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RESEARCH ARTICLE

In vitro clonal propagation of *Coscinium fenestratum* (Gertn.) Colebr. (Weniwel) through nodal explants

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Abstract: An *in vitro* clonal propagation protocol for *Coscinium fenestratum* was developed using shoot explants detached from 1 − 2 year old vines maintained under plant house conditions, by successfully surface sterilising with 0.2 % solution of mercuric chloride for 30 minutes followed by two successive washings with sterilised distilled water. McCowns woody plant medium (WPM) incorporated with 1.0 mgL^{-1} polyvinylpyrrolidone to minimise browning, was the best medium for establishment of nodal cuttings.

Mature double nodal cuttings resulted in the highest shoot proliferation rate (3.90 shoots/explant) when cultured on WPM medium supplemented with 2.0 mgL^{-1} 6-benzylaminopurine, 1.0 mgL^{-1} thidiazuron and 0.4 mgL^{-1} 2,4-dichlorophenoxyacetic acid. Shoots were separated and transferred to WPM medium devoid of plant growth regulators for regeneration into plantlets. The plantlets were successfully acclimatised on coir dust: sand (1:1) potting media with over 60 % survival rate. The results proved that the protocol developed is effective for clonal propagation of *C. fenestratum*.

Keywords: *In vitro* clonal propagation, proliferation, shoot explants, surface sterilisation, WPM medium.

INTRODUCTION

Coscinium fenestratum (Gaertn.) Colebr. (Menispermaceae) is a dioecious large woody climber, which is extensively used in Sri Lanka as a common remedy to heal many diseases. The stems and roots are extensively used in ayurvedic preparations for treating digestive disorders, chronic fevers, wounds, snake bites, ulcers, jaundice, burns, skin diseases, abdominal disorders, diabetes and general debility in South India and Sri Lanka. The root bark is used for dressing wounds, ulcers and in cutaneous leishmaniasis (Tushar *et al*., 2008).

C. fenestratum is indigenous to the Indo-Malayan region and is found in India, Malaysia, Vietnam, Myanmar, Singapore, Thailand and Sri Lanka (Tushar *et al*., 2008). It is naturally abundant in forest fringes and disturbed forests in the low country Wet Zone of Sri Lanka (Jayaweera, 2006).

According to the 1997 IUCN red list of threatened species, *C. fenestratum* is recorded as critically endangered in many countries including Sri Lanka (Walter & Gillett, 1998). As reported by Agusta (2003), and An and Ziegler (2001), *C. fenestratum* has already been listed as an endangered species in India and Sri Lanka. To reach maturity and fruiting stage, the plants take at least 15 years due to its slow growth habit (Tushar *et al*., 2008). Furthermore, destructive harvesting even before maturity, zero cultivation, habitat specificity, and destruction of natural habitats result in a diminishing population of *C. fenestratum.*

Propagation of *C. fenestratum* is commonly done by seeds. There are several limitations for seed propagation; seeds can be seen only once a year and these have hard seed coats and the viability of seeds are lost quickly under low moisture conditions (Senerath, 1991; Kathriarachchi *et al.,* 2004). Within a population, only around 30 % of plants generate flowers during a flowering season (Van & Tap, 2008). Under natural conditions, seed germination was found to be 23 – 26 %, and 12 % of the fresh fruits were found to contain non-viable seeds (Senerath, 1991). Conventional vegetative propagation through stem cuttings and air layering reported were unsuccessful (Gunatillake *et al*., 2002).

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Preliminary studies on *in vitro* multiplication of *C. fenestratum* carried out by Nair and Seeni (2003) was reported by Tushar *et al*. (2008). In a previous study on *in vitro* propagation of *C. fenestratum* epicotyls excised from *in vitro* germinated seedlings were used as explants (Senarath, 2010). However, it is not possible to germinate seeds *in vitro* as described without breaking the dormancy (Warakagoda & Subasinghe, 2014; 2015) and the method is not encountered as a clonal propagation protocol due to the heterogeneous nature of epicotyl explants.

