The Rapid Propagation Technique of the Medicinal Plant *Clinacanthus nutans* by Tissue Culture

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Abstract: The traditional medicinal plant *Clinacanthus nutans* is usually propagated by cutting propagation which has low reproductive capacity. The development of rapid propagation methods for *C. nutans* is needed to satisfy human demand for its medicinal products. This study developed a pratical tissue culture micropropagation technique for *C. nutans* by shoot initiation instead of callus induction. The results showed the optimal proliferation medium for *C. nutans* was Murashige and Skoog (MS) with 1.0 mg L^{-1} BA + 0.02 mg L^{-1} NAA providing 3.9 multiplication rate. A rooting medium composed of ½ MS + 0.25 mg L^{-1} IBA provided 100% rooting and vigourous plantlets. These methods provide reliable mass-propagation of *C. nutans* for medicinal purposes.
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Abbreviation: BA-benzyladenine; NAA-naphthaleneacetic acid; IBA-indole butyric acid

1. Introduction

Clinacanthus nutans (Burm. f.) Lindau (namely Sabah Snake Grass) is a perennial herb belonging to the family Acanthaceae, widely known as a traditional medicinal plant in Southeast Aisa such as Malaysia, Thailand and China (Guangxi, Guangdong, Yunnan, and Hainan Province) due to its medicinal properties in treating skin rashes, insect and snake bits, skin lesions caused by virus, diabetes mellitus, fever and diuretics, and Dengue disease (Lau *et al*., 2014; Kunsorn *et al*., 2013; Goonasakaran, 2013; Sakdarat *et al*., 2006; Sakdarat *et al*., 2009; Shim *et al*., 2013; Sujittapron *et al*., 2010; Tuntiwachwuttikul *et al*., 2004). It grows on the low altitude area from 500 to 620 meters, especially under the scattered forests and bushes on the low altitude. The possibility of employing the *C. nutans* extract acted as an antioxidant substance to ameliorate the oxidative damage (Pannangpetch *et al*., 2007). Eight compounds namely 132-hydroxy-(132-S)-chlorophyll B, 132-hydroxy-(132-R)-chlorophyll B, 132-hydroxy-(132-S)-phaeophytin B, 132-hydroxy-(132-R)-phaeophytin B, 132-hydroxy-(132-S)-phaeophytin A, 132-hydroxy-(132-R)-phaeophytin A, purpurin 18 phytyl ester and phaeophorbide A have been discovered (Sakdarat *et al*., 2006). Recent study found *C. nutans* extracts are antioxidant with antiproliferative effect on cultured human cancer cell lines (Arullappan *et al*., 2014; Yong *et al*., 2013). The *C. nutans* extract had anti-cancer roles was discovered in China (Wang *et al*., 2013). The leaves of *C. nutans* were found rich in amino acids, trace elements and bioactive chemical constituents in China suggesting that *C. nutans* was of high nutritional value (Yi *et al*., 2012).

In China, the rare *C. nutans* is usually propagated by cutting propagation, which is low proliferation freqency. In Malaysia, Ying (2013) and Gunasekaran (2014) succeeded in callus induction but no result in plantlet regeneration. No literature shows the tissue culture propagation technique of *C. nutans* is achieved. Therefore it is necessary to carry out a rapid propagation method for *C. nutans*.

2. Material and Methods

The stocks of *C. nutans* were collected from Shanghai China. The stems with 2-3 nodes from *C. nutans* were used as explants. The explants were immersed in 70% ethanol for 30 seconds, rinsed in sterilized water, transferred to 0.1% $HgCl_2$ for 12 min, and then rinsed in sterilized water four to five times. The explants were cut into pieces with one node each then transferred onto shoot initiation medium. The shoot initiation medium comprised: (1) MS + 1.0 mg L^{-1} BA + 0.1 mg L^{-1} NAA containing 30 g L^{-1} sugar and 5.5 g L^{-1} carrageenan (produced in Quanzhou, Fujian, China), pH 5.8. The explants were incubated in the dark or under weak light for 30 days.

