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Research article

IN VITRO CULTURE OF PETIOLE LONGITUDINAL THIN CELL LAYER EXPLANTS OF VIETNAMESE GINSENG (*PANAX VIETNAMENSIS* HA ET GRUSHV.) AND PRELIMINARY ANALYSIS OF SAPONIN CONTENT

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ABSTRACT: The present work describes a procedure that allows for the easy and rapid induction of somatic embryos, calli, shoots and adventitious roots of Vietnamese ginseng (*Panax vietnamensis* Ha et Grushv.) from longitudinal thin cell layers (lTCLs). In order to investigate the morphogenesis of this medicinal plant, the effect of separately–supplemented plant growth regulators and combinatorial effect of co–supplemented auxins and cytokinins in dark or under 16-hour photoperiod was examined. After eight weeks of culture, the lTCL explants excised from petiole of three-month-old *in vitro* plants and cultured on a semi solid basal Murashige and Skoog (MS) media supplemented with 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/l thidiazuron (TDZ) in dark, 2.0 mg/l α-napththaleneacetic acid (NAA) in dark and 1.0 mg/l 2,4-D under light gave the highest rate of callogenesis (100%), embryogenesis (53.3%) and adventitious root formation (100% with a mean of 16.7 roots), and shoot formation (26.7%), respectively. The metabolite of petiole lTCL-derived calli qualitative and quantitative analyses were performed by using high-performance liquid chromatography and thin layer chromatography. The simple procedure, together with similar saponin profiles between the resulted *in vitro* tissues and plants grown in nature, suggest its potential use in generating Vietnamese ginseng in large amount for medicinal purpose.

Key words: Adventitious root, callus, longitudinal thin cell layer, *Panax vietnamensis*, shoot, somatic embryo.

INTRODUCTION

Thin cell layer (TCL) is a simple but effective system that relies on a small size explant derived from a limited cell number of homogenous tissue. They are excised longitudinally or transversely from different organs ranging from floral parts to root/rhizome of plants. Longitudinal TCL (lTCL) (0.5–1 mm wide and 5–10 mm long) is used when a definite cell type (epidermal, sub-epidermal, cortical, cambial or medullar cell) is to be analysed. TCLs can be excised from stem, leaf, vein, floral stalk, petiole, pedicel, bulb-scale, etc. As for the transverse TCL (tTCL) (0.1–5 mm), other organs (leaf blade, root/rhizome, floral organs, meristems, stem node, etc.) can be used. The reduced cell number in TCL is important for the developmental process or the morphogenetic programme, which can be altered by making changes in organ/tissue and size to be uniformly exposed to the medium (Tran Thanh Van 1980). Thin cell layers were first used to control the development of flowers, roots, shoots and somatic embryos in tobacco pedicels. Since those studies over 30 years ago, TCLs have been successfully used in the micropropagation of many plant species (Altamura *et al.*, 1993; Gozu *et al.*, 1993; Hosokawa *et al.,* 1996; Ozawa *et al.,* 1998; Teixeira da Silva 2003; Falasca *et al.,* 2004; Shinoyama *et al.,* 2006). TCL technology focuses on the size and origin of the explant, which, when appropriately chosen, serves as a fine-scale developmental block for regeneration and transformation (Teixeira da Silva *et al.,* 2007).

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Panax vietnamensis Ha et Grushv. is a famous Vietnamese ginseng. *P. vietnamensis* has not only typical medical effect but also specific physical actions like anti-stress, anti-depression, *in vitro* and *in vivo* anti-oxidation, etc. *P. vietnamensis* possessed the highest dammaran-frame saponin (12–15%) and saponin content among *Panax* genus. With these special features, this ginseng is one of the most precious species not only in Vietnam but also the world. The current supply of *P. vietnamensis* is very limited because of the plant's narrow habitat and lengthy development. Due to excessive harvest, this species is among 250 endangered species, and ranked at high risk of extinction in the Vietnam's Red Data book.

In our previous studies, the morphogenesis including somatic embryogenesis, callus, shoot, and root regeneration (Nhut *et al.*, 2012a), and the effects of exogenous spermidine and proline on enhancement of somatic embryogenesis (Nhut *et al.*, 2012b) from *in vitro* main roots tTCLs of *P. vietnamensis* were investigated. In the present study, the first time the morphogenesis of petiole lTCLs of *P. vietnamensis* was evaluated as a procedure for somatic embryo, shoot, callus and adventitious root production of Vietnamese ginseng, and TLC and HPLC fingerprint methods were utilized for investigating the saponin content biomass of calli.

MATERIALS AND METHODS

Explant source: *In vitro* plants of Vietnamese ginseng grown for three months on Schenk and Hildebrandt (SH) medium supplemented with 30 g/l sucrose, 0.5 g/l activated charcoal, and 8 g/l agar were used as the source of explants. The selected plants were vitrification–free, equally well–growing and healthy with leaves, shoots, and main and fiber roots. Making a vertical cut down the *in vitro* leaf petiole was carried out in oder to have longitudinal thin cell layers (lTCLs) with 10 mm in length as initial explants.

