nhut DT, Teixeira da Silva JA, Van Le B, Tran Thanh Van K (2003b) Organogenesis of cereals and grasses by using thin cell layer technique. In: Nhut DT, Van Le B, Tran Thanh Van K, Thorpe T (eds) Thin cell layer culture system: regeneration and transformation applications. Kluwer Academic Publishers, Dordrecht, The Netherlands. Pp. 427-449

Nhut DT, Van Le B, Tanaka M, Van KT (2001a) Shoot induction and plant regeneration from receptacle tissues of *Lilium longiflorum*. Sci Hortic 87:131-138

Nhut DT, Van Le B, Van KT (2001b) Manipulation of the morphogenetic pathways of *Lilium longiflorum* transverse thin cell layer explants by auxin and cytokinin. In Vitro Cell Dev Biol - Plant 37:44-49

Ogunwande IA, Olawore NO, Usman L (2005) Composition of the leaf oil of *Centratherum punctatum* Cass. growing in Nigeria. J Essent Oil Res 17:496-498

Ohki S (1994) Scanning electron microscopy of shoot differentiation in vitro from leaf explants of the African violet. Plant Cell Tissue Organ Cult 36:157-162

Ohno N, McCormick S, Mabry TJ (1979) Centratherin, a new germacranolide from *Centratherum punctatum*. Phytochemistry 18:681-668

Pawar NK, Arumugam N (2011) Leaf extract of *Centratherum punctatum* exhibits antimicrobial, antioxidant and anti-proliferative properties. Asian J Pharm Clin Res 4: 71-76

Rout GR, Mohapatra A, Jain SM (2006) Tissue culture of ornamental pot plant: A critical review on present scenario and future prospects. Biotechnol Adv 24:531-60

Samantaray MU, Singh MT (2021) Optimization of modified Murashige and Skoog (MS) medium promoting efficient callus induction in *Zinnia elegans*. Int J Res Appl Sci Biotech 8:202-211

Satyal P, Hieu HV, Lai DT, Ngoc NTB, Hung NH, Setzer WN (2018) The essential oil compositions of *Centratherum punctatum* growing wild in Vietnam. Am J Essent Oil 6: 15-18

Sethy R, Kullu B (2022) Micropropagation of ethnomedicinal plant *Calotropis* sp. and enhanced production of stigmasterol. Plant Cell Tissue Organ Cult 149:147-158

Sharma U, Agrawal V (2018) In vitro shoot regeneration and enhanced synthesis of plumbagin in root callus of *Plumbago zeylanica* L.-an important medicinal herb. In Vitro Cell Dev Biol - Plant 54:423-435

Shirin F, Hossain M, Kabir MF, Roy M, Sarker SR (2007) Callus induction and plant regeneration from internodal and leaf explants of four potato (*Solanum tuberosum* L.) cultivars. World J Agric Sci 3:66-72

Shoyeb M, Kanis Fatema M, Sarkar AR, Rahman A, Rahman SM (2020) Efficient regeneration of tobacco (*Nicotiana tabacum* L.) plantlets from cotyledon, hypocotyl and leaf explants: An excellent model plant for gene function analysis. Curr J Appl Sci Tech 39:1-9

Silpa P, Thomas TD (2021) High-frequency shoot regeneration from flower bud derived callus of *Gymnostachyum febrifugum* Benth., an endemic medicinal plant to the Western Ghats. Plant Cell Tissue Organ Cult 147:221-288

Silvertand B, Lavrijsen P, Van Harten A (1992) In vitro multiplication of leek (*Allium peloprasum* L.) by using segments of the flower stalk. Allium Improvement News Lett 2:21-23

Singh SK, Rai MK, Sahoo L (2012) An improved and efficient micropropagation of *Eclipta alba* through transverse thin cell layer culture and assessment of clonal fidelity using RAPD analysis. Ind Crops Prod 37:328-333

Sivasubramanian R, Brindha P (2013) In-vitro cytotoxic, antioxidant and GC-MS studies on *Centratherum punctatum* Cass. Int J Pharm Pharm Sci 5:364-367

Sivasubramanian R, Brindha P (2014) *Centratherum punctatum* Cass. - A herbal dietary supplement in the management of cancer. Int J Pharm Pharm Sci 6:73-74

