Original Article

Current Botany 2014, 5: 22-25
http://scienceflora.org/journals/index.php/cb/

The effect of thin cell layer system in *Vanilla planifolia in vitro* culture

Goh Foong Jing[1], Wan Nur' Aqilah binti Wan AB Razak[1], Zuraida Ab Rahman[1], Sreeramanan Subramaniam[2]*

[1]Biotechnology Research Centre, MARDI Headquarters, MARDI HQ, Persiaran MARDI-UPM, Serdang Selangor, Malaysia, [2]School of Biological Sciences, Universiti Sains Malaysia (USM), 11800 Gelugor, Penang, Malaysia

Received: 11.11.2014
Revised: 09.12.2014
Accepted: 10.12.2014
Published: 10.12.2014

***Address for correspondence:**
Sreeramanan Subramaniam, School of Biological Sciences, Universiti Sains Malaysia (USM), 11800 Gelugor, Penang, Malaysia. E-mail: sreeramanan@gmail.com/ sreeramanan@usm.my

ABSTRACT

This study was conducted using shoots of *Vanilla planifolia* as the explant for shoot induction using two protocols of thin cell layers (TCL) system. Shoots segment of 2-5 mm was cultured on half strength Murashige and Skoog semi-solid medium with of various concentrations of benzylaminopurine (BAP) at 0, 1.0, 2.0, 3.0, and 4.0 mg/L and thidiazuron (TDZ) at 0, 1.0, 2.0, 3.0, and 4.0 mg/L, respectively. Histological analysis of shoot formed showed that there were no differences between cross section and a longitudinal section based on the appearance of the cell structure of the shoots. For longitudinal section TCL system, medium containing 1.0 mg/L BAP and 1.0 mg/L TDZ was the optimal medium for shoot induction of *V. planifolia.* For cross section TCL system, 4.0 mg/L BAP and 3.0 mg/L TDZ were the best concentration for shoots induction of vanilla. Hence, longitudinal section TCL culture system is a more suitable protocol for the micropropagation of *V. planifolia* plantlets.

KEY WORDS: Benzylaminopurine, thidiazuron, thin layer section, *Vanilla planifolia*

INTRODUCTION

Vanilla (*Vanilla planifolia* Andrews) is a creeping plant that can reach up to 20 m in height, and often flowers at a considerable height above the ground (Schlüter *et al.*, 2007). It is a source of natural vanillin plays a major positive role in the economy of some countries (Minoo *et al.*, 2009). The pods of vanilla are valuable due to its aromatic flavor substance called vanillin and is classified as the second most important spice crops. Vanillin is mainly used in food industry for flavoring cakes, ice creams, chocolate, and beverages. Moreover, pharmaceuticals cosmetics and perfume industry are also using it in their products (Janarthanam and Seshadri, 2008; Sasikumar, 2010). According to Minoo *et al.* (2009), vanilla plants are native to the Central America and its primary gene pool is endangered by deforestation and within years has resulted in the loss of origin and natural wild species. Hence, conservation through micropropagation technique is essential for saving the gene pool.

Stem cutting of mature vine is the general propagation method for vanilla. However, this method is slow, high labor, and time-consuming (Geetha and Sheety, 2000; Giridhar *et al.*, 2001, Sasikumar, 2010). By applying aseptic micropropagation techniques, vanilla has been commercial propagated efficient in mass multiplication production such as using nodal segment as explant (Kalimuthu *et al.*, 2006, Zuraida *et al.*, 2013).

According to Swarna (2013), thin cell layers (TCLs) of different plant tissues, such as shoots, stem nodes, and hypocotyls, have been applied to shoot regeneration and somatic embryogenesis. In general, TCL culture system comprised of explants of small size from 0.1 to 5 mm excised either longitudinally or transversely. Protocorm-like bodies (PLBs) of TCL are considered to be developmentally for sensitive explants (da Silva, 2013). Recently, TCLs have been succeeding in used for plantlet regeneration of orchids. However, it is necessary to optimize plant growth regulator used in the regeneration using different propagation protocol. In this present study, the growth rate of the plant using longitudinal section protocol and cross section protocol are being studied as well as the histology structure of the plant after the treatment. This study is important in increase the shoot formation as well as to increase the production of *V. planifolia* plant.

