Tropical Agricultural Research Vol. 21(1): 21 - 29 (2009)

Development of a Successful Protocol for *in vitro* Mass Propagation of *Celastrus paniculatus* Willd. – A Valuable Medicinal Plant

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ABSTRACT. *Celastrus paniculatus Willd. belongs to family Celastraceae. Seeds provide an extremely important medicinal oil which is reported to sharpen the memory and also used to cure a number of diseases. The rate of seed germination is low and other vegetative propagation methods also cannot be used for cultivation. This was recorded as a highly threatened plant in the 1999 list of threatened fauna and flora of Sri Lanka. The objective of this study was to develop a successful protocol for in vitro mass propagation of C. paniculatus and to study the growth and physiology of greenhouse established tissue cultured plants.*

Nodal segments, shoot tips and leaf discs were used as explants. They were cultured in Murashige and Skoog (MS) medium supplemented with different combinations of growth regulators in order to induce callus. It was observed that MS medium supplemented with 5.0 µM 6-benzylaminopurine (BAP) and 7.0 µM indole-3-acetic acids (IAA) was the best medium for callus induction from tested explants. Both shoot tips and nodal segment explants produced multiple shoots in the MS medium in the presence of 10.0 µM BAP and 14.0 µM IAA. Multiple shoots were induced in the MS media with 5.0 µM BAP and 0.5 µM IAA. Elongated shoots developed roots and the highest rooting percentage (73.3%) was obtained in the MS medium supplemented with 5.6 µM IAA and 9.6 µM Indole-3-butyric acid (IBA). Rooted plants were acclimatized in different potting mixtures and a mixture of river sand: top soil: compost (1:1:1) gave the highest survival rate (80.0%). Rate of photosynthesis and the stomatal resistance of in vitro produced plants increased with time indicating that tissue cultured plants adapted to natural environment. Growth of the acclimatized plants had a sigmoid pattern of normal growth in the greenhouse.

INTRODUCTION

Celastrus paniculatus Willd. (family Celastraceae) commonly known as *Duhudu* is a medicinal plant which is believed to sharpen the memory and reduce fatness in the body. This plant is widely used in ayurvedic system of medicine to cure many diseases such as leprosy, leucoderma, skin diseases, paralysis, depression, arthritis, asthma and fever (Sharma *et al.,* 2001). In Sri Lanka, this species is very rare and only few live specimens are to be found. This species has been identified as a threatened plant, requiring scientific efforts for conservation and commercial cultivation (Arya *et al.,* 2001).

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C. paniculatus can be propagated by seeds, but the percentage seed germination is very low due to inhibitory compounds present in the seed coat. Rooting of cuttings is also not successful. There are some records on the microporpagation of this valuable species through callus (Sharada *et al.,* 2003) and through bud differentiation (Rao and Purohit, 2006) with limited success. Therefore, an attempt was made to mass propagate *C. paniculatus* through tissue culture techniques and to establish tissue cultured plants in the greenhouse successfully.

MATERIALS AND METHODS

Field grown mature plants (3-year-old) of *C. paniculatus* were selected as mother plants. Leaf discs (25.0 mm^2) from fully opened second leaf, nodal segments (6.0 mm) and shoot tips (6.0 mm) were used as explants. They were washed under running tap water for 1 h and in 10% Clorox™ for 5 min initially and then in 70% ethanol for 2 min each followed by two successive washings in sterile distilled water. Cultures were incubated at 25±1 ºC at 3000 lx light intensity. Unless otherwise stated, cultures were incubated in 16/8 h light/dark regimes. A completely randomized design (CRD) was used with at least 20 replicates in all experiments. Cultures were randomized weekly.

Callus induction

Explants (leaf discs, nodal segments and shoot tips) were placed on the medium after surface sterilization at a uniform spacing. MS (Murashige and Skoog, 1962) medium was used (Sharada *et al.,* 2003, Rao and Purohit, 2006) as the basal medium. Based on the results of previous experiments, BAP (Benzyl amino purine) (5.0 – 10.0 µM) and IAA (Indole-3-acetic acid) (7.0 – 14.0 µM) were added in different combinations to the culture media. Cultures were incubated in the dark at 25±1 ºC. Colour, texture and mean diameter of the calli were determined over a period of six weeks.

