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SOMATIC EMBRYOGENESIS FROM LEAF TRANSVERSE THIN CELL LAYER DERIVED-CALLUS OF VIETNAMESE GINSENG (*PANAX VIETNAMENSIS* HA *ET* GRUSHV.)

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SUMMARY

No report on plant regeneration via somatic embryogenesis of *P. vietnamensis* has been previously published. In the present study, somatic embryogenesis via callus formation from cultures of leaf transverse thin cell layers (tTCLs) of Vietnamese ginseng (*Panax vietnamensis* Ha *et* Grushv.) was investigated. α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BA) and thidiazuron (TDZ) were added separately and in combination into the culture media. Explant necrosis or low callogenesis rates were observed when 1-mm wide leaf tTCLs were cultured on media with TDZ, BA, 2,4-D or NAA. On the other hand, calli were successfully induced from the tTCL explants cultured on medium supplemented with either 2,4-D and BA or 2,4-D and TDZ. Callogenesis was observed under both light and dark conditions. The highest callogenesis rate (100%) was obtained on Murashige and Skoog (MS) basal medium supplemented with 1.0 mg l^{-1} 2,4-D in combination with 0.1 mg l^{-1} TDZ in darkness after eight weeks of culture. White calli were cut into small pieces (1.0 x 1.0 cm dimension) and placed on MS media containing 1.0 mg l^{-1} 2,4-D, 0.5 mg l^{-1} NAA and TDZ at various concentrations (0.01; 0.1; 0.2; and 0.5 mg l^{-1}), and the best callus proliferation was recorded on medium containing 1.0 mg l^{-1} 2,4-D and 0.2 mg l^{-1} TDZ. Somatic embryogenesis, with a success rate of 53.3% and 35 embryos per explant, was achieved when calli were subcultured onto MS medium supplemented with 1.0 mg l^{-1} 2,4-D, 0.5 mg l^{-1} NAA and 0.2 mg l^{-1} TDZ.

Keywords: *Callogenesis, Panax vietnamensis, somatic embryos, thin cell layers*

INTRODUCTION

Ginseng is a medicinal herb that has long been used in the Far East (*Eleutherococcus senticosus*), America (*Panax quinquefolius*), and in particular Korea and China (*Panax ginseng*) as a respected herbal medicine in maintaining physical vitality. Vietnamese ginseng (*Panax vietnamensis* Ha *et* Grushv., 1985) was found in the central highlands of Vietnam in 1973, and was regarded as a new species belonging to the genus *Panax*.

Investigations of the metabolite constituents of *P. vietnamensis* have identified various chemical constituents including 49 saponins, in which 25 saponins are common to other *Panax* species and 24 new saponins are unique for *P. vietnamensis*, named vina-ginsenoside R_1 to R_{24}. In addition, an extremely high concentration of ocotillol saponins is present, and in particular, majonoside-R_2 ocupies 5.3% of the dried rhizome weight (Duc *et al*., 1999). The main active compounds of *P. vietnamensis* are ginsenosides (Yamasaki, 2000), which have a variety of beneficial effects, including free radical scavenging (Huong *et al*., 1998), anticancer effects (Konoshima *et al*., 1999) and suppressive effects of psychological stress (Yobimoto *et al*., 2000).

The current supply of *P. vietnamensis* is fleeting and this has been attributed to the plant's narrow habitat range, slow growth rate and over-harvesting. Therefore, *P. vietnamensis* has been designated an endangered species (red Data Book of Vietnam, 1996).

One of the most practical and efficient ways to solve the current supply dilemma is to produce plantlets *in vitro* on a large-scale. Our previous report, however, showed that *in vitro* propagation of this species is still limited due to the complicated transplantation process and low survival rate of plantlets after being transferred to *ex vitro* conditions (Nhut *et al*., 2010).

