Plant Tissue Cult. & Biotech. **21**(1): 27-33, 2011 (June)

PTC&B

An Improved Plant Regeneration of *Vanilla planifolia* Andrews

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Key words: Regeneration, Coconut water, Nodal culture, Vanilla

Abstract

Multiple shoots of *Vanilla planifolia* were induced from nodal explants under influence of different concentrations of plant growth regulators and coconut water (CW). A comparison of shoot regeneration between semi solid and liquid culture media was also investigated. Ninety seven per cent of the explants produced a mean number of 9.6 shoots with a mean shoot length of 4.70 cm when cultured on liquid MS supplemented with 1.0 mg/l BAP in combination of 15% CW. Shoots generated were rooted with frequency of 93% on MS supplemented with 1.0 mg/l NAA with a mean number of 2.9 roots per shoot and mean length of 4.0 cm within four weeks. The plantlets were transferred to sand : compost mixture (1 : 2) with 85.0% survival rate recorded after four weeks of acclimation.

Introduction

Vanilla planifolia is a perennial climbing orchid with a thick, cylindrical and succulent green stem (Janarthanam and Seshadri 2008). There are about 110 species of vanilla in Orchidaceae (Divakaran et al. 2008), of which *V. planifolia* Andrews, *V. pompona* Schiede and *V. tahitensis* J.W. Moore are commercially cultivated (Rao and Ravishankar 2000). Conventional propagation of *V. planifolia* using stem cuttings is labour intensive and time consuming (Kalimuthu et al. 2006). In recent years, *V. planifolia* propagated by using tissue culture technique have shown greater advantages than conventional method. A number of studies on multiplication of *V. planifolia* have been reported, through the culture of stem nodes (Giridhar et al. 2001), root tips (Philip and Nainar 1986), shoot tips (Giridhar et al. 2001, Kalimuthu et al. 2006) and callus culture (Janarthanam and

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Seshadri 2008, Tan et al. 2010). However, propagation of *in vitro* cultured plantlets of *V. planifolia* exhibited some drawbacks, such as handling of plantlets and low transplant survival rate. Therefore, the present study was undertaken to improve the prevailing technique and to evaluate the multiplication rate of *V. planifolia* under the influence of plant growth regulators and coconut water.

Materials and Methods

Nodal segments (1.5 - 2.0 cm) from *V. planifolia* obtained from the nursery of Kuasa Sekata Sdn. Bhd., Selangor, Malaysia, were surface disinfected according to the protocol of Tan et al. (2010). The selected dehusked six-month-old coconut fruits were purchased from local market at Semenyih, Malaysia. The coconut water was filtered through several layers of cheese cloth and refiltered using 0.2 μm filters (Minisart, Sartorius).

All explants were cultured on MS containing 3% (w/v) sucrose and 0.55% (w/v) agargel (Sigma, USA) unless otherwise stated. The media were adjusted to pH 5.8 ± 0.2 with 1 N NaOH or 1 N HCl and autoclaved at 121ºC for 15 min. The cultures were incubated at 25 ± 2ºC under 16 hrs light and 8 hrs dark cycle with a light intensity of 40 μmol/m^2/s provided by cool white fluorescent lights.

Cleaned nodal explants were cultured on MS supplemented with different concentrations of BAP (0.5, 1.0 and 2.0 mg/l) and Kn (0.5, 1.0 and 2.0 mg/l) for shoot initiation and proliferation.

To determine the effect of CW on shoot initiation and proliferation, nodal explants were inoculated on semi solid or liquid medium of MS supplemented with 1.0 mg/l BAP and CW at five concentrations (0, 5, 10, 15 and 20% v/v). Agargel at 0.55% (w/v) was only added into semi solid culture media. For the liquid MS, the explants were cultured into 100 ml conical flasks under continuous shaking condition of 100 rpm. The percentage of explants forming shoots, number of shoots per explants and shoot length were recorded after 60 days of culture.