Therefore, identification of feasible propagation techniques is crucial to fulfill the unlimited demand in the island for ayurvedic drug, cosmetic and personal care product manufacturing companies as well as for the conservation of this valuable species. The present investigation was aimed at developing a protocol for *in vitro* clonal propagation of *C. fenestratum* as an alternative method to the conventional propagation methods, so as to ensure conservation and sustainable use of this valuable plant.

METHODOLOGY

All the experiments were carried out at the Tissue Culture Laboratory, Department of Crop Science, Faculty of Agriculture, University of Ruhuna, Mapalana, Kamburupitiya, Sri Lanka.

Surface sterilisation of shoots

Shoot tips (≤ 1.0 cm) and subsequent single (1.0 – 2.0 cm) and double nodal cuttings (≥ 2.0 cm) having dormant axiliary buds were excised from 2-wk old fresh and young axiliary shoots initiated from nodes on the stems of mother plants maintained at the plant house. The explants were immediately dipped in distilled water after excision. Shoot tips and nodal segments, after removing the leaflets, were treated with liquid detergent (Teepol 5 % v/v) for 5 min and placed under running tap water for 30 min followed by immersion in a 3 % (w/v) Topsin solution (fungicide) for 1 h. Then the explants were treated with 3 different Clorox™ (5.25 % NaOCl) concentrations [10, 15, 20 % (v/v)] for 3 different exposure times (10, 15, 20 min) inside a lamina air flow cabinet. To enhance the activity of the detergent, 2 drops of Tween 20 (polyoxyethelene sorbitanmonolaurate), a surfactant, was mixed with the Clorox™ solutions. Then they were dipped in 70 % (v/v) ethanol for 2 min. After exposing to each solution the explants were thoroughly washed twice using sterilised distilled water. Finally, cut ends of the explants were trimmed and inoculated vertically on a basal MS (Murashige & Skoog, 1962) medium.

Since negative results were obtained, the surface sterilisation procedure was redesigned by using 2 concentrations [0.1 % and 0.2 % (w/v)] of mercuric chloride ($HgCl_2$) for 2 exposure time durations (15 and 30 min) as the disinfectant. To see the effect of ethanol on controlling contaminations, surface sterilised explants with $HgCl_2$ were introduced either to 50 or 70 % (v/v) solutions of ethanol for 30 s or 2 min. After exposing to each solution the explants were thoroughly washed using sterilised distilled water twice. Numbers of aseptic cultures were recorded during the culture period up to 4 wks.

In vitro establishment of shoots

Surface sterilised shoot tips and subsequent single and double nodal cuttings were cultured on MS and McCown's woody plant medium (WPM) (Lloyd & Mc Cown' 1981) media vertically and horizontally. Activated charcoal (AC) (1.0, 5.0 and 10 gL^{-1}) and polyvinylpyrrolidone (PVP) (0.5, 1.0 and 3.0 gL^{-1}) as absorbents, and ascorbic acid (0.1, 0. 25 and 0.5 gL^{-1}) and citric acid (0.1, 0.5 and 1.0 gL^{-1}) as antioxidants were added to culture media separately. Numbers of culture media that turned brown were assessed during the 4-wk culture period.

In vitro shoot proliferation

For all the experiments, WPM medium was used as the basal medium as it is superior to MS medium during culture establishment. All the media contained 1.0 gL^{-1} PVP as the anti-browning agent.

Effects of plant growth regulator (PGR) combination on multiple shoot induction

Four weeks after the establishment of aseptic cultures, buds which initiated double nodal explants on basal WPM medium were transferred to WPM media containing different concentrations of either 6-benzylaminopurine (BAP) or kinetin (Kin) (1.0, 3.0 or 5.0 mgL^{-1}) combined with either indole-3-acetic acid (IAA), naphthaleneacetic acid (NAA) or 2, 4-dichlorophenoxyacetic acid (2, 4-D) (0.0, 0.2 or 0.4 mgL^{-1}). Since negative results were obtained, the experiment was rearranged using the above mentioned concentrations of BAP with Kin (0.5, 1.0, 2.0 or 4.0 mgL^{-1}) and IAA, NAA or 2, 4-D (0.0 or 0.4 mgL^{-1}). With repeated negative results, the experiment was redesigned using thidiazuron (TDZ) (0.5, 1.0, 2.0 mgL^{-1}) in combination with BAP (0.0, 1.0, 2.0, 3.0 mgL^{-1}) and IAA, NAA or 2, 4-D (0.0, 0.2 or 0.4 mgL^{-1}), which resulted in positive results. The number of shoots and leaves per explant were evaluated up to 12 wks.