MATERIALS AND METHODS

Plant Material and Culture Condition

V. planifolia plants that have been propagated *in vitro* were obtained from the Plant Biotechnology Laboratory, School of Biological Sciences, USM and was used as the plant material for shoot induction (Figure 1a). The plants were cultured on half strength of Mutashige and Skoog's (MS) medium supplemented with 10% coconut water. The media was gelled with 0.28% gelrite, supplied 2.0% sucrose plants, and 1 mg/L benzyl aminopurine (BAP). The experiment was carried out in culture jar each containing 40 ml of medium. The pH of the medium was adjusted to 5.75 ± 0.05 before adding gelrite. The medium was autoclaved at 121°C for 15 min. The plants cultured were then transferred to an aseptic culture room at temperature of 25 ± 2°C at 150 µmol/m^{-1}/s under 16 h photoperiod.

TCL of *V. planifolia*

The shoot explants of *V. planifolia* were cultured on MS basal medium supplemented with 1 mg/L BAP for multiplication purpose. The shoots with 0.8-1.0 cm were selected for TCL system. The cross section and longitudinal section TCL system were used in this experiment with 2-5 mm shoot section. The shoot section was then cultured for 8 weeks on ½ MS medium supplemented with 0.0-4.0 m/L BAP or TDZ placed in culture room condition with 16 h photoperiod.

Histology Analysis

The four weeks old shoots formed from the TCL, were fixed in FAA (40% formalin:acetic acid:70% ethanol = 1:1:18 v/v/v). The shoot samples were embedded in paraffin wax and slided with microtome before stained with Safranin and Fast Green. The slide observation was done using a light microscope (Olympus, Japan). For scanning electron microscope (SEM) observation, the shoot samples derived from the two protocols of TCL systems were freeze-dried processed before observe in SEM.

Statistical Analysis

The induction of shoots was analyzed using independent sample t-test and analysis of variance (ANOVA). The independent sample t-test was used to compare the means scores between the two groups on a given variable while one-way ANOVA used to test the significant differences in the means. *Post-hoc* comparisons using Tukey honestly significant difference were performed to check the significant differences at the significant level of 0.05. The software used for statistical analysis was Statistical Package for Social Sciences, version 20.0.

RESULTS

After 4 weeks of culture, for the TCL longitudinal section protocol, the shoots start to form a plantlet, and some of them come along with root with hairy root hair (Figure 1b). Besides, there was also plantlet form had the whitish appearance in the state of green. The highest percentage of shoot formation for TCL longitudinal section protocol was obtained at 0 mg/L of BAP. The medium that acts as a control shows the highest percentage of shoot formation that is 28.39% and the lowest percentage of shoot formation was MS supplemented with 3.0 mg/L with 11.88% (Table 1).

For TCL cross section protocol, the shoot formation after four weeks has a green structure with whitish hairy roots on some of them instead of the formation of whitish structure only. The highest percentage is 23.85% on the medium supplemented with 1.0 mg/L and the