Shoots of 2 - 4 cm in height were transferred for root induction on half strength or full strength of MS supplemented with different concentrations of IBA (0.0, 1.0 and 2.0 mg/l) and NAA (0.0, 1.0 and 2.0 mg/l). The percentage of shoots with roots, number of roots per shoot and root length were recorded after 30 days of culture. Plantlets with roots 2 - 4 cm in length were washed thoroughly in running tap water to remove residual agar medium and dipped in 0.2% (v/v) carbendazim for 1 min before transfer to sand : compost mixture (1 : 2) in plastic pots. The plants were grown in a shade house under 80% shading.

The experiments were conducted with a total of 20 explants per treatment and were repeated thrice. The percentage data were subjected to square root

transformation before analysis. The data were recorded and analysed statistically by two-way ANOVA followed by DMRT at a significance level of $p < 0.05$.

Results and Discussion

The shoot proliferation of *V. planifolia* under influence of different concentrations of BAP and Kn was investigated (Table 1). Among the treatments tested, the highest number of shoots per explants (5.03 shoots) with a mean length of 3.9 cm was observed from the medium supplemented with 1.0 mg/l BAP while the PGR-free medium gave the lowest number of shoots as well as shoot length.

Table 1. Effect of different concentrations of BAP and Kn on shoot proliferation of *V. planifolia*.

Plant growth regulators (mg/l)		Percentage of explants forming shoots	Mean number of shoots per explant	Mean shoot length (cm)
BAP	Kn			
0.0	0.0	90.0 ± 0.03^{a}	2.80 ± 0.21^{d}	2.74 ± 0.06^{d}
0.5	0.0	95.0 ± 0.03^{a}	3.47 ± 0.26^{bcd}	3.29 ± 0.18^{bcd}
1.0	0.0	92.0 ± 0.02^{a}	5.03 ± 0.30^{a}	3.87 ± 0.18^{a}
2.0	0.0	90.0 ± 0.03^{a}	4.03 ± 0.34^{b}	3.77 ± 0.18^{ab}
0.0	0.5	88.0 ± 0.03^{a}	2.97 ± 0.29^{cd}	3.01± 0.17^{cd}
0.0	1.0	92.0 ± 0.02^{a}	3.73 ± 0.29^{bcd}	3.14 ± 0.16^{cd}
0.0	2.0	93.0 ± 0.04^{a}	3.82 ± 0.23^{bc}	3.32 ± 0.21^{bc}

Results represent mean ± standard error mean (SEM) of three replicated experiments after 60 days of culture. Difference letters within a column indicates a significantly different at $p < 0.05$ level.

The proliferation rate of explants cultured on the medium supplemented with Kn was generally lower than BAP. BAP has been considered to be one of the most effective cytokinins for the induction of shoot regeneration in plant tissue culture (Janarthanam and Seshadri 2008). Furthermore, a few studies showed that BAP was more effective than Kn in enhancing shoot multiplication on several plant species, such as *Crossandra infundibuliformis* (Girija et al. 1999), *Geoderum purpureum* (Mohapatra and Rout 2004) and *Curculigo orchioides* (Nagesh 2008).

The explants were cultured on either semi solid or liquid MS supplemented with various concentrations of CW in combination with BAP for shoot initiation and elongation (Table 2). In the present study, more than 88.0% of explants formed shoots. The presence of CW in both types of media has shown significantly different in the number of shoots formed per explant compared to CW-free medium. Among the treatments tested, the liquid medium containing

1.0 mg/l BAP and 15% CW was more effective as it induced the highest mean number of shoots per explant (9.6 shoots) with a mean length of 4.7 cm (Fig. 1A).

Table 2. Effect of BAP and CW on shoot proliferation of *V. planifolia*.