Somatic embryogenesis is used as a tool for micropropagation of herbaceous plants, including ginseng (Monteiro *et al*., 2002). There have been a number of studies on somatic embryogenesis of *P. ginseng* and *P. quinquefolius* (Chang, Hsing, 1980; Choi *et al*., 1982; Shoyama *et al*., 1988; Lee *et al*., 1990; Arya *et al*., 1991; Kishira *et al*., 1992; Jiu 1992; Arya *et al*., 1993; Benkrima *et al*., 1994; Wang, 1990; Tirajoh, Punja, 1994; Nhut *et al*., 2011). However, to the best of our knowledge, no report on plant regeneration via somatic embryogenesis of *P. vietnamensis* has been published.

The aim of the current study was to create an *in vitro* protocol for somatic embryogenesis of *P. vietnamensis* from callus cultures of tTCL.

MATERIAL AND METHODS

Callus induction

Vietnamese ginseng plants grown for three months on MS (Murashige, Skoog 1962) medium supplemented with 2.0 mg l^{-1} BA, and 1.0 mg l^{-1} NAA (Chien *et al*., 2011) were used as the source of explants (Fig. 1a). The selected plants were vitrification–free with healthy leaves and shoots. tTCLs of 1 mm in width were cut from *in vitro* leaves as initial explants and used for callus induction. Plant growth regulators (PGRs) including NAA, 2,4-D, BA and TDZ were added separately and in combination into culture media for different experiments.

Callus proliferation

Calli formation stage were cultured in MS media supplemented with 0.2 mg l^{-1} TDZ and different concentrations mg l^{-1} of the auxins 2,4-D, indole-3-butyric acid (IBA) and NAA with different concentrations (0.5; 1.0; 2.0; 3.0; and 5.0 mg l^{-1}) in a 16 hours/day photoperiod. After 8 weeks of culture, the white calli were used as primary explants to establish embryogenic cultures.

Embryogenesis

White calli derived from *in vitro* leaves were cut into small pieces (1.0 x 1.0 cm dimension) and placed on MS media containing 1.0 mg l^{-1} 2,4-D, 0.5 mg l^{-1} NAA and TDZ at various concentrations (0.01; 0.1; 0.2; and 0.5 mg l^{-1}).

Culture condition and statistical analysis

All experiments were in triplicate and each replicate with 15 explants in five culture vessels per replicate and under environment. 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.

The data obtained from the present investigation were subjected to analysis of variance (ANOVA) and Duncan Multiple Range Test (Duncan 1995) at $p < 0.05$ was carried out to determine differences in the means using SPSS Software package (SPSS version 16.0)

RESULTS AND DISCUSSION

Callus induction

TCL technology originated almost 30 years ago with the controlled development of various organs on tobacco pedicel (Tran Thanh Van, 1973). tTCLs have been successfully used in the micropropagation of vegetable, leguminous, and medicinal plants, including *Amaranthus edulis* (amaranth), *Beta vulgaris* (sugar beet), *Brassica napus* (oilseed rape), *Lupinus* spp. (lupin), *Panax ginseng* (ginseng), and *Phaseolus vulgaris* (common bean) (Nhut *et al*., 2003b); cereals and grasses, including *Digitaria sanguinalis* (large crabgrass), *Oryza sativa* (rice), *Sorghum bicolor* (sorghum), and *Zea mays* (corn) (Nhut *et al*., 2003a); fruits, including *Musa* sp. (banana), *Citrus* spp. (orange, lemon, mandarin), *Poncirus trifoliata* (trifoliate orange), *Cocos nucifera* (coconut palm), *Garcinia mangostana* (mangosteen), *Lycopersicon esculentum* (tomato) (Nhut *et al*., 2003c); woody plants, including *Bambusa* spp. And *Dendrocalamus* spp. (bamboo), *Manihot esculenta* (cassava), *Pinus radiata* (Monterey pine), *Paulownia fortunei* (paulownia), *Populus* spp. (poplar), *Pseudotsuga manziesii* and *Sequoiadendron* spp. (conifers), *Garcinia mangostana* (garcinia/kokum), and *Rosa* spp. (rose) (Nhut *et al*., 2003c; 2003d).

The tTCls have also been successfully applied to *Lilium longiflorum* (Bui *et al*., 1999) or *Oryza sativa* L. (Nhut *et al*., 2000). This culture system proved to be more efficient than other *in vitro* culture methods with regard to the total output of plantlets in several plant species (Lakshmanan *et al*., 1995). In order to obtain rapid plant regeneration, the tTCL culture method was exploited for somatic embryogenesis from leaf derived-callus of *P. vietnamensis* Ha *et* Grushv.