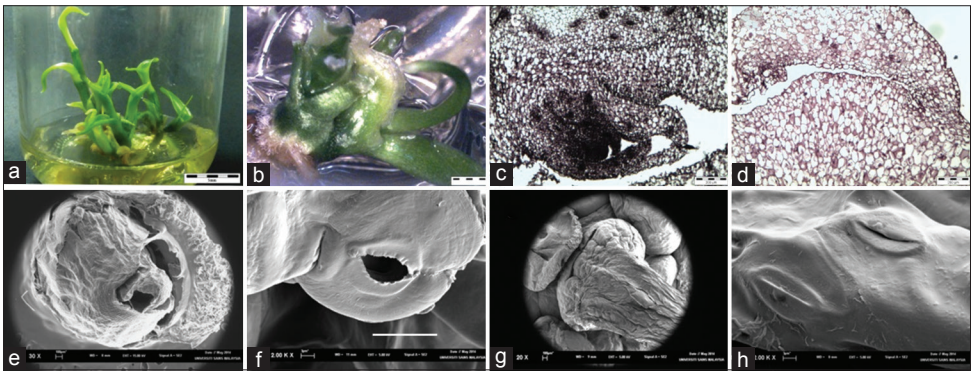

Figure 1: (a) *In vitro Vanilla planifolia* plantlets. The bar represents 5.0 mm. (b) The *V. planifolia* shoot regenerated from longitudinal section thin cell layer (TCL). The bar represents 2.0 mm. (c) The shoot of *V. planifolia* from longitudinal section TCL protocol. The bar represents 200 µm. (d) *V. planifolia* from the cross section. The bar represents 200 µm. leaf primordial, shoot apical meristem. Upper view of the sample from scanning electron microscope photograph of *V. planifolia* from control plantlets (e) and stoma (f), longitudinal section TCL protocol (g) and stoma (h)

lowest in the percentage of shoot formation was on the medium contained 3.0 mg/L concentration at 17.67%. The medium supplemented with 2.0 mg/L obtained the highest percentage of shoot formation at 28.40%, and the lowest percentage of shoots obtained on medium contained 4.0 mg/L within 11.56% (Table 2).

In the TCL longitudinal section protocol, the highest multiple shoot formation showed in ½ MS supplemented with 0 mg/L of BAP with means shoot number of 2.68. ½ MS supplemented with various concentrations of TDZ showed no significant different of multiple shoot formation. For TCL cross section protocol, both plant growth regulators with various concentrations did not provide a significant effect to the multiple shoot formations.

Figure 1c and d indicating some cells that were actively dividing. The shoot apical meristem can be observed at 1.00 mg/L of TDZ cross section using the protocol as well as leaf primordial. Figure 1d has showed the shoot tip and the active dividing meristem region. SEM provided the observation of the surface of the shoot excised using TCL longitudinal section protocol and cross section protocol. Figure 1e and f showed the shoot-derived from TCL system had grown normally. Histological analysis indicated that the TCL protocol derived plant shoots did not promote any abnormal growth or somaclonal variation.

Table 1: Effects of BAP and TDZ on shoots induction from TCL longitudinal section protocol of *Vanilla planifolia*

Plant growth regulator	Concentrations (mg/L)	Mean number of shoots produced (*mean±SE)
BAP	0	2.68 ± 0.16^{c}
	1.0	2.54 ± 0.31^{bc}
	2.0	1.30 ± 0.18^{a}
	3.0	1.13 ± 0.16^{a}
	4.0	1.73 ± 0.20^{ab}
TDZ	0.0	1.30 ± 0.14^{ac}
	1.0	1.93 ± 0.20^{a}
	2.0	1.57 ± 0.17^{a}
	3.0	1.43 ± 0.21^{a}
	4.0	1.88 ± 0.21^{a}

*Mean within a column followed by the same letter are not significantly different with Tukey Test $P=0.05$. TDZ: Thidiazuron, BAP: Benzylaminopurine, TCL: Thin cell layer, SE: Standard error

Table 2: Effects of BAP and TDZ on shoots induction from TCL cross section protocol of *Vanilla planifolia*

Plant growth regulator	Concentrations (mg/L)	Mean number of shoots produced (*mean±SE)
BAP	0	1.20 ± 0.29^{a}
	1.0	1.37 ± 0.13^{a}
	2.0	0.92 ± 0.17^{a}
	3.0	1.30 ± 0.29^{a}
	4.0	1.60 ± 0.27^{a}
TDZ	0.0	0.95 ± 0.19^{ab}
	1.0	0.83 ± 0.19^{ab}
	2.0	1.48 ± 0.23^{b}
	3.0	1.35 ± 0.26^{ab}
	4.0	0.60 ± 0.16^{a}