Shoot initiation

For shoot induction, calli, shoot tips and nodal segments were used. They were cultured in MS and WPM (Mc Cown and Lloyd, 1981) media supplemented with different combinations of BAP (5.0 – 10.0 µM) and IAA (14.0 – 28.0 µM) and incubated in 16/8 h light/dark regime for six weeks. Number of shoots per explant, mean shoot length and mean number of leaves per shoot was observed.

Shoot multiplication

Although multiple shoots were induced from shoot tips and nodal segments in the shoot induction medium (MS + 10.0 µM BAP + 14.0 µM IAA), the rate of multiplication was low. Therefore *in vitro* produced shoots (10.0 – 15.0 mm long) were separated and were cultured in MS medium supplemented with a combination of BAP (5.0 – 10.0 µM) and IAA (0.1 – 0.5 µM) for rapid multiplication. Mean number of shoots per shoot, mean shoot length and mean number of leaves per shoot were measured over a period of six weeks.

Root induction

Elongated shoots (4.0 – 5.0 cm long) were transferred into a MS medium supplemented with different combinations of IAA (5.6 – 11.2 µM) and IBA (Indole-3-butyric aid) (4.8 – 9.6 µM). Rooting percentage, number of roots per shoot and mean root length were measured after a period of 12 weeks. Rooting percentage was calculated by taking the proportion between the number of rooted shoots and total number of shoots per treatment and mean root length was measured at the time of transferring them into soil for acclimatization.

Determination of the suitable potting mixture for acclimatization and greenhouse establishment of tissue cultured plants

Rooted plantlets were removed from vessels without damaging the roots and washed carefully to remove all traces of agar using sterile distilled water. They were then dipped in a solution of Captan® (1.0 g/L) for 5 minutes. Initially these plantlets were transferred into river sand sterilized in a hot air oven at 162±1 ºC for 2 h. Sand trays were kept inside a humid chamber. After three weeks, plants were transferred into four different potting mixtures - i) top soil: coir dust: compost, ii) river sand: coir dust: compost, iii) river sand: top soil: compost and iv) river sand: top soil: coir dust - all in 1:1:1 ratio. Survival percentage was measured after six weeks.

Determination of the growth and physiology of tissue cultured plants

After transplanting the tissue cultured plants in a mixture of river sand: top soil: compost (1:1:1), they were transferred into the greenhouse. Plants were watered daily and fertilizers were added after 2 months of growth. Height of plants, number of leaves per plant and number of branches per plant were measured fortnightly over a period of six months. Rate of photosynthesis (LI-COR Inc. USA) and stomatal resistance (Delta-T devices Ltd., UK) were measured in the morning (10.00 am) and afternoon (2.00 pm) at three months and six months after transplanting.

Data Analysis

The effect of different treatments was quantified and the level of significance was determined by analysis of variance (ANOVA) and least significant difference (LSD) at α=0.05 probability level.

RESULTS AND DISCUSSION

Callus induction

Cultures from calli are potentially useful as a method for commercial propagation due to its ability of achieving high multiplication rate (Hartmann *et al.,* 1997). Callus induction was observed in all tested explants in all tested media after 7 days. Colour of the callus varied from yellow to green. Calli obtained from leaf discs were green and hard in texture while other two explants produced yellowish brittle calli. The best growth regulator combination for callus induction was 5.0 µM BAP and 7.0 µM IAA. Mean callus diameter was significantly higher in leaf discs (2.06±0.33) than those in nodal segments and shoot tips (0.81±0.12 and 0.70±0.10 respectively) (Table 1). Therefore, leaf discs were selected as the best explant to produce callus.