Sreekumar S, Seeni S, Pushpangadan P (2000) Micropropagation of *Hemidesmus indicus* for cultivation and production of 2-hydroxy 4-methoxy benzaldehyde. Plant Cell Tissue Organ Cult 62:211-218

Sudhakaran S, Teixeira da Silva JA, Sreeramanan S (2006) Test tube Bouquets - in vitro flowering. In: Teixeira da Silva JA (ed) Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues, 1st edn. (Vol II), Global Science Books, London, UK. Pp. 336-346

Swarna J, R Ravindharan (2013) In vitro organogenesis from leaf and transverse thin cell layer derived callus cultures of *Talinum triangulare* (Jacq.) Willd. Plant Growth Regul 70:79-87

Taylor NJ, van Staden JA (2006) Towards an understanding of the manipulation of in vitro flowering. In: Teixeira da Silva JA (ed) Floriculture, ornamental and plant biotechnology: Advances and topical issues, 1st edn. (Vol IV), Global Science Books, London, UK. Pp. 1-22

Teixeira da Silva JA, Nhut DT (2003) Thin cell layers and floral morphogenesis, floral genetics and in vitro flowering. In: Nhut DT, Van Le B, Tran Thanh Van K, Thorpe T (eds) Thin cell layer culture system: regeneration and transformation applications. Kluwer Academic Publishers, Dordrecht, The Netherlands. Pp. 65-134

Teixeira da Silva JA, Tanaka M (2011) Thin cell layers: The technique. In: Davey M, Anthony P (eds) Plant cell culture: methods express. Wiley-Blackwell, Chichester, UK. Pp. 25-37

Thomas TD, Philip B (2005) Thidiazuron-induced high-frequency shoot organogenesis from leaf-derived callus of a medicinal climber, *Tylophora Indica* (Burm. F.) merrill. In Vitro Cell Dev Biol - Plant 41:124-128

Thomas TD, Yoichiro H (2010) In vitro propagation for the conservation of a rare medicinal plant *Justicia gendarussa* Burm. f. by nodal explants and shoot regeneration from callus. Acta Physiol Plant 32:943-950

Tran Thanh Van M (1973) In vitro control of de novo flower, bud, root, and callus differentiation from excised epidermal tissues. Nature 246:44-45

Tubić L, Savić J, Mitić N, Milojević J, Janošević D, Budimir S, Zdravković-Korać S (2016) Cytokinins differentially affect regeneration, plant growth and antioxidative enzymes activity in chive (*Allium schoenoprasum* L.). Plant Cell Tissue Organ Cult 124:1-14

Vaidya BN, Jackson CL, Perry ZD, Dhekney SA, Joshee N (2016) *Agrobacterium*-mediated transformation of thin cell layer explants of *Scutellaria ocmulgee* small: a rare plant with anti-tumor properties. Plant Cell Tissue Organ Cult 127:57-69

Vdawale AV, Mehta-bhatt P, Dave AM (2004) Rapid in vitro propagation of Ashwagandha (*Withania somnifera*) through axillary bud multiplication and indirect organogenesis. Phytomorphology 54:59-64

Welander M (1988) Plant regeneration from leaf and stem segments of shoots raised in vitro from mature apple trees. J Plant Physiol 132:738-744

Whistler W A (2000) Tropical ornamentals: a guide. Timber Press, Portland, Oregon. Pp. 541-542

World Health Organisation (2000) Quality control methods for medicinal plant materials, WHO, Geneva, Switzerland

Wu Q, Yang H, Sun Y, Hu J, Zou H (2021) Organogenesis and high-frequency plant regeneration in *Caryopteris terniflora* Maxim. using thidiazuron. In Vitro Cell Dev Biol - Plant 57:39-47

Yeshi K, Crayn D, Ritmejerytė E, Wangchuk P (2022) Plant secondary metabolites produced in response to abiotic stresses has potential application in pharmaceutical product development. Molecules 27:313-344

Zhao P, Wang W, Feng FS, Wu F, Yang ZQ, Wang WJ (2007) High-frequency shoot regeneration through transverse thin cell layer culture in *Dendrobium candidum* Wall Ex Lindl. Plant Cell Tissue Organ Cult 90:131-139