Effects of explant type on multiple shoot induction

A separate experiment was conducted to evaluate the influence of explant type on *in vitro* shoot proliferation ability. Explants were collected from 2-wk old fresh and young axiliary shoots initiated from nodes on the pruned stems of mother plants and collected into separate beakers labelled as shoot tips, immature and mature nodal and double nodal cuttings. Single and double nodal cuttings were divided into 2 maturity stages as immature and mature. Easy to excise cuttings using a scalpel were considered as immature and hard to excise were considered as mature cuttings. Excised cuttings adjoining the shoot apexes were usually immature and those which were excised further from the shoot tips were mature in nature.

After surface sterilisation, the shoots were inoculated on WPM media containing 1.0 gL^{-1} PVP for 4 wks while maintaining the identity of the explant type. Then they were transferred to WPM media containing 2.0 mgL^{-1} BAP, 1.0 mg L^{-1} TDZ and 0.4 mg L^{-1} 2, 4-D for multiplication. Number of shoots and leaves per explant were assessed up to 12 wks.

Effects of BAP spray on mother plants on multiple shoot induction

Mother plants (1 − 2 year old) were grouped into 5 and treated separately with one of the concentrations of (25, 50, 100, 200 and 500 mgL^{-1}) BAP at 2 wk intervals as a foliar spray to evaluate its effects on axillary shoot induction from cultured explants. Another group of 5 mother plants were treated as mentioned above weekly. Mature double nodal cuttings were collected for *in vitro* culturing after 1, 3, 5 and 7 days after BAP spray. After surface sterilisation they were introduced to WPM media containing 1.0 gL^{-1} PVP for 4 wks while maintaining the above labelling system. Then they were introduced to WPM media containing 2.0 mgL^{-1} BAP, 1.0 mgL^{-1} TDZ and 0.4 mgL^{-1} 2, 4-D for multiplication. Time taken for initiation of the first shoot bud and the number of shoots formed per explant were measured up to 12 wks.

Effects of pulse treatment on multiple shoot induction

To evaluate the effect of pulse treatment on adventitious shoot induction, higher concentrations of BAP alone and in combination with Kin in similar concentrations (25, 50, 100, 150 and 200 mgL^{-1}) were tested. After 1, 3, 6, and 24 h the treated shoots were transferred to solid basal WPM medium.

A separate experiment was conducted using the best plant growth regulator combination (2.0 mgL^{-1} BAP, 1.0 mgL^{-1} TDZ and 0.4 mgL^{-1} 2, 4-D), which gave the highest shoot proliferation rate as the pulse treatment. Shoots were slightly shaken at 50 rpm for 6 h, 1, 3, 7, 14 and 21 ds followed by transferring to solid basal WPM media or WPM media containing the same PGR combination.

Mature double nodal cuttings were used as the explants. To minimise any adverse effect of phenolic exudates secreted into the solutions used for pulse treatments, an anti-browning agent (1.0 gL^{-1} PVP) was introduced. Time taken for the initiation of first shoot bud and the number of shoots formed per explant were measured up to 12 wks of observation period.

***In vitro* elongation of plantlets**

During the proliferation stage, inter nodal length of the shoots produced from the nodes of the double nodal cuttings were very low forming shoot clumps. Since it was very difficult to separate the shoots due to their low height, sub culturing aiming further multiplication was not practiced. Instead, to enhance inter nodal length, 4 different concentrations of GA_3 (0.5, 1.0, 1.5 and 2.0 mgL^{-1}) were incorporated into the proliferation medium. Another set of multiplied shoots without separation were transferred to WPM media containing the above concentrations of GA_3. As the control, WPM basal medium was used.

Acclimatisation procedure

Acclimatisation stage 01

After 4 wks from root formation, culture bottles containing *in vitro* rooted plants were taken out from the culture room and placed under normal laboratory conditions for a week. The culture bottles were then placed in a shade house for 3 ds.