The multiplication media comprised: (2) MS + 1.0 mg L^{-1} BA + 0.1 mg L^{-1} NAA; (3) ½MS + 1.0 mg L^{-1} BA + 0.1 mg L^{-1} NAA; (4) MS + 1.0 mg L^{-1} BA + 0.02 mg L^{-1} NAA; or (5) ½ MS + 1.0 mg L^{-1} BA + 0.02 mg L^{-1} NAA, each containing 30 g L^{-1} sugar and

5.5 g L^{-1} carrageenan, pH 5.8. The shoots were subcultured every 30 days. There were 30 jars of each medium and 3 replication for the same experiment. The shoots were maintained under 15–25 µmol m^{-2} s^{-1} irradiance (12 h d^{-1}) (Chen, 2009, 2012; Chen *et al*., 2014) with a room temperature of 26 ± 2°C. Shoot number and average shoot length were measured after each passage, and shoot vigour was observed macroscopically. Shoot multiplication rate was calculated as the average coefficient of multiplication (Sánchez and Vieitez, 1991; Hung and Trueman, 2011).

Shoots of 2.5-cm length were then transferred to one of four rooting media: (6) ½MS + 0.1 mg L^{-1} IBA; (7) ½ MS+ 0.25 mg L^{-1} IBA; (8) ½ MS+ 0.5 mg L^{-1} IBA; or (9) ½ MS+ 1.0 mg L^{-1} IBA, each containing 20 g L^{-1} sugar and 6.0 g L^{-1} carrageenan, pH 5.8. There were 30 jars of each medium and 3 replication for the same experiment. The shoots were maintained under 15–25 µmol m^{-2} s^{-1} irradiance (12 h d^{-1}) with a room temperature of 26 ± 2°C. Rooting percentage, root number, root length and plantlet height were recorded at the end of 60 days in rooting medium. Plantlets were then transplanted into rectangle plastic baskets containing red core soil (natural local soil) in greenhouse.

Data were analysed by analysis of variance (ANOVA) (for 3–6 means), with a post-hoc Tukey's test if the ANOVA was significant. Means are provided with standard errors, and means were considered significantly different at $P < 0.05$.

3. Results

The induction rate of the explants was 63.6%, i.e. 21 of total 33 explants were initiated (Figure 1).

Shoot multiplication rate was highest (3.90 ± 0.06 shoots per passage) in full-strength MS medium (Table 1). Shoot multiplication rate and shoot growth vigour did differ between MS and ½ MS media in either 0.1 mg L^{-1} NAA or 0.02 mg L^{-1} NAA, but 0.1 mg L^{-1} NAA reduced the multiplication rate and caused roots. However, continuous culture in full-strength MS medium supplemented with 0.1 mg L^{-1} NAA caused numerous roots. continuous culture in half-strength MS medium supplemented with either 0.1 mg L^{-1} NAA or 0.02 mg L^{-1} NAA caused slight yellowing and limited shoot elongation (1.87 ± 0.03 cm). Rooting in the multiplication passage was not encouraged bacause it caused the proliferation rate decreased. Shoot length and vigour were greatest in MS medium containing 1.0 mg L^{-1} BA and 0.02 mg L^{-1} NAA (double 'a'), and no root was found in this medium (Table 1) (Figure 2).

Table 1. Effect of basal medium and plant growth regulators on proliferation, length and growth vigour of *C. nutans* shoots

Medium	BA (mg L^{-1})	NAA (mg L^{-1})	Average multiplication rate	Average shoot length (cm)	Shoot growth vigour
MS	1.0	0.1	2.73 ± 0.12 b	3.53 ± 0.09 a	All leaves green. Vigorous shoots. Roots. ++
½MS	1.0	0.1	1.63 ± 0.09 d	1.87 ± 0.03 b	Slight yellowing of lower leaves. Slow shoot growth. Roots. +
MS	1.0	0.02	3.90 ± 0.06 a	3.33 ± 0.03 a	All leaves green. Vigorous shoots. No root. +++
½MS	1.0	0.02	2.13 ± 0.09 c	1.60 ± 0.06 c	Slight yellowing of lower leaves. Slow shoot growth. No root. +

Means (± SE) with different letters within a column are significantly different (ANOVA and Tukey's test; $P < 0.05$; n = 30 jars). '+++': good; '++': intermediate; '+': poor

Table 2. Effect of plant growth regulators on rooting frequency, root number, root length and plantlet height of *C. nutans*