Culture media: The basic medium for all experiments was MS medium supplemented with 30 g/l sucrose and 8 g/l agar. Plant growth regulators (PGRs) including NAA, 2,4-D, BA and TDZ were added separately and in combination into culture media for different experiments. All culture media were adjusted to pH 5.7–5.8 before autoclaving.

Experimental design: Callogenesis, direct embryogenesis, and root and shoot formation of lTCL explants from *in vitro* Vietnamese ginseng petioles were investigated. The appropriate medium for each morphogenesis process was determined based on evaluating the individual and combinatorial effect of TDZ (0.01, 0.05, 0.1, 0.2, 0.5, or 1 mg/l), BA (0.1, 0.2, 0.5, 1, or 2 mg/l), NAA (0.1, 0.2, 0.5, 1, or 2 mg/l), and 2,4-D (0.1, 0.2, 0.5, 1, or 2 mg/l) after eight weeks of culture.

Culture condition and statistical analysis: All treatments were in triplicate and each replicate each with 15 explants in five culture vessels. Morphogenesis conditions were: 25 ± 2°C, 80% relative humidity, and under regular lighting conditions with a 16-h photoperiod (2,000–2,500 lux) or darkness. Data were analyzed by analysis of variance and the means were compared using Duncan's Multiple range Test using SPSS (SPSS version 16.0) at α 0.05.

Histological studies: Histological analysis was performed, according to Gonzalez and Cristóbal (1997), for explants at 15 days after culture initiation. Samples of cultured explants were fixed in FAA (formaline:acetic acid:70% ethanol – 5:5:90), dehydrated with Deshidratante histológico BIOPUR®, embedded in paraffin wax as described by Johansen (1940), and sectioned into 8–10 µm thick serial sections with a rotary microtome. Sections were mounted on glass slides and stained with safranin-Astra blue (Luque *et al.*, 1996), and observed under a light microscope.

Qualitative and quantitative saponin analysis: Calli derived from petiole lTCL explants of *in vitro* Vietnamese ginseng were used for saponin analysis. The procedures for saponin extraction, HPLC and TLC analyses were described by Zhai *et al.*, (2001) and Odani *et al.*, (1983a, b).

Calli of *Panax vietnamensis* were collected after 8 weeks of culture. Collected samples were cleaned, dried at 60°C, grounded to give powder and stored at room temperature until utilization. Reference samples of *Panax vietnamensis* and standard compound MR_2 were supported by Research Center of Ginseng and Medicinal Materials; ginsenoside-Rb_1 (G-Rb_1), ginsenoside-Rg_1 (G-Rg_1) were purchased from Wako Pure Chemical Industries, Ltd, Japan.

HPLC system: Supelco RP C18 column (250 mm x 4.6 mm; I.D. 5 µm), SPD-M20A-PDA detector (Shimadzu). HPLC parameters: volume injection: 20 µl; flow rate: 0.5 ml/min. Column temperature was held at 25°C.

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Sample (0.5 g) was exhaustively extracted in methanol in sonicator (10 ml methanol x 6 times). The extracts were pooled together and concentrated by evaporator to give dried residue, dissolved the residue with 20 ml water and fractionated extracted with ether ethylic and n-butanol, respectively. The ether ethylic fraction was discarded, and the n-butanol was collected and evaporated under vacuum pressure to yield the dried extract. The resulting dried extract was continuously dissolved with mixture of acetonitrile:water solvent (7:3, v/v) and fixed in 5 ml, passed through 0.45 µm membrane, and the filtrate was injected to HPLC system for quantitative determination of saponins by using calibration curves method.

RESULTS AND DISCUSSION

Effect of separately–supplemented plant growth regulators on the morphogenesis of petiole lTCLs

The formation of floral buds, vegetative buds, and roots has been demonstrated in thin cell layer explants of several species by regulating the auxin:cytokinin ratio, carbohydrate supply, and environmental conditions (Tran Thanh Van *et al.*, 1974; Tran Thanh Van and Trinh 1978). Certain isolated tissue layers in species that readily regenerate organs *in vivo* showed a remarkable potential to form organs during culture. Morphogenesis through lTCLs of Vietnamese ginseng, however, has not been studied. In this study, high rate of embryogenesis, callogenesis, shoot formation and adventitious root formation were achieved directly from petiole lTCLs of Vietnamese ginseng.

It has been demonstrated that 2,4-D has a critical role in the induction of somatic embryogenesis in many plant species (Halperin and Whetherell 1964; Ammirato 1983). However, a two-step culture is generally required for completion of somatic embryogenesis in carrot (Borkid *et al.*, 1986). That is, calli with embryogenic potential were induced on a medium containing 2,4-D, but a 2,4-D-free medium was required in order to obtain somatic embryos. Direct somatic embryos were differentiated on cotyledon tTCLs of *Panax ginseng* after nine weeks in MS medium containing 2,4-D (5 □M) under 16-hour photoperiod and 100 □$Mm^{-2}s^{-1}$ light intensity. In present experiment, petiole lTCLs of Vietnamese ginseng cultured on MS media supplemented with 2,4-D induced shoot formation, callogenesis and adventitious root formation, but did not induce embryogenesis. After eight weeks of culture, both calli (53.3%) and adventitious root (20% and 0.3 root per explant) but somatic embryo were obtained on medium with 1.0 mg/l 2,4-D in dark. Although callogenesis and adventitious shoot formation rates were lower, shoot formation was observed (26.7%) on the same medium under light while PGR-free medium and media with TDZ, BA, and NAA alone did not induce shoot formation (Table 1, 2).