Acclimatisation stage 02

Lids of the culture vessels were opened and a mixture of 0.6 mgL^{-1} Captan™ and 0.7 mgL^{-1} Thiram™ fungicides was sprayed on plants inside the containers. They were placed inside a propagator and kept inside the shade house for another 3 ds. To maintain high humidity, regular spraying of water was needed at least twice a day.

Acclimatisation stage 03

Plantlets were taken out from the bottles and the roots were thoroughly washed using lukewarm water at least thrice. Then the plantlets were dipped in a mild fungicide (0.6 mgL^{-1} Captan™) solution for 15 min and transferred to poly bags containing sand and coir dust (1:1 ratio). Potting medium was mixed with 'Osmocot', a slow release high nitrogen fertiliser, a week before usage. After 8 wks, the plants were transferred to poly bags containing cow dung: top soil: sand (1:1:1 ratio) and kept under shade house conditions for another 4 wks before introducing to the field. Survival rates were measured throughout the experimental period of 14 wks.

Experimental design and statistical analysis

All the experiments were designed according to the factorial randomised complete block design (RCBD) with 10 replicates. Each experiment was repeated 3 times. Non contamination percentages, browning percentages, number of new shoot buds and leaves appeared and survival rates were recorded weekly. Data were analysed using SAS statistical software.

RESULTS AND DISCUSSION

Surface sterilisation of shoots

All the explant types were severely contaminated with bacterial and fungal contaminants within a week even after treating with 20 % Clorox™ for 20 minutes. Bacterial oozes secreted to culture medium and fungal mycelia were prominent on the surface of the medium after the second week from culture establishment.

However, for surface sterilisation of both shoot tips and nodal cuttings, interactions of the $HgCl_2$ concentration and exposure time were significantly effective for controlling contaminations at $p \leq 0.05$ probability level. The treatment with 0.2 % $HgCl_2$ for 30 minutes was found to be the best for shoot tips and nodal cuttings with 99 % and 88 % mean non contamination rates, respectively (Table 1). It was further observed that double nodal cuttings were contaminated more frequently compared to single nodal cuttings.

Dipping the explants either in 50 % or 70 % ethanol for 30 seconds or two minutes after treating the explants with 0.2 % $HgCl_2$ for 30 minutes was not significantly effective at $p \leq 0.05$ level on mean non-contamination percentages of shoot tips and nodal cuttings (Figure 1).

Table 1: Mean non-contamination rates resulted with different concentrations of $HgCl_2$ and exposure times

Mercuric chloride concentration	Exposure time	Mean non contamination rate (%) ± SE	
		Shoot tips	Nodal cuttings
0.1 %	15 minutes	20.50 ^{d} ± 3.87	11.00 ^{d} ± 4.39
0.1 %	30 minutes	59.00 ^{c} ± 4.39	38.66 ^{c} ± 5.03
0.2 %	15 minutes	79.00 ^{b} ± 4.39	79.00 ^{b} ± 4.39
0.2 %	30 minutes	99.00 ^{a} ± 4.39	88.00 ^{a} ±7.08
		CV = 5.77, LSD = 2.32	CV = 10.92, LSD = 2.81

Means represented by the same letter in each column are not significantly different at $p \leq 0.05$ probability level (SE = standard error, CV = coefficient of variation, LSD = least significant difference)

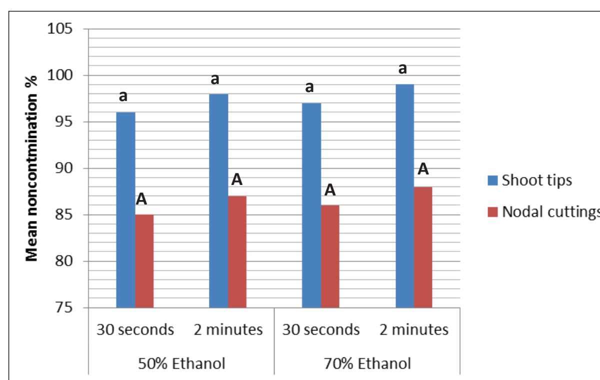

Figure 1: Effect of ethanol on mean non-contamination percentages of shoot tips and nodal cuttings
Means represented by the same letter-case on the bars are not significantly different at $p \leq 0.05$ probability level

$HgCl_2$ has been used as a surface sterilant in plant tissue culture since decades especially when microbial contaminations are very difficult to control. During the study to avoid bacterial and fungal contaminations, the only effective treatment procedure was found to be dipping the explants in 0.2 % $HgCl_2$ solution for 30 minutes.