Figures

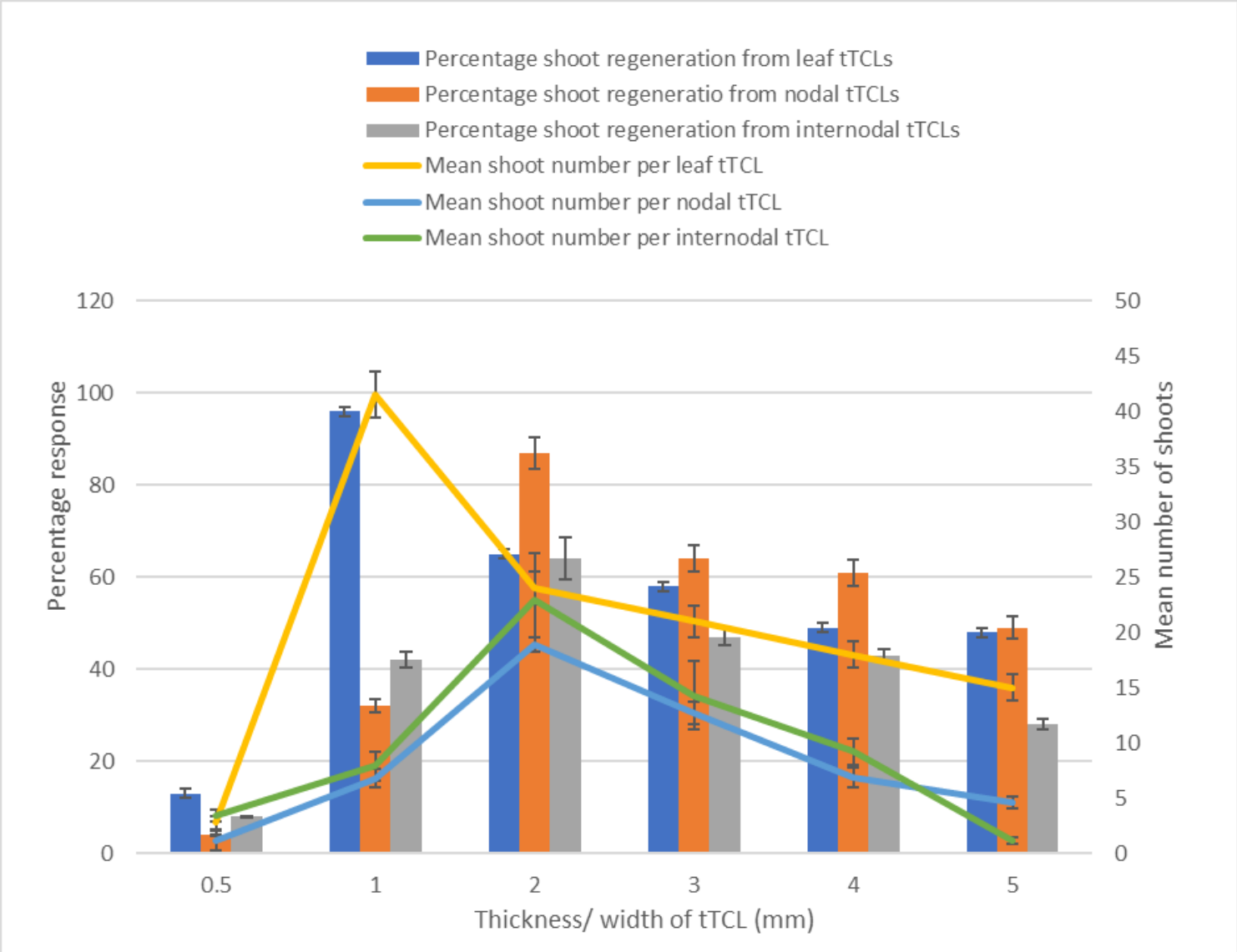

Figure 1

Effect of tTCL thickness/width on percentage shoot regeneration and mean shoot number. MS medium supplemented with 1.5 mg/l TDZ, 0.5 mg/l TDZ and 1.0 mg/l BAP in combination with 0.2 mg/l NAA were used for tTCLs of leaf, internode and node respectively.