Compared to media with 2,4-D, the same morphogenesis was obtained when NAA was used, except for shoot formation. While explants cultured on PGR-free medium and media supplemented with TDZ or BA alone died off (Table 1, 2).

Moreover, there are some significant differences between the morphogenesis placed under 16-hour photoperiod and in dark, especially in embryogenesis, root formation, and adventitious shoot formation. It was observed that the highest embryogenesis rate (53.3%) was obtained on medium with 2.0 mg/l NAA in dark (Fig. 1b, 1c), and higher than that under 16-hour photoperiod (20%) (Table 1, 2). These embryos were transferred to PGR-free MS medium, and some of them germinated within two weeks (Fig. 1d, 1e), and formed complete plantlets (Fig. 1f).

The maximum callogenesis (60%) was recorded on medium supplemented with 1.0 mg/l NAA in dark and medium with 2.0 mg/l NAA placed under 16-hour photoperiod (Table 1, 2). Most calli induced in dark were soft with transparent-white color while those placed under 16-hour photoperiod were hard, greenish yellow, brownish yellow, greenish white and transparent-white.

Medium with 2.0 mg/l NAA in dark gave not only the highest embryogenesis rate but also the highest adventitious root formation rate (100%) and the maximum number of roots per explant (16.7 roots). It could be observed that these roots were thin and long with milk- and transparent-white colors. Media supplemented with 2,4-D also induced root formation. The root formation rate and number of roots per explant on these media, however, were considerably lower than those on media with NAA (Table 2).

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Table 1: Effect of separately–supplemented PGRs on the morphogenesis of *P. vietnamensis* petiole lTCLs after 8 weeks of culture under 16h photoperiod

PGRs (mg/l)				Morphogenesis					Comments
TDZ	BA	2,4-D	NAA	Embryo (%)	Shoot (%)	Callus (%)	Adventitious roots (%)	Adventitious roots Roots/ explant	
-	-	-	-	0.0c*	0.0b	0.0e	0.0e	0.0c	Explant died off
0.01	-	-	-	0.0c	0.0b	0.0e	0.0e	0.0c	Explant died off
0.05	-	-	-	0.0c	0.0b	0.0e	0.0e	0.0c	Explant died off
0.10	-	-	-	0.0c	0.0b	0.0e	0.0e	0.0c	Explant died off
0.20	-	-	-	0.0c	0.0b	0.0e	0.0e	0.0c	Explant died off
0.50	-	-	-	0.0c	0.0b	0.0e	0.0e	0.0c	Explant died off
1.00	-	-	-	0.0c	0.0b	0.0e	0.0e	0.0c	Explant died off
-	0.1	-	-	0.0c	0.0b	0.0e	0.0e	0.0c	Explant died off
-	0.2	-	-	0.0c	0.0b	0.0e	0.0e	0.0c	Explant died off
-	0.5	-	-	0.0c	0.0b	0.0e	0.0e	0.0c	Explant died off
-	1.0	-	-	0.0c	0.0b	0.0e	0.0e	0.0c	Explant died off
-	2.0	-	-	0.0c	0.0b	0.0e	0.0e	0.0c	Explant died off
-	-	0.1	-	0.0c	0.0b	0.0e	0.0e	0.0c	Explant died off
-	-	0.2	-	0.0c	0.0b	0.0e	0.0e	0.0c	Explant died off
-	-	0.5	-	0.0c	0.0b	13.3d	13.3d	0.3bc	Small, hard, brownish yellow calli Long and white roots
-	-	**1.0**	-	0.0c	**26.7a**	20.0c	33.3c	1.0bc	White and green shoot clusters Friable and transparent-white calli Small, long roots with milk- and transparent-white colors
-	-	2.0	-	0.0c	0.0b	0.0e	0.0e	0.0c	Explant died off
-	-	-	0.1	0.0c	0.0b	0.0e	0.0e	0.0c	Explant died off
-	-	-	0.2	0.0c	0.0b	0.0e	0.0e	0.0c	Explant died off
-	-	-	0.5	0.0c	0.0b	0.0e	0.0e	0.0c	Explant died off
-	-	-	1.0	6.7b	0.0b	53.3b	40.0b	2.0b	Milk white globular embryos Small, hard, brownish yellow calli, few in number Thin, long roots with milk- and transparent-white colors
-	-	-	2.0	20.0a	0.0b	60.0a	100.0a	9.7a	Milk-white globular embryos Hard, greenish calli emerging from both the proximal and distal ends of lTCL Short, green and white roots

Different letters (*) in the same column indicate significantly different means using Duncan's test ($p < 0.05$)

In previous studies with *P. ginseng*, somatic embryos were obtained on calli derived from root explants after 12 to 32 weeks of culture (Chang and Hsing 1980; Ahn and Kim 1992). In this study, embryos were observed to form directly on the surface of lTCLs after eight weeks. Directly-formed somatic embryos have also been obtained from TCLs of *Helianthus* (Pelissier *et al.*, 1990) and a *Nicotiana* hybrid (Tran Thanh Van 1980). By using a lTCL system, which presents the advantage that most cells are in contact with nutrients and PGRs, direct somatic embryos can be obtained within a short period.