Browning of explant tissues due to any surface sterilant was not detected since the shoots and immature leaves of this species are naturally brown in colour.

It has been reported by Rodriguez *et al*. (1989) that during surface sterilisation of adult material (shoot segments) of *Juglans* spp., the last step was to submerge them in $HgCl_2$ (0.05 % w/v) for 5 minutes, and in certain

cases submerging in $HgCl_2$ (1 − 2 mgL^{-1}) for 10 minutes was the best. In another experiment, the suitability of Clorox™ and $HgCl_2$ as sterilising agents was tested on seeds collected from wild mature trees of *Aquilaria malaccensis* and leaf and nodal explants excised from shade house grown 4 − 5 year old plants. The best treatment identified for seeds, leaves and nodal explants was dipping in 0.2 % $HgCl_2$ for 12 minutes, 0.1 % $HgCl_2$ for 15 and 30 seconds, respectively (Daud *et al*., 2012).

In vitro establishment of shoots

Browning of the culture medium was not significantly affected at $p \leq 0.05$ level by the three factor interactions of type of the explant, culture medium and anti-browning agents. However, anti-browning agents present in the culture media significantly ($p \leq 0.05$) reduced browning as a single factor. Phenolic exudates released into the culture media by shoot tips and nodal cuttings were significantly minimised up to 80 %, 79 % and 78 % by 3.0 gL^{-1} and 1.0 gL^{-1} PVP followed by 10.0 gL^{-1} AC, respectively in both MS and WPM media at $p \leq 0.05$ probability level (Figure 2).

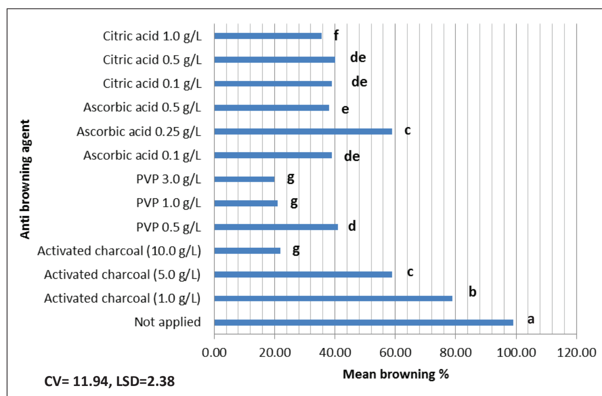

Figure 2: Effect of anti-browning agents on mean browning rates of culture media
Means represented by the same letter are not significantly different at $p \leq 0.05$ probability level

Although not significant at $p \leq 0.05$ level, the WPM medium (45.46 %) is less prone to browning compared to MS medium (46.33 %) (data not shown). Further, axillary buds were initiated only from the nodes of the double nodal cuttings cultured on WPM medium (Figure 3a).

Explants cultured horizontally on both media were easily contaminated by bacterial and fungal infections. When horizontally cultured, the surface area of explants touching the nutrient medium is much higher than when placed vertically, which may promote the growth of many suppressed contaminants.

Figure 3: (a) Buds initiated from nodes of mature double nodal explant on WPM medium; (b) single shoot transferred into same new medium for further multiplication after excision from a shoot clump; (c) brown colour leaves of the shoot eventually turned into green colour after 8 weeks; (d) micro cutting with elongated roots six weeks after introducing to WPM medium; (e) plantlets at acclimatisation stage 3 (after introducing to sand: coir dust medium); (f) plants in poly bags containing cow dung: top soil: sand (1: 1: 1)

In vitro shoot proliferation

Effect of plant growth regulator combinations on multiple shoot induction

As it has been recorded that some auxins in minor concentrations promote shoot proliferation when applied with cytokinins (Machakova *et al*., 2008), three concentrations of IAA, NAA and 2, 4-D were introduced to BAP or/and Kin containing media. However, up to six months of observation period none of the shoots produced new shoots, branches or leaves.