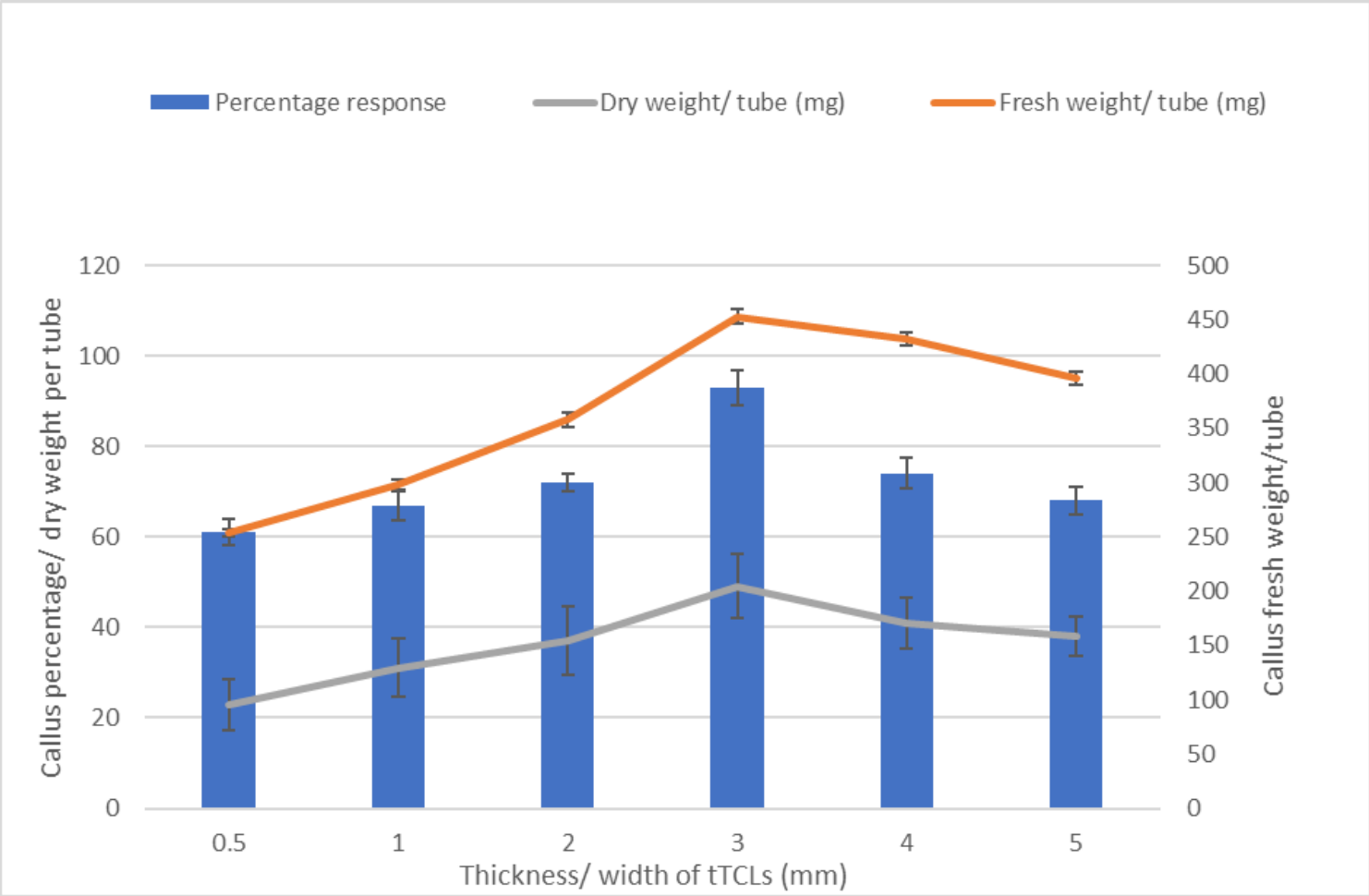

Figure 2

Effect of internodal tTCL thickness on percentage callus induction, fresh and dry weight of callus per tube after 45 d of culture on MS medium supplemented with 1.5 mg/l 2, 4-D.