In general, 2,4-D and NAA have significant influence on the morphogenesis of Vietnamese ginseng petiole lTCLs. Optimal conditions for embryogenesis and adventitious root formation, callogenesis, and shoot formation were 2.0 mg/l NAA in dark, 2.0 mg/l NAA under light, and 1.0 mg/l 2,4-D under light, respectively (Table 1, 2).

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Table 2: Effect of separately–supplemented PGRs on the morphogenesis of *P. vietnamensis* petiole lTCLs after 8 weeks of culture in dark

PGRs (mg/l)				Morphogenesis				Comments
TDZ	BA	2,4-D	NAA	Embryo (%)	Callus (%)	Adventitious roots		
						(%)	Roots/ explant	
-	-	-	-	0.0c*	0.0d	0.0e	0.0c	Explant died off
0.01	-	-	-	0.0c	0.0d	0.0e	0.0c	Explant died off
0.05	-	-	-	0.0c	0.0d	0.0e	0.0c	Explant died off
0.10	-	-	-	0.0c	0.0d	0.0e	0.0c	Explant died off
0.20	-	-	-	0.0c	0.0d	0.0e	0.0c	Explant died off
0.50	-	-	-	0.0c	0.0d	0.0e	0.0c	Explant died off
1.00	-	-	-	0.0c	0.0d	0.0e	0.0c	Explant died off
-	0.1	-	-	0.0c	0.0d	0.0e	0.0c	Explant died off
-	0.2	-	-	0.0c	0.0d	0.0e	0.0c	Explant died off
-	0.5	-	-	0.0c	0.0d	0.0e	0.0c	Explant died off
-	1.0	-	-	0.0c	0.0d	0.0e	0.0c	Explant died off
-	2.0	-	-	0.0c	0.0d	0.0e	0.0c	Explant died off
-	-	0.1	-	0.0c	0.0d	0.0e	0.0c	Explant died off
-	-	0.2	-	0.0c	0.0d	26.7c	0.2c	Short, milk-white and green roots
-	-	0.5	-	0.0c	33.3b	20.0d	0.5c	Soft calli with transparent-white colors Small, long roots with transparent-white colors
-	-	1.0	-	0.0c	53.3a	20.0d	0.3c	Soft calli with transparent-white colors Small, long roots with transparent-white colors
-	-	2.0	-	0.0c	0.0d	40.0b	1.5c	Small, long roots with white and yellow colors
-	-	-	0.1	0.0c	0.0d	0.0e	0.0c	No callogenesis
-	-	-	0.2	0.0c	0.0d	0.0e	0.0c	No callogenesis
-	-	-	0.5	0.0c	20.0c	20.0d	0.4c	Small, hard, brownish yellow calli, few in number Small, short roots with transparent-white colors
-	-	-	1.0	20.0b	60.0a	100.0a	7.3b	Two-cotyledon embryos Soft, brownish yellow calli Small, long roots with milk- and transparent-white colors
-	-	-	**2.0**	**53.3a**	**53.3a**	**100.0a**	**16.7a**	Two-cotyledon embryos Soft calli with transparent-white colors Thin, long roots with milk- and transparent-white colors

Different letters (*) in the same column indicate significantly different means using Duncan's test ($p < 0.05$)

Combinatorial effect of co–supplemented auxins and cytokinins on the morphogenesis of petiole lTCLs

TCL systems allow for the isolation of specific cells or tissue layers, which, depending on the genetic state and epigenetic requirements, and in conjunction with strictly controlled growth conditions (light, temperature, pH, PGRs, media additives and others) may lead to the *in vitro* induction of specific morphogenetic programs. Within the TCL system the morphogenetic and developmental pathways of specific organs – derived from other specific or non-specific cells, tissues or organs – may be clearly directed and controlled (Teixeira da Silva JA and Nhut 2003). In the present experiment, PGRs and light conditions showed their strong effect on morphogenesis from petiole lTCL explants.

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As showed in Table 3 and 4, media supplemented with 2,4-D in combination with BA at various concentrations in dark gave the higher callogenesis rate than those placed under 16-hour photoperiod. Six out of nine treatments in dark gave the callogenesis rate of 100% (Table 4). Among them, the highest number and uniform quality of calli were obtained on medium supplemented with 1.0 mg/l 2,4-D and 0.2 mg/l BA in dark (data not show). These calli were friable, numerous and milk-white in color. Whereas, all calli formed under light were rigid with brownish red, red, green, and yellow in colors.