Medium	IBA (mg L^{-1})	Average rooting rate (%)	Average root number per plantlet	Average length per root (cm)	Average plantlet height (cm)	Growth vigour
½MS	0.1	100.0	2.23 ± 0.11 b	4.73 ± 0.74 a	10.43 ± 0.44 a	All plantlet grew vigourly; leaves green; shoot elongating. +++
½MS	0.25	100.0	2.47 ± 0.13 ab	4.64 ± 0.29 a	9.39 ± 0.43 ab	All plantlet grew vigourly; leaves green; shoot elongating. +++
½MS	0.5	100.0	2.33 ± 0.06 ab	5.49 ± 0.23 a	9.54 ± 0.17 ab	All plantlet grew vigourly; leaves green; shoot elongating. +++
½MS	1.0	100.0	2.73 ± 0.03 a	4.44 ± 0.13 a	7.32 ± 0.81 b	All plantlet grew vigourly; leaves green; shoot elongating. +++

Means (± SE) with different letters within a column are significantly different (ANOVA and Tukey's test; $P < 0.05$; n = 30 jars). '+++': good; '++': intermediate; '+': poor

Figure 1. The explant initiated of *C. nutans*

Figure 2. Proliferation of *C. nutans* shoots in MS medium containing 1.0 mg L^{-1} BA, 0.02 mg L^{-1} NAA

Figure 3. Rooting of *C. nutans* shoots in ½MS medium containing 0.25 mg L^{-1} IBA

Figure 4. Survival plantlets of *C. nutans* in the greenhouse

Conversion to plantlets was high (100.0%) in all rooting media (Table 2). However, the optimal combination of highest rooting frequency (100.0%), avearge root number per plantlet (2.47 ± 0.13), root length (5.49 ± 0.23 cm) and plantlet height (9.54 ± 0.17 cm) was obtained with the ½ MS medium supplemented with 0.25 or 0.5 mg L^{-1} IBA. The plantlets on the two media grew vigourly with normal green leaves and elongating shoots (Figure 3). The plantlets acclimatized readily to glasshouse conditions, with 100% survival (Figure 4).

4. Discussion

The optimal medium for *C. nutans* propagation was MS + 1.0 mg L^{-1} BA + 0.02 mg L^{-1} NAA, which provided a multiplication rate of 3.9 shoots per passage

during passages of 30-d duration. Annual shoot production was, therefore, approximately 3.9^{12}=12,381,557, which allows mass-propagation of *C. nutans* in a tissue culture facility. The shoot multiplication rate increases, but shoot size diminishes, during long-term production possibly due to cytokinin accumulation. Thus, BA and NAA concentrations could be adjusted during long-term propagation to maximize shoot production and quality (Chen *et al*, 2014).

The low inorganic salt and nitrogen concentrations of hall-strength MS medium reduced shoot elongation and caused slightly yellowing of leaves. The high inorganic salt concentration of full-strength MS medium accelerated shoot elongation and the shoots grew with normal green leaves. The medium containing 0.1 mg L^{-1} NAA caused rooting in the proliferation stage indicated 0.1 mg L^{-1} NAA was too much for the propagation of *C. nutans*.

The use of ½ MS as the basal medium supplemented with IBA from 0.1 to 1.0 mg L^{-1} provided virtually 100% rooting but the optimal combination of highest avearge root number per plantlet, root length and plantlet height was obtained with the ½ MS medium supplemented with 0.25 or 0.5 mg L^{-1} IBA. There were not significant different between ½MS medium supplemented with 0.25 and 0.5 mg L^{-1} IBA. Considering the economic factor, ½ MS medium supplemented with 0.25 mg L^{-1} IBA was selected as the rooting medium, which could lower the production cost during mass propagation.

The callus induction and plantlet regeneration for *C. nutans* is not easy to achieved (Ying, 2013; Gunasekaran, 2014). The extracts from the callus and suspension cells used for drugs are controversial since it may contain residual composition of plant growth regulators.

Compared to other medicinal plants, tissue culture technique of *C. nutans* achieved more easily than that of *Tripterygium wilfordii* and *Dendrobium officinale* (Chen, 2009; Chen *et al*, 2014).

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