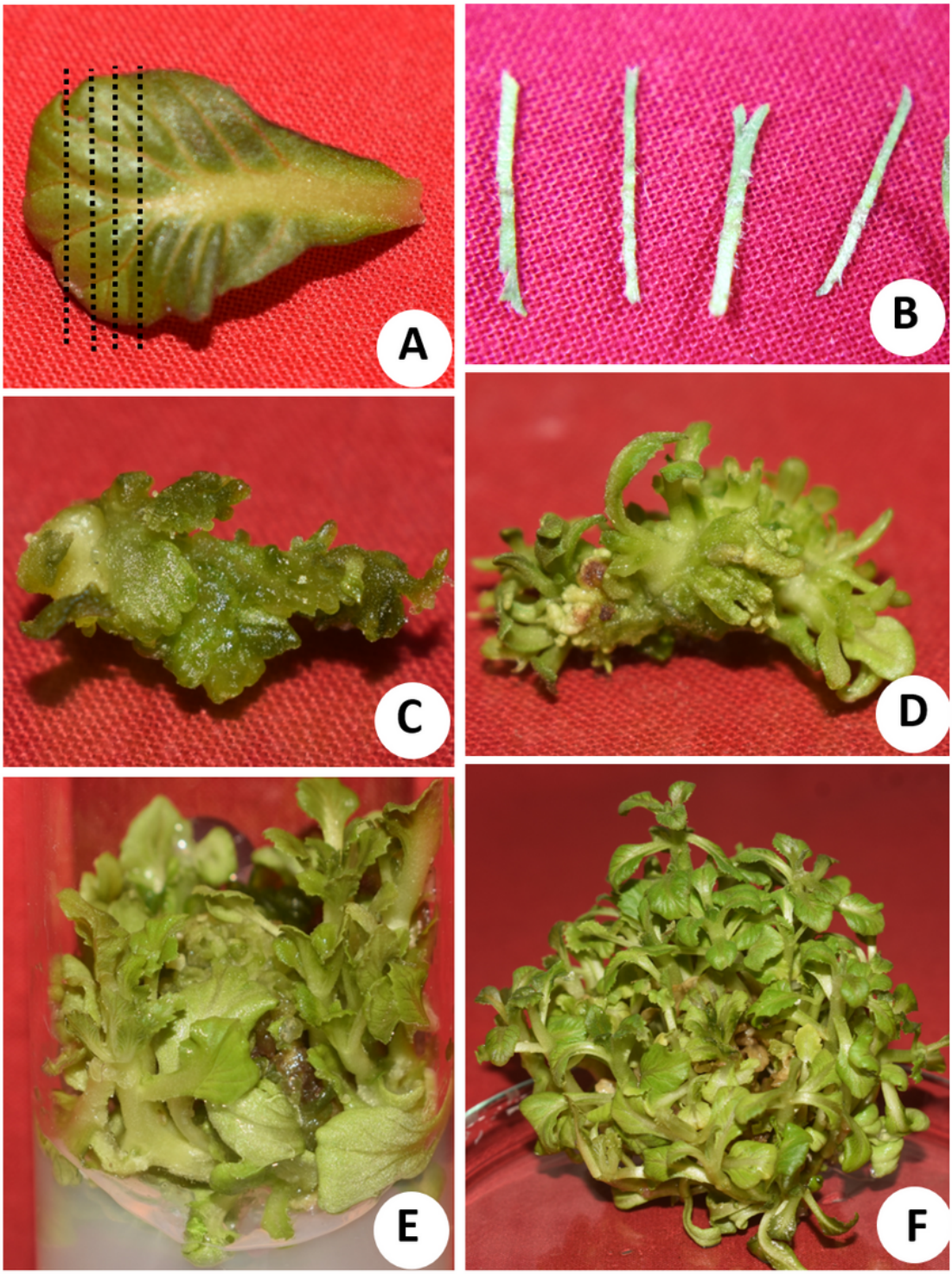

Figure 3

Direct shoot induction from leaf tTCLs of *C. punctatum* on MS medium supplemented with 1.5 mg/l TDZ.