*Mean within a column followed by the same letter are not significantly different with Tukey test $P=0.05$. TDZ: Thidiazuron, BAP: Benzylaminopurine, TCL: Thin cell layer, SE: Standard error

DISCUSSION

In this study, the number of shoots increases according to the concentrations of cytokinin. In the previous study carried out by Kaur and Bhutani (2011), the induction of shoot bud of *Malaxis acuminata* orchid was successful in an individual treatment of BAP or NAA (1.0 mg/L each). The combination of BAP and NAA (1.0 mg/L BAP + 1.0 mg/L NAA) induced the formation of PLBs of *M. acuminate* orchid. The increasing levels BAP concentration caused shoot length to continue developing, but at higher levels of this decreased BAP at 4.00 mg/L due to the toxic regulators accumulate (Otroshy, 2013).

According to Roy *et al*. (2012), absorption of TDZ was in good at a dual phase culture system where a thin layer of the liquid medium overlaid the semi-solid medium. In this experiment, 4 weeks of exposure time to TDZ did not adversely affect healthy plantlet development from the cut shoots using longitudinal protocol when transferred to the basal medium. Most of the culture using TDZ did not induce more shoot to compare to BAP. The present study carried out by El-Mahrouk *et al.* (2010) suggested that TDZ at high concentration can produce the highest percentage of explants forming shoots. Palama *et al*. (2010) reported that the removal of TDZ may increase the shoot differentiation instead of NAA or other endogenous cytokinins. A study carried out by Lakshmanan (1995) showed that a thin section culture system obtained by transverse sectioning of *Aranda* Deborah had more significant effect on callus production compared to longitudinal sectioning. According to Monja-Mio (2013), *Agave fourcroydes* plants gave the best embryogenic response with BAP hormone using transverse TCL protocol. However, *V. planifolia* yield better result using longitudinal section.

The histological observation obtained from four weeks culture of shoot segments cut with longitudinal section protocol TCL are compared with cross section protocol TCL of *V. planifolia*. According to Scherwinski-Pereira *et al*. (2010), longitudinal section protocol TCL showned the transversal section of apical meristem along with cytoplasm and nucleus. Cross section protocol TCL of *V. planifolia* shows the cross section of shoot cutting. The

histological analysis also shows the same structure like nucleus and cytoplasm. Compared to longitudinal section protocol, cross section protocol shows dense cytoplasm and clear nucleus structure (Scherwinski-Pereira *et al*., 2010). In this study, the presence of apical meristem and leaf primordial was confirmed using cross section protocol in histological analysis. This indicated that the TCL system did not damage the meristem region.

SEM was used to observe the direction of shoot formation for explants in longitudinal section and cross section protocols TCL of *V. planifolia* as well as to analyze the common features appearing on the sample (Borowska-Wykręt *et al*., 2013). The most common feature in shoots that can be observed in both protocols was the presence of stomata (Figure 1f and h) which indicates the healthiness of the explants. In addition, while using SEM, the upper view of shoot using longitudinal section protocol showed that the direction of shoot growth growing upward (apically) (Figure 1e and g). This is because longitudinal section protocol involved transverse cutting. In contrast, cross section protocol TCL involves cutting of the shoot longitudinally. Therefore, the upper view of SEM showed the shoots growing toward the edge (laterally).

CONCLUSION

The study on the effect of TCL on *V. planifolia in vitro* culture system confirmed that longitudinal section protocol is better than cross section protocol. Besides, protocol section is the most favorable method of TCL for *V. planifolia* either using BAP or TDZ for shoots multiplication. The highest number of shoots obtained at media supplemented with BAP of 1.00 mg/L and for TDZ at 3.00 mg/L. Histological analysis and scanning electron microscope confirmed that longitudinal section protocol could minimize damage or differences on the plant cell structure.