Type of medium	Treatment		Percentage of explants forming shoots	Mean number of shoots per explant	Mean shoot length (cm)
	BAP (mg/l)	CW (%)			
Semi solid	0.0	0	97.0 ± 0.02[a]	3.02 ± 0.20[h]	2.98 ± 0.17[c]
	1.0	0	97.0 ± 0.02[a]	5.03 ± 0.38[f]	4.20 ± 0.14[b]
	1.0	5	93.0 ± 0.03[a]	5.22 ± 0.31[f]	4.00 ± 0.19[b]
	1.0	10	88.0 ± 0.04[a]	7.10 ± 0.46[d]	3.89 ± 0.23[b]
	1.0	15	93.0 ± 0.03[a]	8.42 ± 0.47[c]	4.08± 0.19[b]
	1.0	20	98.0 ± 0.02[a]	6.90 ± 0.34[d]	3.82 ± 0.14[b]
Liquid	0.0	0	93.0 ± 0.03[a]	3.77 ± 0.25[g]	3.11 ± 0.21[c]
	1.0	0	97.0 ± 0.02[a]	6.45 ± 0.29[e]	5.04 ± 0.22[a]
	1.0	5	95.0 ± 0.03[a]	7.03 ± 0.30[d]	5.03 ± 0.24[a]
	1.0	10	97.0 ± 0.02[a]	8.72 ± 0.36[bc]	4.91 ± 0.21[a]
	1.0	15	97.0 ± 0.02[a]	9.58 ± 0.35[a]	4.70 ± 0.22[a]
	1.0	20	93.0 ± 0.03[a]	9.00 ± 0.29[b]	4.70 ± 0.26[a]

Results represent mean ± SEM of three replicated experiments after 60 days of culture.

In the present study, the inclusion of CW in the liquid MS generally showed a higher multiplication rate compared to semi-solid medium (Fig. 1B). CW is known as a natural substance with high levels of zeatin in its composition and has been found to be beneficial in micropropagation protocols. Several reports have also revealed the positive effects of the CW supplement (5 - 20%) for the *in vitro* growth and development of different orchid species, including *Dendrobium* species, *Vanda sanderiana* and *Grandiflora* spp. (Rethinam and Kumar 2001). The effects of liquid culture in enhancing shoot proliferation are probably due to the submergence of the whole explants in the liquid medium facilitating the uptake of nutrients and growth regulators compared to semi-solid media. Liquid medium based culture has been reported to provide more economically beneficial method for mass propagation in a number of plant species (Hung et al. 2006). Hung et al. (2006) proposed that the liquid culture system could be used to save labour and expense by eliminating or reducing gelling agents in large scale *in vitro* propagation, thereby reducing the cost of micropropagated plantlets.

In vitro derived shoots separated from multiple shoot clusters successfully developed into rooted plantlets after two weeks of culture (Table 3).