A. A 10-d-old leaf taken for thin cell layer culture. The line of excision shown as dotted lines.

B. Leaf tTCLs just before transfer to media. Each tTCL has a width of 1.0 mm.

C. Shoot initiation from cultured tTCL 7 d after culture on MS medium supplemented with 1.5 mg/l TDZ.

D. Same as in C after 14 d. More shoots developed from the explant.

E. Formation of elongated shoots 30 d after culture from the explant.

F. The emergence of larger number of shoots from cultured tTCL 45 d after culture.

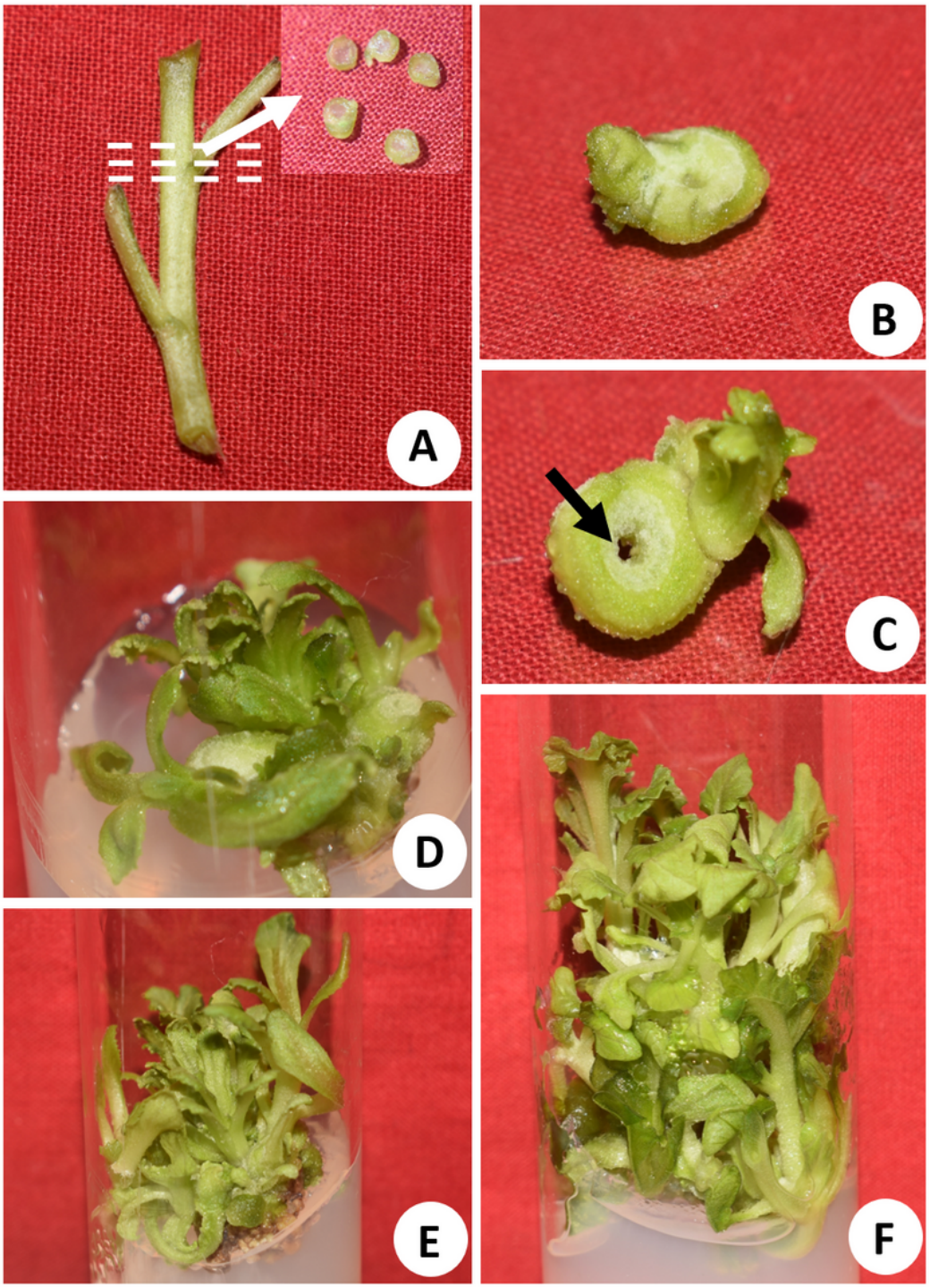

Figure 4

Various stages of direct shoot induction from nodal tTCLs on MS medium supplemented with 1.0 mg/l BAP in combination with 0.2 mg/l NAA.

A. Excision of tTCLs from nodal region of *C. punctatum* (Dotted lines). Inset shows tTCLs after excision from nodal region before inoculation.

B. Shoot initiation from nodal tTCL7 d after culture.

C. Same as in B 14 d after culture. A single shoot developed further. Note the formation of a hollow region at the centre of tTCL (arrow).