Table 1. Effect of PGRs on the callogenesis of *P. vietnamensis* leaf tTCLs after 8 weeks of culture under 16-h photoperiod.

PGRs (mg l^{-1})				Callogenesis (%)	Comments on callus appearance
TDZ	BA	2,4-D	NAA		
-	-	-	-	0.0^{e}*	Necrosis
0.01	-	-	-	0.0^{e}	Necrosis
0.05	-	-	-	0.0^{e}	Necrosis
0.10	-	-	-	0.0^{e}	Necrosis
0.20	-	-	-	0.0^{e}	Necrosis
0.50	-	-	-	0.0^{e}	Necrosis
1.00	-	-	-	0.0^{e}	Necrosis
-	0.1	-	-	0.0^{e}	Necrosis
-	0.2	-	-	0.0^{e}	Necrosis
-	0.5	-	-	0.0^{e}	Necrosis
-	1.0	-	-	0.0^{e}	Necrosis
-	2.0	-	-	0.0^{e}	Necrosis
-	-	0.1	-	0.0^{e}	Necrosis
-	-	0.2	-	6.7^{d}	Small, brownish yellow, hard, and very few in number
-	-	0.5	-	13.3^{c}	Transparent white, and soft
-	-	1.0	-	46.7^{a}	Milk white, yellow, and friable
-	-	2.0	-	40.0^{b}	Transparent yellow, and soft
-	-	-	0.1	0.0^{e}	Necrosis
-	-	-	0.2	0.0^{e}	Necrosis
-	-	-	0.5	0.0^{e}	No callogenesis
-	-	-	1.0	40.0^{b}	Brownish red, and very few in number
-	-	-	2.0	40.0^{b}	Brownish red, hard, and very few in number

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

Table 2. Effect of PGRs on the callogenesis of *P. vietnamensis* leaf tTCLs after 8 weeks of culture under total darkness.

PGRs (mg l^{-1})				Callogenesis (%)	Comments on callus appearance
TDZ	BA	2,4-D	NAA		
-	-	-	-	0.0^{e}*	Necrosis
0.01	-	-	-	0.0^{e}	Necrosis
0.05	-	-	-	0.0^{e}	Necrosis
0.10	-	-	-	0.0^{e}	Necrosis
0.20	-	-	-	0.0^{e}	Necrosis
0.50	-	-	-	0.0^{e}	Necrosis
1.00	-	-	-	0.0^{e}	Necrosis
-	0.1	-	-	0.0^{e}	Necrosis
-	0.2	-	-	0.0^{e}	Necrosis
-	0.5	-	-	0.0^{e}	Necrosis
-	1.0	-	-	0.0^{e}	Necrosis
-	2.0	-	-	0.0^{e}	Necrosis
-	-	0.1	-	0.0^{e}	Necrosis
-	-	0.2	-	0.0^{e}	No callogenesis
-	-	0.5	-	13.3^{d}	Transparent white, soft, and very few in number
-	-	1.0	-	33.3^{c}	Milk white, yellow, and soft
-	-	2.0	-	66.7^{a}	Milk-, transparent-white, and friable
-	-	-	0.1	0.0^{e}	No callogenesis
-	-	-	0.2	0.0^{e}	No callogenesis
-	-	-	0.5	0.0^{e}	No callogenesis
-	-	-	1.0	46.7^{b}	Brownish yellow, hard, and few in number
-	-	-	2.0	33.3^{c}	Brownish yellow, hard, and few in number

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

Explants from *P. vietnamensis* leaf tTCL explants were necrotic when cultured on PGR-free medium and media containing different concentrations of TDZ (0.01-1.0 mg l^{-1}) or BA (0.1-2.0 mg l^{-1}) under either 16-h photoperiod or total darkness. tTCLs cultured on media supplemented with different concentrations of 2,4-D (0.2-2.0 mg l^{-1}) and NAA (1.0-2.0 mg l^{-1}) resulted in callogenesis stemming from the edges of explants (Table 1, 2).