Therefore, the experiment was redesigned using TDZ, which gave promising results only in the presence

of 0.4 mgL^{-1} 2, 4-D. Two factor interactions of BAP and TDZ significantly ($p \leq 0.05$) affected the mean number of shoots produced by double nodal cuttings within the 8 week observation period. During the multiplication phase, the highest number of shoots (3.90 shoots/ explant) was observed in 0.4 mgL^{-1} 2, 4-D containing WPM medium when 2.0 mgL^{-1} BAP and 1.0 mgL^{-1} TDZ were fortified in addition (Table 2).

Table 2: Effect of concentration and combination of cytokinins on mean number of shoots and leaves produced by mature double nodal cuttings cultured on WPM medium containing 0.4 mgL^{-1} 2, 4-D (after 8 weeks)

Cytokinin (mgL^{-1})		Mean number of shoots ± SE	Mean number of leaves ± SE
BAP	TDZ		
0.00	0.20	0.80 ^{g} ± 0.41	0.75 ^{e} ± 0.44
	0.50	0.85 ^{g} ± 0.41	0.80 ^{e} ± 0.41
	1.00	1.25 ^{f} ± 0.44	2.20 ^{d} ± 0.41
	2.00	0.84 ^{g} ± 0.37	0.84 ^{e} ± 0.37
1.00	0.20	0.85 ^{g} ± 0.36	0.90 ^{e} ± 0.30
	0.50	0.90 ^{g} ± 0.30	0.95 ^{e} ± 0.22
	1.00	1.80 ^{c} ± 0.41	2.80 ^{c} ± 0.41
	2.00	1.15 ^{f} ± 0.37	1.05 ^{e} ± 0.40
2.00	0.20	1.25 ^{f} ± 0.55	1.00 ^{e} ± 0.32
	0.50	1.55 ^{e} ± 0.51	1.50 ^{e} ± 0.51
	1.00	3.90 ^{a} ± 0.30	6.10 ^{a} ± 0.30
	2.00	1.63 ^{d} ± 0.49	1.42 ^{e} ± 0.50
3.00	0.20	1.30 ^{f} ± 0.73	1.20 ^{e} ± 0.41
	0.50	1.20 ^{f} ± 0.61	1.20 ^{e} ± 0.41
	1.00	2.95 ^{b} ± 0.22	3.75 ^{b} ± 0.44
	2.00	1.05 ^{g} ± 0.22	1.10 ^{e} ± 0.30
		CV = 9.89, LSD = 0.26	CV = 11.34, LSD = 0.22

Means represented by the same letter in each column are not significantly different at $p \leq 0.05$ probability level (n = 10)

The tested concentrations of Kin even when combined with TDZ were not effective in multiple shoot production (data not shown). Produced shoots were separated after 8 weeks and transferred to the same media (1st sub culture) for further multiplication. They were not multiplied further, however, brown colour minute leaf primodia turned into light green colour and leaf laminas were enlarged (Figures 3b and 3c).

The number of leaves produced were significantly ($p \leq 0.05$) affected by the concentrations of BAP and TDZ incorporated into 0.4 mgL^{-1} 2, 4-D fortified WPM culture media. The highest number of leaves (6.10 leaves per shoot) was recorded in the presence of 2.0 mgL^{-1} BAP and 1.0 mgL^{-1} TDZ (Table 2). Tested concentrations of IAA and NAA did not form leaves in the presence of cytokinins. Other than 0.4 mgL^{-1}, rest of the tested concentrations of 2, 4-D were not successful in forming leaves (data not shown).