D. Multiple shoots initiation from cultured tTCL after 20 d.

E. Cultured nodal tTCLs producing more healthy shoots after 30 d.

F. Large number of elongated fully developed shoots from tTCL after 45 d of culture.

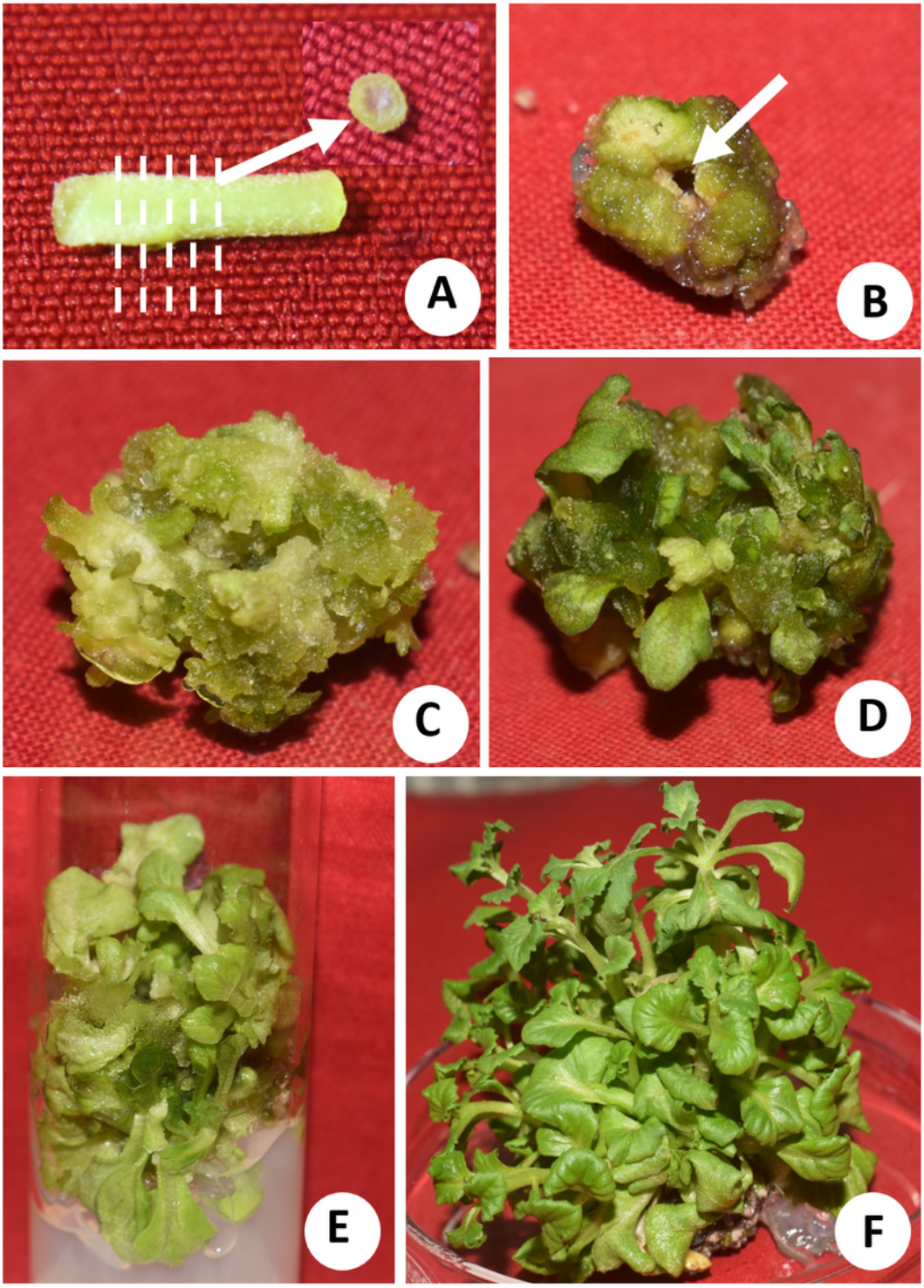

Figure 5

Callus induction and shoot organogenesis from internodal tTCLs of *C. punctatum*.

A. Explant excision from internodal region (dotted lines). Inset shows an excised tTCL from internodal region.

B. Callus initiation from cultured internodal tTCL after 7 d of culture on MS medium supplemented with 1.5 mg/l 2, 4-D. Note the formation of a hollow region at the centre of the callus (arrow).

C. Emergence of several minute shoot buds from cultured calli on MS medium supplemented with 0.5 mg/l TDZ 15 d after culture.