Soft friable and hard non-friable calli, were obtained on media supplemented with 2,4-D and NAA, respectively. The highest rate of callogenesis was obtained on medim supplemented with 2.0 mg l^{-1} 2,4-D under total darkness (66.7%). 2,4-D is usually the most effective auxin for callus induction of species belonging to the genus *Panax* (Choi *et al*., 1994). Our result also support the conclusion that in the present study, after 8 weeks of culture, 2,4-D was the most effective PGR at promoting callus induction. NAA also induced callus formation while media containing TDZ and BA resulted in necrotic explants.

After 8 weeks of culture, under both 16-h photoperiod and total darkness *P. vietnamensis* leaf tTCL explants regardless under light or dark conditons cultured on media supplemented with 2,4-D in combination with BA induced callus formation. Initial callus tissue emerged from the edges of explants followed by the surface. 16-h photoperiod callogenesis rates were similar to those under total darkness, and six out of eighteen treatments gave callogenesis rate of 100% (Table 3, 4).

Table 3. Effect of 2,4-D and BA on the callogenesis of *P. vietnamensis* leaf tTCLs under 16-h photoperiod.

PGRs (mg l^{-1})		Callogenesis (%)	Callus charateristics
2,4-D	**BA**		
1.0	0.1	100.0^{a}*	Greenish white, and hard
1.0	0.2	100.0^{a}	Greenish yellow, and hard
1.0	0.5	93.3^{b}	Milk white, yellow, and friable
1.0	1.0	93.3^{b}	Greenish yellow, yellow, hard, and few in number
1.0	2.0	90.0^{b}	Bright yellow, friable, and few in number
0.1	1.0	46.7^{e}	Brownish yellow, and very few in number
0.2	1.0	60.0^{d}	Green, brownish yellow, hard, and very few in number
0.5	1.0	80.0^{c}	Brownish yellow, hard, and few in number
2.0	1.0	100.0^{a}	Milk white, yellow, and friable

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 callogenesis of *P. vietnamensis* leaf tTCLs under total darkness.

PGRs (mg l^{-1})		Callogenesis (%)	Callus charateristics
2,4-D	**BA**		
1.0	0.1	90.0^{b}*	Milk white, yellow, and friable
1.0	0.2	100.0^{a}	Milk white, and friable
1.0	0.5	100.0^{a}	Milk-, transparent-white, and friable
1.0	1.0	100.0^{a}	Milk-, transparent-white, and friable
1.0	2.0	93.3^{b}	Brownish yellow, soft, and few in number
0.1	1.0	73.3^{d}	Brownish yellow, hard, and few in number
0.2	1.0	80.0^{c}	Brownish yellow, hard, and few in number
0.5	1.0	93.3^{b}	Milk-, transparent-white, and friable
2.0	1.0	93.3^{b}	Small, white, brownish red, and soft

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

Among them, the maximum number of callus induction was achieved from explants cultured on media supplemented with 1.0 mg l^{-1} 2,4-D and 0.2 mg l^{-1} BA under 16-h photoperiod (data not show). Explants cultured under 16-h photoperiod induced green hard calli, while milk, transparent-white and brownish yellow friable calli were observed when explants were maintained under dark conditions.

Eleven of the eighteen media treatments supplemented with 1.0 mg l^{-1} 2,4-D in combination with various concentrations of TDZ (0.01-1.0 mg l^{-1}) under 16-h photoperiod, and in the darkness with various concentrations of TDZ (0.01-0.5 mg l^{-1}) gave callogenesis rates of 100% (Table 5, 6).

Table 5. Combinatorial effect of 2,4-D and TDZ on the callogenesis of *P. vietnamensis* leaf tTCLs under 16-h photoperiod.