TDZ is among the most active cytokinin-like substances for woody plant tissue culture. It facilitates efficient micropropagation of many recalcitrant woody species (Parthasarathy, 2007). Although low concentrations (< 0.25 mgL^{-1}) of TDZ can induce greater axillary bud proliferation than many other cytokinins it may inhibit shoot elongation (Sathyanarayana & Varghese, 2007). In some cases it is necessary to transfer the shoots to an elongation medium containing a lower level of TDZ and/or a less active cytokinin (Huetteman & Preece, 1993). However, it was mentioned that most commonly used cytokinins in plant tissue culture including Zeatin, Kin, BA, TDZ and 2-iP can be applied in higher concentrations 1 − 10 μM (0.2 − 2.0 mgL^{-1}) for adventitious shoot formation, while suppressing the apical dominance regulated by auxins (Staden *et al*., 2008).

Effect of explant type on multiple shoot induction

It was observed that only mature double nodal cuttings produced multiple shoots, while shoot tips and other cutting types did not produce any shoots. Similar results were shown by some other plants (e.g. *Eucalyptus*), where a bud bearing node is a more reliable initial explant for shoot cultures than a dissected shoot apex (Gahan & George, 2008). It was also recorded that two-node explants for all cultivars of *Buddleia* produced higher multiplication rates compared to those of single nodes and apical tips (Phelan *et al*., 2005).

It is important to identify the correct maturity stage of the shoots prior to excision of explants from mother plants to achieve highest shoot proliferation. However, the selection of an explant of the optimum ‘age’ for micropropagation can sometimes be complex. In *Rosa hybrida*, single node explants (1.0 cm of stem plus a bud) taken from new shoots, which grew after the bushes were pruned, where the new shoots reached 10 cm height and having six axillary buds were the best. All the lateral buds were then capable of growing *in vitro*. Much less reliable explants were obtained from shoots, which had extended until they bore terminal flower buds. Explants, which grew well and produced vigorous shoots could then only be obtained from middle order buds. By the time the shoots were over 60 cm in length, growth of cultured buds was erratic. Similarly when single node

explants from tomato plants at the seventh leaf stage were cultured, buds from nodes 1 − 3 grew slowly, those from nodes 4 and 5 grew most rapidly and those from node 6 grew slightly less quickly (Gahan & George, 2008).

Therefore, based on the results it can be concluded that after two weeks from axillary shoot initiation on pruned mother plants, the distal nodes must be at the correct maturity stage with the capacity to produce *in vitro* growing buds. After this stage inter nodal length of the shoots increased and brown colour immature leaves eventually turned into light green colour.

Effect of BAP spray on mother plants on multiple shoot induction

In commercial *in vitro* propagation a major disadvantage identified was the dependence on a special development phase of the mother plant especially when organs are used for explantation (Sathyanarayana *et al*., 2007). To break dormancy of the explants cytokinins can be applied to the mother plants. A 100 mgL^{-1} solution of BAP or more can be sprayed or 500 mg L^{-1} can be injected to the mother plants (Neumann *et al*., 2009). However, during this study either shoot bud initiation or the number of shoots produced per explant was not significantly affected at $p \leq 0.05$ level by the excised date of explants from mother plants, after spraying of any tested concentrations of BAP weekly or at two week intervals to the mother plants (data not shown). As the sprayings were not successful, injection of BAP into the mother plants was not practiced.

Effect of pulse treatment on multiple shoot induction

It was recorded that exposure of explants to higher concentrations of cytokinins for a short period of time (pulse treatment) is resorted to before transferring into either basal culture media or media consisting the same cytokinin in lower concentrations to enhance shoot multiplication ability (Staden *et al*., 2008). In certain occasions it was recorded that the combination of cytokinin and auxin for a period of up to 7 − 14 days followed by transferring to PGR free media is more effective (Beyl, 2011). However, during the present study none of the treatment combinations used as pulse treatment was effective (data not shown).

***In vitro* elongation of plantlets**

During the multiplication stage inter nodal length of the shoots produced from the nodes of the double nodal cuttings were very low forming shoot clumps with a few prominent shoot apices. Therefore, it was difficult to separate the shoots to be introduced into proliferation media for further multiplication. Even when separated from shoot clumps and introduced to the proliferation medium they never multiplied may be due to their minute size. It was reported that shoots should be treated with GA_3 to increase the length of the shoots during multiplication, or prior to rooting. Further, GA_3 was beneficial especially when higher levels of cytokinins has resulted in many short shoots (Moshkov *et al*., 2008).