D. Proliferation of shoots from calli 20 d after culture.

E. Same as in D 30 d after culture. The shoots developed further.

F. Luxuriantly growing callus derived shoots on MS medium supplemented with 0.5 mg/l TDZ 45 d after culture.

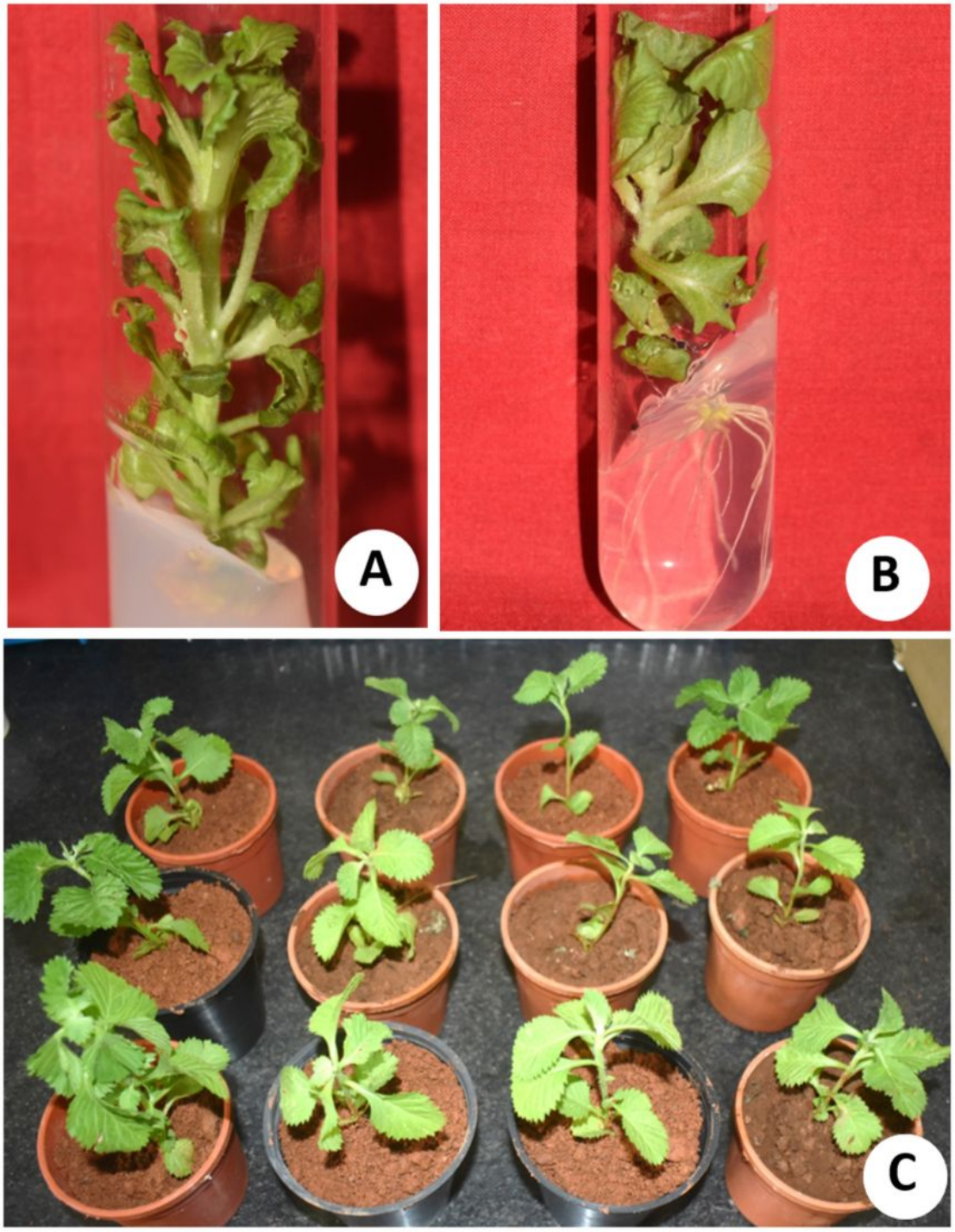

Figure 6

Rooting and acclimatization of shoots.

A. A well-developed elongated shoot on ½ MS medium supplemented with 1.0 mg/l BAP 30 d after culture.

B. Rooted shoot 45 d after culture in half strength MS medium supplemented with 0.5 mg/l IBA.

C. Successfully acclimatized tTCL derived 2-month-old *C. punctatum* plants.