PGRs (mg l^{-1})		Callogenesis (%)	Comments on callus appearance
2,4-D	TDZ		
1.0	0.01	100.0[a]*	White, yellow, and friable
1.0	0.05	100.0[a]	Greenish white, brownish yellow, and hard
1.0	0.10	100.0[a]	Greenish white, reddish yellow, and hard
1.0	0.20	100.0[a]	Greenish white, yellow, soft, and few in number
1.0	0.50	100.0[a]	Greenish white, reddish yellow, hard, and few in number
1.0	1.00	100.0[a]	White, brownish yellow, and friable
0.1	0.20	80.0[b]	White and friable, green and hard, and few in number
0.2	0.20	80.0[b]	Green, hard, and few in number
0.5	0.20	100.0[a]	White, brown, and friable
2.0	0.20	73.3[c]	Greenish white, brownish yellow, and soft

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 callogenesis of *P. vietnamensis* leaf tTCLs under total darkness.

PGRs (mg l^{-1})		Callogenesis (%)	Comments on callus appearance
2,4-D	TDZ		
1.0	0.01	100.0[a]*	Brownish yellow, and friable
1.0	0.05	100.0[a]	Milk white, yellow, and friable
1.0	0.10	100.0[a]	Milk white, and friable
1.0	0.20	100.0[a]	White, brownish yellow, and few in number
1.0	0.50	100.0[a]	Milk-, transparent-white, and friable
1.0	1.00	86.7[c]	Milk white, brownish yellow, soft, and few in number
0.1	0.20	80.0[d]	Transparent white, brown, soft, and few in number
0.2	0.20	93.3[b]	White, brown, and soft
0.5	0.20	93.3[b]	Milk white, brownish yellow, friable, and few in number
2.0	0.20	0.0[e]	Necrosis

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

In comparison with media containing 2,4-D and BA, media supplemented with 2,4-D and TDZ promoted greater callus induction (data not show). Darkness was as suitable as light for callogenesis, however calli produced under 16-h photoperiod were green and hard, while explants they were white, yellow and friable calli in the darkness. Under total darkness, medium containing 1.0 mg l^{-1} 2,4-D and 0.1 mg l^{-1} TDZ yielded milk white friable calli emerging from

the edges (Fig 1b), and was the most suitable for callogenesis with the maximum callus induction (data not show).

NAA combined with BA was less effective at inducing callogenesis compared with 2,4-D and BA or 2,4-D and TDZ. Explants were necrotic in six of eighteen treatments, and callogenesis was not observed in two other treatments even though explants were still green (Table 7, 8).

Table 7. Combinatorial effect of NAA and BA on the callogenesis of *P. vietnamensis* leaf tTCLs under 16-h photoperiod.

PGRs (mg l^{-1})		Callogenesis (%)	Comments on callus appearance
NAA	BA		
1.0	0.1	13.3^{c*}	Green, hard, and very few in number
1.0	0.2	0.0^{d}	Necrosis
1.0	0.5	0.0^{d}	Necrosis
1.0	1.0	33.3^{b}	Brown, and very few in number
1.0	2.0	0.0^{d}	No callogenesis
0.1	1.0	0.0^{d}	Necrosis
0.2	1.0	0.0^{d}	Necrosis
0.5	1.0	0.0^{d}	Necrosis
2.0	1.0	60.0^{a}	Green, hard, and very few in number

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

Table 8. Combinatorial effect of NAA and BA on the callogenesis of *P. vietnamensis* leaf tTCLs under total darkness.

PGRs (mg l^{-1})		Callogenesis (%)	Comments on callus appearance
NAA	BA		
1.0	0.1	40.0^{d*}	Transparent white, brownish yellow, soft, and few in number
1.0	0.2	33.3^{e}	Brown, and very few in number
1.0	0.5	53.3^{c}	Brown, hard, and few in number
1.0	1.0	93.3^{b}	White, brownish yellow, and soft
1.0	2.0	13.3^{f}	Brownish yellow, and few in number
0.1	1.0	0.0^{g}	No callogenesis
0.2	1.0	0.0^{g}	Necrosis
0.5	1.0	90.0^{b}	Greenish white, brownish yellow, hard, and few in number
2.0	1.0	100.0^{a}	White, brown, and soft

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

Total darkness was more suitable to callus formation than the 16-h photoperiod (Table 7, 8), and explants cultured on media supplemented with 2.0 mg l^{-1} NAA and 1.0 mg l^{-1} BA under total darkness gave the best rate of callogenesis (100%), while 60% was achieved on the same media formulation under 16-h photoperiod. Calli emerged from the edges of explants and were few in number.