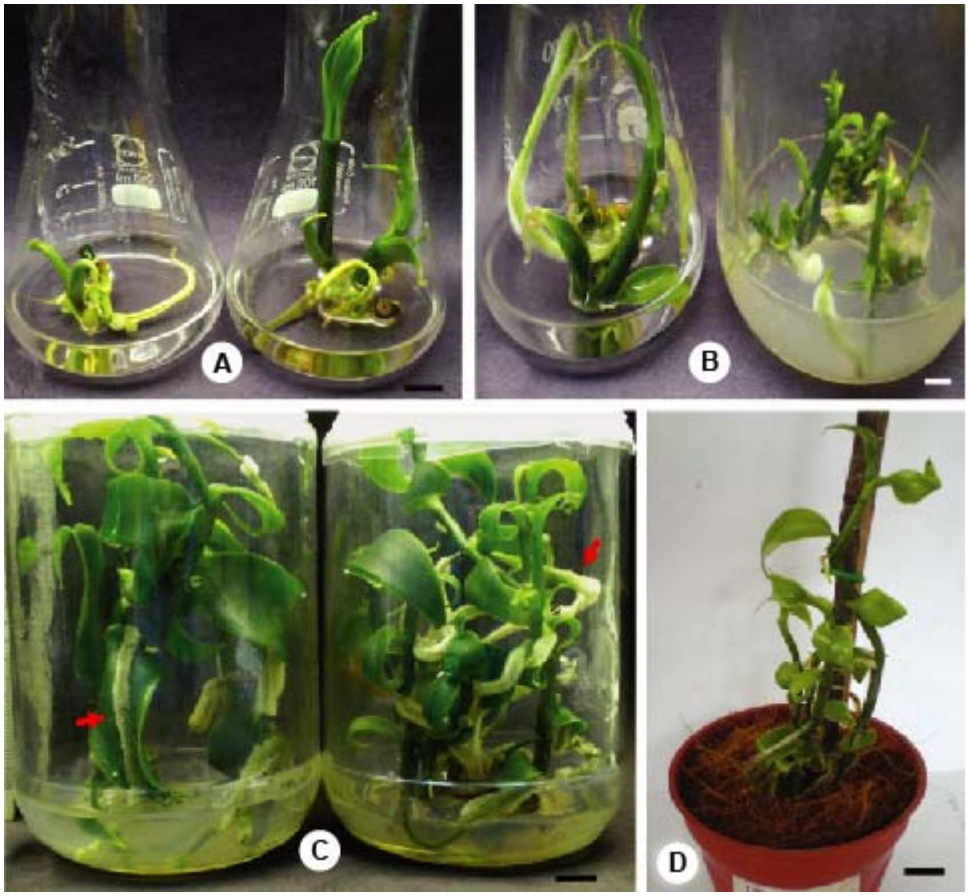

Fig. 1. Proliferation of multiple shoots on (A) PGR-free liquid MS (left) and medium containing 1.0 mg/l BAP and 15% CW (right); (B) Liquid MS containing 1.0 mg/l BAP and 15% CW and semi-solid MS supplemented with 1.0 mg/l BAP. (C) Multiple root development from shoot on MS containing 1.0 mg/l NAA. (D) A well established and hardened plant. (bar = 1 cm).

High rooting response was achieved on the MS supplemented with 1.0 mg/l NAA (93.0%) with a mean number of 2.9 roots per shoot and mean length of 4.0 cm (Fig. 1C). The results showed that the roots regeneration response in full strength MS was higher compared to MS. Media supplemented with IBA alone was observed to have increased in mean number of roots per shoot at a higher concentration (2.0 mg/l) whereas the opposite effect was observed for NAA. In the present study, NAA was found to be more effective than IBA. This has been in agreement with the studies carried out on several plant species, such as *Ocimum sanctum* Linn. (Banu and Bari 2007), *Dioscorea oppositifoliaa* L. (Behera et al. 2009) and *Morus alba* L. (Balakrishnan et al. 2009).

Table 3. Effect of IBA and NAA on root formation from regenerated shoots of *V. planifolia*.

MS	Plant growth regulators (mg/l)		Percentage of shoots forming roots	Mean number of roots per shoot	Mean root length (cm)
	IBA	NAA			
Half strength	0.0	0.0	90.0 ± 0.04^{ab}	1.83 ± 0.15^{c}	1.96 ± 0.14^{d}
	1.0	0.0	90.0 ± 0.04^{ab}	2.13 ± 0.19^{bc}	2.27 ± 0.15^{cd}
	2.0	0.0	85.0 ± 0.05^{ab}	2.32 ± 0.22^{bc}	2.49 ± 0.18^{c}
	0.0	1.0	85.0 ± 0.05^{ab}	2.17 ± 0.20^{bc}	2.65 ± 0.18^{c}
	0.0	2.0	80.0 ± 0.05^{b}	2.27 ± 0.23^{bc}	2.58± 0.20^{c}
Full strength	0.0	0.0	85.0 ± 0.05^{ab}	2.17 ± 0.20^{bc}	2.54 ± 0.19^{c}
	1.0	0.0	92.0 ± 0.04^{a}	2.45 ± 0.20^{ab}	3.18 ± 0.21^{b}
	2.0	0.0	90.0 ± 0.04^{ab}	2.93 ± 0.26^{a}	3.81 ± 0.21^{a}
	0.0	1.0	93.0 ± 0.03^{a}	2.92 ± 0.23^{a}	4.01 ± 0.22^{a}
	0.0	2.0	87.0 ± 0.04^{ab}	2.43 ± 0.26^{ab}	3.77 ± 0.25^{a}

Results represent mean ± SEM of three replicated experiments after 30 days of culture.

Sixty rooted plantlets were transplanted to sand:compost mixture (1 : 2) in plastic pots (Fig. 1D). Survival rate of 85.0% was recorded during hardening for the first four weeks under 80% shading. These results provide an efficient *in vitro* method for rapidly inducing shoots and roots in *V. planifolia*.

Acknowledgement

This work was supported by an EScience Fund from Ministry of Science, Technology and Innovation (MOSTI), Malaysia. The first author (BCT) thanks Nguyen Phan Nguyen Nhi for her technical assistance.

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