Table 1. Callus induction from different types of explants after 6 weeks of incubation (± SE)

Growth regulators	Mean Callus diameter (cm)		
BAP : NAA (µM)	Nodal segment explants	Shoot tip explants	Leaf pieces
5.0 : 7.0	0.81±0.12	0.70±0.10	
5.0 : 14.0	0.41±0.19	0.65±0.17	2.10±0.14
10.0 : 7.0	0.21±0.11	0.55±0.10	1.80±0.44
10.0 : 14.0	0.51±0.11	0.50±0.89	1.80±0.59
LSD 5%	0.21	0.04	0.18

Callus growth within a plant may vary depending on factors such as the original position of explant within the plant, which reflects the endogenous hormone level (Auge, 1995). Leaves give the best response for callus initiation in *Munronia pinnata* (Senarath *et al.,* 2007) and *Cajanus cajan* (Pierik, 1997). There is no literature available on the use of leaf explants of *C. paniculatus* for plant regeneration except cotyledonary leaf explants (Sharada *et al.,* 2003) where MS medium supplemented with 5.0 µM NAA and 5.0 µM Kinetin was used as the medium. However, it has been observed that the growth regulator combination which gave the highest callus production was different from what was reported by them.

Shoot initiation

No shoots were induced from the calli in any of the tested media but the calli enlarged. Shoots were initiated from shoot tips and nodal segments after 20 – 25 days of incubation. Highest mean shoot length (3.10±0.81 cm) was observed from nodal segments in MS medium supplemented with 10.0 µM BAP and 14.0 µM IAA. Lowest (1.90± 0.26 cm) was observed when BAP level was decreased to 5.0 µM. Although lowering BAP level significantly affects shoot length, lowering the IAA level had no significant effect on shoot length. Mean shoot lengths were almost the same (ranging from 2.50±0.77 to 2.65±0.94 cm) in all tested combinations of growth regulators when WPM was used as the basal medium. Differences among mean shoot number and mean number of leaves per shoot were non significant when MS was used as the basal medium. The highest mean number of leaves per shoot (7.00±0.89) was observed in WPM supplemented with 10.0 µM BAP and 14.0 µM IAA. However, this was not significantly different from the values obtained (6.60±1.03) in MS medium with the same growth regulator combination (Table 2).

In the experiment where shoot tips were used for shoot induction, MS medium supplemented with 10.0 µM BAP and 14.0 µM IAA gave the highest values for mean number of shoots and mean number of leaves per shoot and this was significantly different from those of other treatments (Table 3).

Mean shoot length was also higher in the same treatment but the difference was non-significant. Thus, it suggests that MS medium supplemented with 10.0 µM BAP and 14.0 µM IAA is the best medium for shoot induction from both shoot tips and nodal segments.

Table 2. Shoot initiation from nodal segment explants after 6 weeks of incubation (±SE)

Basal medium	BAP : IAA (µM)	Mean shoot length±SE (cm)	Mean number of shoots ±SE	Mean number of leaves per shoot±SE
MS	5.0 : 14.0	1.90±0.26	4.00±0.89	4.30±1.03
MS	5.0 : 28.0	2.00±0.33	2.80±0.75	5.30±1.36
MS	10.0 : 14.0	3.10±0.81	5.30±1.21	6.60±1.03
MS	10.0 : 28.0	2.90±0.34	3.60±1.36	5.10±1.60
WPM	5.0 : 14.0	2.60±0.79	3.60±1.03	4.60±1.21
WPM	5.0 : 28.0	2.65±0.94	2.80±0.75	5.60±1.96
WPM	10.0 : 14.0	2.65±0.93	2.50±0.83	7.00±0.89
WPM	10.0 : 28.0	2.50±0.77	1.80±0.75	5.30±1.03
	LSD 5%	0.38	0.41	1.15

Table 3. Shoot initiation from shoot tip explants after 6 weeks of incubation (± SE)