Therefore, in this study to enhance inter nodal length, three different concentrations of GA_3 were applied into the proliferation medium. However, as there was no effect of the above media on shoot length, multiplied shoots were transferred to WPM media containing three different concentrations of GA_3. As the control, WPM basal medium was used. Within four weeks, one prominent shoot elongated (up to 2.5 cm) from the shoot clumps on basal WPM media and roots were formed from the lower node of the initial explant. Therefore, it was decided to separate multiplied shoots from the initial explant as much as possible (maximum up to three) before introducing to basal WPM media. Shoots were elongated up to a mean height of 3.0 cm and mean three roots per plantlet (4.3 cm mean root length) were formed within four weeks from culture initiation (Figure 3d). It is a must to excise shoots immediately after a node, and at least leaving two nodes per shoot to facilitate shoot elongation and rooting after introducing to basal WPM medium.

Elongated shoots on basal WPM media did not multiply when transferred to the proliferation medium after removing the roots, emphasising the necessity of proper maturity level to form new shoots since mature double nodal cuttings only succeeded as an initial explant. On the other hand this may be due to the removal of habituation effect of cytokinins while on the basal WPM medium. Therefore, with the present findings it was not possible to maintain several multiplication cycles when micropropagating *C. fenestratum*.

It was reported that among the three concentrations of GA_3 (0.0, 0.5 and 2.0 mgL^{-1}), 0.5 mgL^{-1} had no effect on elongating *in vitro* cultured shoots of *Hevea*, while 2.0 mgL^{-1} was inhibiting shoot growth. A level of GA_3 between these two concentrations may facilitate shoot elongation (Gunatilleke & Samaranayake, 1988). Further, the support of GA_3 up to 0.1 mgL^{-1} on *in vitro* shoot elongation was identified, where the highest shoot length (5.6 cm/shoot) was observed with 0.1 mgL^{-1} within 15 days of culture (Arumugam *et al*., 2003). Increased concentrations of GA_3 suppressed shoot elongation. Similarly during the current study, concentrations

lower than 0.5 mgL^{-1} (the lowest concentration of GA_3 tested during the study) enhanced shoot elongation of *C. fenestratum*. However, since the shoots reached 3.0 cm height within four weeks without GA_3 further attempts were not taken to identify such a concentration.

Acclimatisation procedure

Plants, after 14 weeks of three stage-acclimatisation procedure during which micro plantlets were gradually adapted to normal environmental conditions with over 60 % survival rate, are transferrable to normal field conditions (Figures 3e and 3f).

In vitro grown species require a properly designed acclimatisation process in order to ensure a sufficient number of plants to be survived after transferring to soil, since the plant house and field has comparatively lower relative humidity, higher light conditions and septic environment that are stressful to micropropagated plants (Hazarika, 2003). Hence plantlets should be slowly acclimatised to *ex vitro* conditions with high light intensity and low humidity conditions (Chandra *et al.*, 2010). Under such situation delaying the removal of plantlets from highly humid conditions inside the culture bottles until reaching the mid stage of the acclimatisation process was useful to slowly adapt the plants to low humidity conditions. Further, the plantlets were kept in a propagator and then in a shade house up to 14 weeks with gradual increments of light intensity prior to exposing to field conditions.

CONCLUSION

Shoot tips and nodal cuttings of *C. fenestratum* can be successfully surface sterilised by dipping the explants for 30 minutes in 0.2 % solution of $HgCl_2$ followed by two successive washings with sterilised distilled water.

McCowns woody plant medium is the best establishment medium for shoot tips and nodal cuttings. Incorporation of 1.0 mgL^{-1} PVP into culture media is needed to minimise the browning effect.

Mature double nodal cuttings resulted in the highest shoot proliferation rate (3.90 shoots/ explant) when cultured on WPM medium supplemented with 2.0 mgL^{-1} BAP, 1.0 mgL^{-1} TDZ and 0.4 mgL^{-1} 2, 4- D. Shoots separated and transferred to basal WPM medium for regeneration into plantlets can be successfully acclimatised on coir dust: sand (1:1) potting media with over 60 % survival rate.

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