REFERENCES

Borowska-Wykret D, Elsner J, De Veylder L, Kwiatkowska D. Defects in leaf epidermis of *Arabidopsis thaliana* plants with CDKA;1 activity reduced in the shoot apical meristem. Protoplasma 2013;250:955-61.

da Silva JA. The role of thin cell layers in regeneration and transformation in orchids. Plant Cell Tissue Organ 2013;113:1-13.

El-Mahrouk ME, Dewir YH, Omar AM. *In vitro* propagation of adult strawberry tree (*Arbutus unedo* L.) through adventitious shoots and somatic embryogenesis. Propag Ornament Plants 2010;10:93-8.

Geetha S, Shetty SA. *In vitro* propagation of Vanilla planifolia, a tropical orchid. Curr Sci 2000;79:886-9.

Giridhar P, Reddy BO, Ravishankar GA. Silver nitrate influences *in vitro* shoot multiplication and root formation in *Vanilla planifolia* Andrapradesh. Curr Sci 2001;81:1166-70.

Janarthanam B, Seshadri S. Plantlet regeneration from leaf derived callus of *Vanilla planifolia* Andrew. *In vitro* Cell Dev Biol Plant 2008;44:84-9.

Kalimuthu K, Senthilkumar R, Murugalatha N. Regeneration and mass multiplication of *Vanilla planifolia* Andr. - A tropical orchid. Curr Sci 2006;91:1401-3.

Kaur S, Bhutani KK. Micropropagation of *Malaxis acuminata* D. Don: A rare orchid of high therapeutic value. Open Access J Med Aromat Plants 2011;1:29-33.

Lakshmanan P, Loh CS, Goh CJ. An *in vitro* method for rapid regeneration of a monopodial orchid hybrid Aranda Deborah using thin section culture. Plant Cell Rep 1995;14:510-4.

Minoo D, Babu KN. Micropropagation and *in vitro* conservation of Vanilla (*Vanilla planifolia* Andrews) Protocols for *In vitro* Cultures and Secondary Metabolite Analysis of Aromatic and Medicinal Plants. New York: Springer; 2009. p. 129-38.

Otroshy M, Khalili Z, Ebrahimi MA, Nekoui MK, Moradi K. Effect of growth regulators and explant on plant regeneration of *Solanum lycopersicum* L. var. cerasiform. Russian Agric Sci 2013;39:226-35.

Palama TL, Menard P, Fock I, Choi YH, Bourdon E, Govinden-Soulange J, *et al*. Shoot differentiation from protocorm callus cultures of *Vanilla planifolia* (Orchidaceae): Proteomic and metabolic responses at early stage. BMC Plant Biol 2010;10:82.

Roy AR, Sajeev S, Pattanayak A, Deka BC. TDZ induced micropropagation in Cymbidium giganteum Wall. Ex Lindl. and assessment of genetic variation in the regenerated plants. Plant Growth Regul 2012;68:435-45.

Sasikumar B. Vanilla breeding - A review. Agric Res Commun Centre 2010;31:139-44.

Scherwinski-Pereira JE, da Guedes RS, Fermino PC, Jr, Silva TL, d Costa FH. Somatic embryogenesis and plant regeneration in oil palm using the thin cell layer technique. *In vitro* Cell Dev Biol Plant 2010;46:378-85.

Schlüter PM, Soto-Arenas, MA, Harris SA. Genetic variation in *Vanilla planifolia* (Orchidaceae). Econ Bot 2007;61:328-36.

Zuraida, AR, Izzati KH, Nazreena OA, Zaliha WS, Radziah CM, Zamri Z, *et al*. A simple and efficient protocol for the mass propagation of *Vanilla planifolia*. Am J Plant Sci 2013;4:1685-92.