Basal medium	BAP : IAA (µM)	Mean shoot length (cm) ±SE	Mean number of shoots ±SE	Mean number of leaves per shoot ±SE
MS	5.0 : 14.0	2.10±0.53	2.60±0.81	3.80±0.75
MS	5.0 : 28.0	2.90±0.98	3.10±1.16	5.20±1.04
MS	10.0 : 14.0	3.30±1.02	4.50±1.51	6.20±0.7
MS	10.0 : 28.0	1.90±0.40	4.10±1.16	4.80±1.16
WPM	5.0 : 14.0	2.50±0.58	3.00±0.63	4.30±1.03
WPM	5.0 : 28.0	2.60±1.13	2.60±1.21	5.60±1.50
WPM	10.0 : 14.0	2.60±1.13	2.50±0.83	6.60±1.21
WPM	10.0 : 28.0	2.40±0.42	2.60±0.81	5.10±0.98
	LSD 5%	0.22	1.10	0.68

Organogenesis depends upon hormonal balance and other factors such as plant tissue, environmental conditions, composition of the medium, polarity and growth substances (Razdan, 1994). Shoots were induced in MS medium supplemented with 8.44 µM BAP in *C. paniculatus* (Rao and Purohit, 2006) while in another study MS medium supplemented with 1.5 mg/L BAP, 0.1 mg/L NAA, 50.0 mg/L ascorbic acid, 25.0 mg/L each of adenine sulfate, arginine and citric acid as additives (Arya *et a*l., 2001). Although MS medium as well as

WPM were used as basal media, it was observed that MS provides better medium for *C. paniculatus*. Shoots were initiated in the presence of comparatively higher concentrations of IAA (14.0 µM) compared to BAP (10.0 µM) and the results were not comparable with previous studies. This may be due to some other factors which may change the internal growth regulator level.

Shoot multiplication

When BAP and IAA levels were lowered than those present in the induction medium, multiple shoots were initiated within 6 - 10 days. BAP and IAA at concentration of 5.0:0.5 µM produced the highest number of shoots (8.30±0.60) but took about 10 days for multiple shoot induction, whereas in the presence of 10.0 µM BAP and 0.1 µM IAA, multiple shoots were produced within 6 days but number of shoots produced were low (6.10±0.75). After six weeks mean shoot length was highest in the presence of 10.0 µM BAP and 0.5 µM IAA (4.70±1.12 cm) while the explants in BAP: IAA at 10.0:0.1 µM and 5.0:0.5 µM showed second and third highest lengths (4.45±1.12 cm and 3.45±0.55 cm respectively) but the difference was not significant. Mean number of leaves per shoot was significantly higher in the presence of BAP and IAA at the level of 5.0:0.5 µM (6.30±1.36) (Table 4). In the presence of NAA, 4-6 shoots per explant was produced but they were vitrified (Gerald *et al.,* 2006). Multiple shoots were successfully induced directly from nodal segments of *C. paniculatus* by culturing on MS medium containing 2.22 µM BAP but incorporation of IAA or NAA did not improve the response (Rao and Purohit, 2006). However, in the present study, mean of 8.6 shoots per shoot was obtained in the MS medium supplemented with 5.0 µM BAP and 0.5 µM IAA suggesting that as the best medium for multiple shoot induction of *C. paniculatus.*

Table 4. Multiple shoot induction in different culture media (± SE)

Growth regulators (µM) BAP: IAA	Mean number of shoots per shoot	Mean shoot length (cm)	Mean number of leaves per shoot
5.0:0.1	4.80±0.98	2.06±0.81	4.10±0.75
5.0:0.5	8.60±0.81	3.45±0.55	6.30±1.36
10.0:0.1	6.10±0.75	4.45±0.99	5.80±1.16
10.0:0.5	5.50±1.04	4.70±1.12	4.30±1.63
LSD 5%	1.61	0.87	0.60

Root induction

Mean numbers of roots, mean root length and rooting percentage were significantly different among tested treatments (Table 5). Roots were initiated after 15 days in the MS medium with 5.6 µM IAA and 9.6 µM IBA while all other tested treatments took more than 28 days. Mean number of roots per shoot (5.60±0.81), mean root length (3.20±0.40) and rooting percentage (73.3%) was significantly higher in this medium suggesting that it is the best medium for rooting of *in vitro* propagated shoots of *C. paniculatus*.