Table 3: Combinatorial effect of 2,4-D and BA on the morphogenesis of *P.vietnamensis* petiole lTCLs under 16h photoperiod

PGRs (mg/l)		Morphogenesis (%)	Comments on callus appearance
2,4-D	BA	Callus	
1.0	0.1	93.3ab*	Brownish red, green, yellow and few in number
1.0	0.2	100.0a	Milk-white, red and friable
1.0	0.5	93.3ab	Green, red, opalescent, hard and few in number
1.0	1.0	86.7b	Red, brownish yellow, light green and few in number
1.0	2.0	66.7c	Light yellow, red and very few in number
0.1	1.0	13.3d	Very few in number
0.2	1.0	73.3c	Brown, hard and very few in number
0.5	1.0	86.7b	Yellow, green calli emerging from the distal end of lTCL and few in number
2.0	1.0	100.0a	Milk-white, yellow, friable calli emerging from all the surface

Different letters (*) in the same column indicate significantly different means using Duncan's test ($p < 0.05$)

Table 4: Combinatorial effect of 2,4-D and BA on the morphogenesis of *P. vietnamensis* petiole lTCLs in dark

PGRs (mg/l)		Morphogenesis (%)	Comments on callus appearance
2,4-d	BA	Callus	
1.0	0.1	100.0a*	Brownish red, brownish yellow, milk-white and friable
1.0	0.2	100.0a	Milk-white, friable and numerous
1.0	0.5	100.0a	Milk-white and friable
1.0	1.0	100.0a	Milk-white, yellow, friable and few in number
1.0	2.0	100.0a	Light yellow and friable
0.1	1.0	46.7d	Brown, hard and very few in number
0.2	1.0	80.0c	Brown, yellow, hard calli emerging from both the proximal and distal ends of lTCL and scattering on the explant surface
0.5	1.0	93.3b	Milk-white, friable calli emerging from either proximal or distal ends of lTCL and scattering on the explant surface
2.0	1.0	100.0a	Yellow, brown, friable and few in number

Different letters (*) in the same column indicate significantly different means using Duncan's test ($p < 0.05$)

Kurilcik (2008) reported that a change of the photoperiod influences morphological and biometric parameters and concentration of photosynthetic pigments in different ways in *in vitro Chrysanthemum* plantlets. For the plantlet height and root development, the optimum photoperiod of 16 hours was established. Kozai *et al.*, (1995) also showed suppressed root growth of potato plantlets under conditions of 8-hour photoperiod in comparison to 16-hour photoperiod. In the present experiment, morphogenesis from Vietnamese ginseng petiole lTCLs was also strongly affected by different light conditions.

The results showed that the combinations of 2,4-D and TDZ lead to high callogenesis rate. Six out of twenty treatments gave callogenesis rate of 100% (Table 5, 6). Especially, higher number and more uniform quality of calli were observed on media supplemented with 2,4-D and TDZ in dark (Fig. 1a). Medium with 1.0 mg/l 2,4-D and 0.1 mg/l TDZ in dark was most effective for callogenesis. Calli emerged from the proximal and distal ends of lTCL and the explant surface on this medium. Besides, milk-white, yellow and friable soft calli were observed in dark while calli under light were green and hard.

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Table 5: Combinatorial effect of 2,4-D and TDZ on the morphogenesis of *P. vietnamensis* petiole lTCLs under 16h photoperiod

PGRs (mg/l)		Morphogenesis (%)	Comments on callus appearance
2,4-D	TDZ	Callus	
1.0	0.01	86.7c*	Soft, milk- and transparent-white calli emerging from the distal end of lTCL and few in number
1.0	0.05	93.3b	Milk-white, friable calli emerging on the explant surface Red, soft calli emerging from the proximal and distal ends of lTCL
1.0	0.10	93.3b	Greenish white, transparent-white and hard
1.0	0.20	100.0a	Large, soft, friable, milk-white, brownish red calli emerging from the proximal and distal ends of lTCL and the explant surface
1.0	0.50	100.0a	Hard, greenish calli emerging from the distal end of lTCL
1.0	1.00	93.3b	Friable, soft, milk-white, brownish red calli almost emerging from the distal end of lTCL
0.1	0.20	73.3d	Hard, green calli emerging from the distal end of lTCL, very few in number
0.2	0.20	73.3d	Friable, milk-white calli emerging from the distal end of lTCL, very few in number
0.5	0.20	93.3b	Friable, greenish white calli emerging from the distal end of lTCL and scattering on the explant surface
2.0	0.20	0.0e	Explant died off

Different letters (*) in the same column indicate significantly different means using Duncan's test ($p < 0.05$)

Table 6: Combinatorial effect of 2,4-D and TDZ on the morphogenesis of *P. vietnamensis* petiole lTCLs in dark

PGRs (mg/l)		Morphogenesis (%)	Comments on callus appearance
2,4-D	TDZ	Callus	
1.0	0.01	93.3b*	Friable, soft, milk-white and brownish yellow calli almost emerging from the distal end of lTCL
1.0	0.05	100.0a	Friable, milk-white and yellow calli almost emerging from the distal end of lTCL
1.0	**0.10**	**100.0a**	Friable, milk- and transparent-white, yellow calli emerging from the proximal and distal ends of lTCL and the explant surface
1.0	0.20	100.0a	Friable, soft, milk-white and brownish yellow calli emerging from the distal end of lTCL many more than those from the proximal end
1.0	0.50	93.3b	Friable, milk-white and yellow calli almost emerging from the distal end of lTCL
1.0	1.00	86.7c	Friable, white and yellow calli emerging from the proximal and distal ends of lTCL and the explant surface
0.1	0.20	0.0e	No callogenesis
0.2	0.20	80.0d	Friable, soft, white and brownish yellow calli emerging from the proximal and distal ends of lTCL and the explant surface
0.5	0.20	100.0a	Soft, white and brownish yellow calli emerging from the proximal and distal ends of lTCL and the explant surface
2.0	0.20	86.7c	Soft, white and brownish yellow calli emerging from the distal end of lTCL, few in number