Table 5. Root induction from shoots after 6 weeks of incubation (± SE)

Growth regulators (µM) IAA:IBA	Mean number of roots/ shoot±SE	Mean root length±SE (cm)	Percentage rooting
5.6:4.8	3.60±0.81	1.80±0.16	66.6
5.6:9.6	5.60±0.81	3.20±0.44	73.3
11.2:4.8	2.80±0.98	1.80±0.20	66.6
11.2:9.6	2.30±1.36	1.03±0.23	50.0
LSD 5%	0.90	0.15	-

Callus induction at the base which limits root growth and browning of the medium was observed in all other tested media. Rooting in WPM supplemented with 5.0 µM IBA was achieved with limited success (Sharada *et al.*, 2003). However, in the present study, MS was found to be the best medium with 5.6 µM IAA and 9.6 µM IBA for rooting of *C. paniculatus.*

Determination of the suitable potting mixture for acclimatization and greenhouse establishment of tissue cultured plants

Longer roots could easily break while removing from the agar. Thus, plants with roots around 5.0 mm long were used for transplanting. It was observed that a mixture of river sand:top soil:compost (1:1:1) was the best potting mixture for acclimatization with 80% survival. However, a mixture of top soil: coir dust: compost also gave a significantly higher survival percentage (75%) suggesting both mixtures could be used as substrates for accilimatization of tissue cultured *C. paniculatus* plants. Use of a mixture of black soil: fine sand: farmyard manure: soilrite (4:2:1:1) gave only 53% survival (Arya *et al.,* 2001).

Determination of the growth and physiology of tissue cultured plants

Introduction of micropropagated plants to the natural environment is a critical step because of the malformed leaf structures and malfunctioning stomata (Khautan *et al*., 1998). Mean height, mean number of leaves and mean number of branches per plant showed a sigmoid pattern of growth in first six months when plants were established in the greenhouse.

Plants showed the normal pattern of stomatal resistance and photosynthesis from the beginning. Stomatal resistance was high in the morning and lowered by noon while increasing again in the afternoon. Rate of photosynthesis was also low in the morning and increased by noon. Stomatal resistance and the rate of photosynthesis were low after three months of transplanting but significantly increased within next three months (Table 6) indicating tissue cultured plants adapted to the natural environmental conditions well.

Table 6. Stomatal resistance and the rate of photosynthesis of tissue cultured plants of *C. paniculatus* after three and six months (± SE)

Age	Stomatal resistance (scm^{-1})			Rate of photosynthesis ($\mu mol\ CO_2 m^{-2} s^{-1}$)	
	Morning	**Noon**	**Evening**	**Morning**	**Noon**
Three months	4.78±0.4	3.21±0.3	4.30±0.4	5.66±0.30	6.67±0.20
Six months	7.04±0.7	5.02±0.4	6.84±0.6	6.01±0.30	7.01±0.30
LSD 5%	0.31	0.10	0.02	0.22	0.84

CONCLUSIONS

MS medium supplemented with 10.0 µM BAP and 14.0 µM IAA could be used for shoot induction and lower BAP and IAA (5.0 and 0.5 µM, respectively) induces multiple shoots. MS medium supplemented with 5.6 µM IAA and 9.6 µM IBA is the best medium for rooting. Rooted plants could successfully be acclimatized in a mixture of river sand: top soil: compost (1:1:1) with 80.0% survival. Since the growth of tissue cultured plants expressed normal growth up to six months in the greenhouse, it could be concluded that the tissue culture protocol used in this study could successfully be applied for mass propagation of *C. paniculatus.*

ACKNOWLEDGEMENT

Authors would like to acknowledge Bandaranaike Memorial Ayurvedic Research Institute (BMARI), Nawinna for providing facilities to conduct the research. They also wish to thank Mr. Gunarathna Silva (Former Head, Botany Division, BMARI) and Mrs. S. Sugathadasa for their help in numerous ways.

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