Different letters (*) in the same column indicate significantly different means using Duncan's test ($p < 0.05$)

The effect of NAA in combination with BA on morphogenesis was considerably different from the combinations of 2,4-D and BA, and 2,4-D and TDZ. Media with 1.0 mg/l NAA and 0.1–0.5 mg/l BA in dark induced adventitious root formation. The highest root formation rate and the highest number of roots per explant were achieved on medium supplemented with 1.0 mg/l NAA and 0.2 mg/l BA in dark, 93.3% and 3.9 roots, respectively (Table 8). Root formation rate and number of roots per explant on this medium, however, were significantly lower than media supplemented with NAA alone in dark, and under light. Media with concentration of BA at 1.0 mg/l and higher in dark, and all media with different concentrations of NAA and BA under light did not show adventitious root formation (Table 7, 8).

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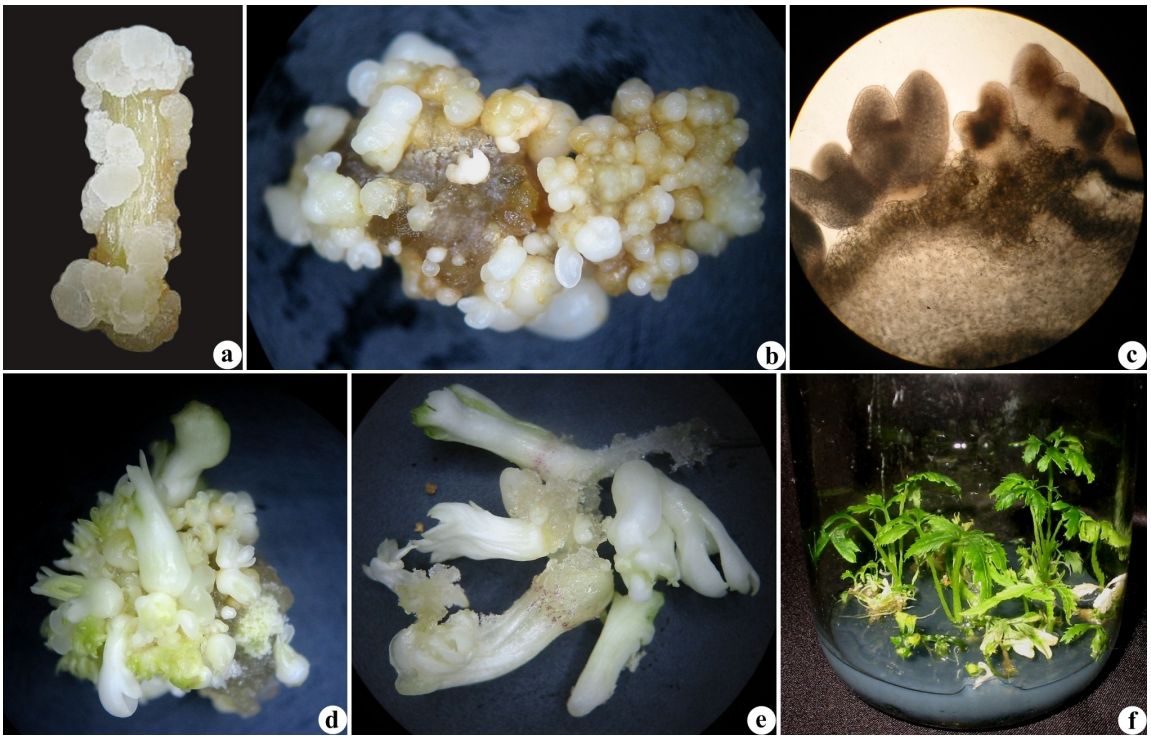

Figure 1: Callogenesis and somatic embryogenesis of *Panax vietnamensis* Ha et Grushv. (a) Petiole lTCL-derived calli (b) Embryo cluster (c) Embryo structure (d) Embryo maturation (e) Single embryos (f) Vigorous embryo-derived plantlets after 2 months cultured on MS½ medium supplemented with 0.5 mg/l NAA, 1.0 mg/l BA and 2.0 mg/l activated charcoal

Table 7: Combinatorial effect of NAA and BA on the morphogenesis of *P. vietnamensis* petiole lTCLs under 16h photoperiod

PGRs (mg/l)		Morphogenesis (%)	Comments on callus appearance
NAA	BA	Callus	
1.0	0.1	60.0c*	Hard, brownish green calli emerging from the proximal and distal ends of lTCL, few in number
1.0	0.2	53.3cd	Hard, brownish red, green calli emerging from the distal end of lTCL, few in number
1.0	0.5	13.3e	Brown, very few in number
1.0	1.0	46.7d	Hard, brown calli emerging from the proximal and distal ends of lTCL, very few in number
1.0	2.0	80.0b	Hard, brownish yellow, green calli emerging from the distal end of lTCL and scattering on explant surface
0.1	1.0	0.0f	Explant died off
0.2	1.0	0.0f	Explant died off
0.5	1.0	0.0f	Explant died off
2.0	1.0	93.3a	Hard, white, brownish yellow calli almost emerging from the distal end of lTCL

Different letters (*) in the same column indicate significantly different means using Duncan's test ($p < 0.05$)

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Table 8: Combinatorial effect of NAA and BA on the morphogenesis of *P. vietnamensis* petiole lTCLs in dark

PGRs (mg/l)		Morphogenesis			Comments
NAA	BA	Callus (%)	Adventitious roots		
			(%)	Roots/explant	
1.0	0.1	86.7c*	80.0b	1.1b	Soft, brownish yellow calli almost emerging from the distal end of lTCL Small, long roots with milk- and transparent-white colors
1.0	0.2	100.0a	93.3a	3.9a	Soft, yellow and brownish yellow calli emerging from the proximal and distal ends of lTCL and the explant surface Thin, long roots with milk- and transparent-white colors
1.0	0.5	93.3b	6.7c	0.1c	Soft, transparent-white and yellow calli, few in number Thin, small, milk-white roots
1.0	1.0	86.7c	0.0d	0.0c	Soft, yellow calli emerging from the distal end of lTCL, few in number
1.0	2.0	86.7c	0.0d	0.0c	Soft, brownish yellow calli emerging from the distal end of lTCL
0.1	1.0	0.0d	0.0d	0.0c	Explant died off
0.2	1.0	0.0d	0.0d	0.0c	Explant died off
0.5	1.0	0.0d	0.0d	0.0c	No callogenesis
2.0	1.0	100.0a	0.0d	0.0c	Friable, soft, milk- and transparent-white calli almost emerging from the proximal and distal ends of lTCL and scattering on the explant surface

Different letters (*) in the same column indicate significantly different means using Duncan's test ($p < 0.05$)

Compared to the combinatorial effect of 2,4-D and BA, and 2,4-D and TDZ, combination of NAA and BA gave the lower rate of callogenesis. Thin cell layer systems could be used as a tool for *in vitro* regeneration and micropropagation. The efficiency is very high compared to conventional techniques of tissue culture. Recent progress in thin cell layer technology has opened new possibilities for improvement of ornamental and floricultural crops.

In this study, various patterns of morphogenesis displayed (callus, shoot, root, somatic embryo) could be induced either separately or in combination when petiole lTCL explants of Vietnamese ginseng were cultured on media supplemented with PGRs alone or in combination in dark, and under light. The results showed that media supplemented with 1.0 mg/l 2,4-D and 0.1 mg/l TDZ in dark, 2.0 mg/l NAA in dark and 1.0 mg/l 2,4-D under light were the most effective culture conditions for callogenesis, embryogenesis and adventitious root formation, and shoot formation, respectively.

Qualitative and quantitative analyses of saponins

Metabolite extracts from Vietnamese ginseng tissue grown in nature (1), and biomass of calli (2) were run on TLC plate along with authentic standards of majonoside-R_2 (MR_2), ginsenosides G-Rb_1 and G-Rg_1 (Table 9, Fig. 2). Results showed that biomass of calli included MR_2, G-Rb_1 and G-Rg_1. Furthermore, metabolite extracted from biomass of calli also had the other bands corresponding to those present in extract from plant grown in nature, suggesting that petiole lTCL-derived calli had similar chemical profile with those in natural environment.

Table 9: Result of the saponin content in petiole lTCL-derived callus sample of *P. vietnamensis*

Weight of sample (mg)	Standard	Peak area	Content of saponin in sample	
			(µg)	(%)
565.3	G-Rg_1	924368 ± 23	0.320324 ± 0.00001	0.061
	MR_2	483832 ± 28	2.213238 ± 0.00013	0.424
	G-Rb_1	378858 ± 25	0.454827 ± 0.00003	0.087

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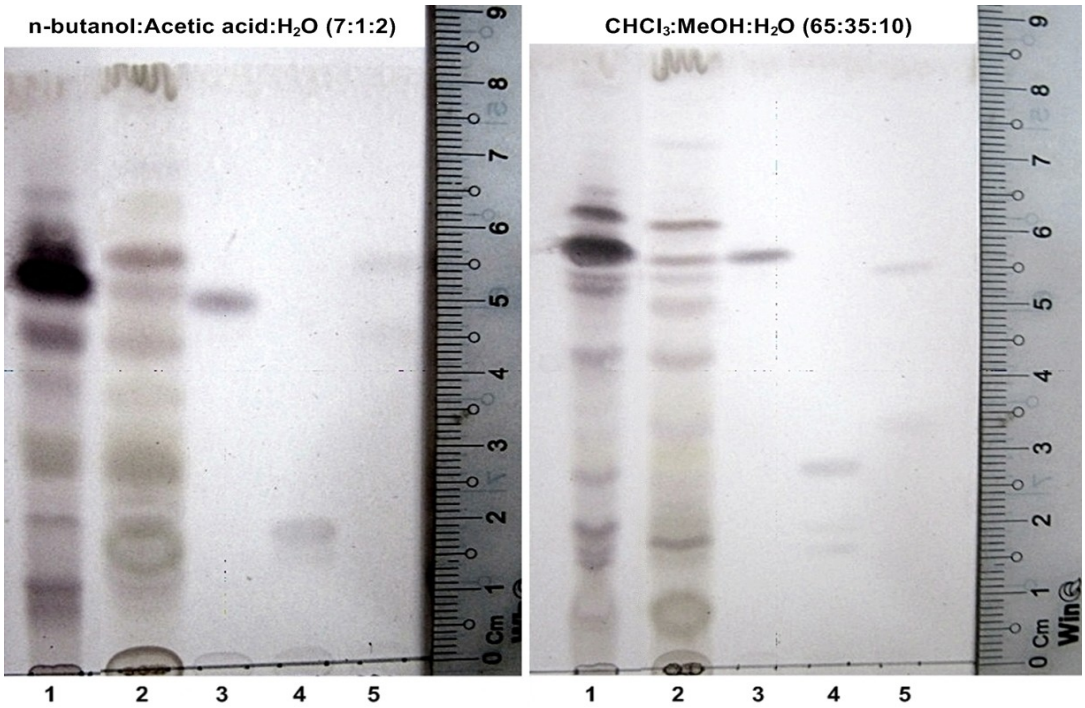

Figure 2: Fractions eluted from petiole lTCL-derived callus sample and reference sample (1) Reference sample (2) Biomass of calli (3) MR_2. 4 G-Rb_1. 5 G-Rg_1

Saponins from *in vitro* Vietnamese ginseng calli were also analyzed using HPLC with photodiode array detector at 190 nm (for MR_2), and 203 nm (for G-Rb_1, and G-Rg_1) (Fig. 3, 4). With authentic saponin standards, HPLC analysis revealed that all three important saponins of Vietnamese ginseng were present in the petiole lTCL-derived calli at high abundance. They included MR_2 (0.424%), G-Rg_1 (0.061%), and G-Rb_1 (0.087%).

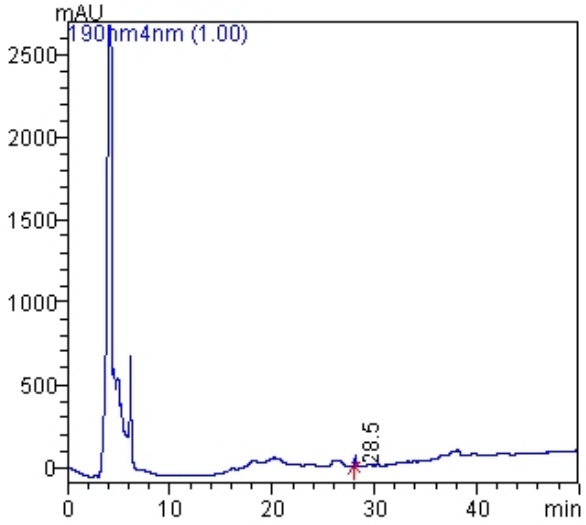

Figure 3: HPLC analysis of *in vitro* Vietnamese ginseng callus with PDA detection at UV wavelength 190 nm

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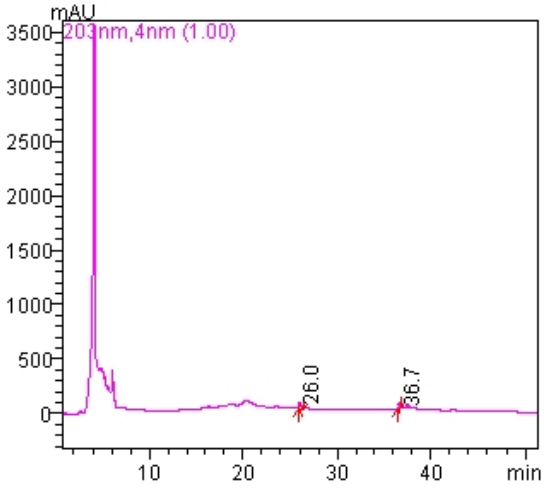

Figure 4: HPLC analysis of *in vitro* Vietnamese ginseng callus with PDA detection at UV wavelength 203 nm

The present study suggests that the lTCL morphogenesis protocol developed is feasible for *in vitro* Vietnamese ginseng, and there is no difference in profile of saponins in the obtained calli compared to that in plants grown in nature.

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Abbreviations

2,4-D 2,4-dichlorophenoxyacetic acid
BA 6-benzylaminopurine
HPLC High-performance liquid chromatography
ITCLs Longitudinal thin cell layers
MS Murashige and Skoog medium
NAA α-napththaleneacetic acid
PGR Plant growth regulator
SH Schenk and Hildebrandt medium
TDZ Thidiazuron
TLC Thin layer chromatography

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