The rate of callogenesis was increased when using one auxin in combination with one cytokinin, and this was apparent in media supplemented with 2,4-D in combination with TDZ, which was the most suitable combination for callus formation. This result is consistent with callus formation in *P. ginseng* and *P. quinquefolius, which was* most successful on MS media supplemented with 2,4-D in combination with kinetin (KIN) or with BA (Furuya *et al*., 1986; Wang 1990; Jiu, 1992).

Previous studies reported that dark conditions are the most suitable for callogenesis in species

belonging to the genus *Panax* (Furuya *et al*., 1986; Wang 1990; Choi *et al*., 1994; Tirajoh, Punja 1994). In this study, explants cultivated under dark and light conditions induced callus formation. No significant difference ($p > 0.05$) in the rate of callus initiation was observed in cultures incubated under total darkness compared with 16-h photoperiod. The calli formed under total darkness were milk, transparent-white to white, and brownish yellow to brown in color while calli induced under 16-h photoperiod were white to greenish white and green, and yellow to brownish yellow in color. Under dark conditions, two types of calli were formed: hard calli, and soft and friable calli whereas the 16-h photoperiod conditions yielded mostly hard and friable calli.

Callus proliferation

Auxin/cytokinin ratio is important for growth of cells *in vitro* (Rita *et al*., 1991). In the present work, three sets of treatments were explored to study the combined effect of auxins and cytokinins on callus proliferation. Calli derived from leaf tTCLs of Vietnamese ginseng were sub-cultured on media supplemented with 2,4-D, IBA and NAA at either 0.5, 1.0, 2.0, 3.0 or 5.0 mg l^{-1} in combination with TDZ at 0.2 mg l^{-1}. Callus pieces continued to proliferate on all tested media and produced fresh biomass between 0.5 to 0.8 g and a dry biomass between 0.035 to 0.066 g from the initial inoculum of approximately 0.2 g callus after 4 weeks of culture (Table 9).

Most of the media containing 2,4-D stimulated higher callus induction than those with IBA or NAA (Table 9). Callus exhibited good growth on the medium supplemented with 1.0 mg l^{-1} 2,4-D with approximately 4-fold fresh weight increase after 4 weeks of culture (Table 9). The higher concentration of 2,4-D (5 mg l^{-1}) was not suitable for callus growth.

Our results also showed that the combination of TDZ and auxins, especially 2,4-D, significantly improved the callus growth of *P. vietnamensis*. TDZ is classified as a type of cytokinin; however, it has shown both auxin and cytokinin like effects to induce and maintain a number of biological events in cells (Guo *et al*., 2011). It is thought that TDZ enhances the accumulation and transport of auxin in cultured tissues.

Table 9. Effect of the combinations of 0.2 mg l^{-1} TDZ and 2,4-D, IBA or NAA on callus proliferation of *P. vietnamensis* after 4 weeks of culture.

PGRs (mg l^{-1})			Final fresh weight – FW (mg)	Dry weight - DW (mg)
2,4-D	**IBA**	**NAA**		
-	-	-	424^{ij*}	31.4^{h}
0.5	-	-	584^{cde}	43.3^{ef}
1.0	-	-	809^{a}	66.2^{a}
2.0	-	-	711^{b}	52.4^{b}
3.0	-	-	508^{fghi}	36.6^{g}
5.0	-	-	493^{ghi}	34.6^{gh}
-	0.5	-	474^{ij}	45.6^{cde}
-	1.0	-	532^{efgh}	48.6^{bcd}
-	2.0	-	631^{c}	49.5^{bc}
-	3.0	-	552^{def}	41.1^{f}
-	5.0	-	531^{efgh}	35.3^{gh}
-	-	0.5	485^{hi}	41.2^{f}
-	-	1.0	548^{defg}	45.0^{def}
-	-	2.0	588^{cd}	46.6^{cde}
-	-	3.0	602^{cd}	45.7^{cde}
-	-	5.0	720^{b}	51.6^{b}

Initial fresh weight was 205 ± 8 mg. Different letters (*) in the same column indicate significantly different means using Duncan's test ($p < 0.05$).

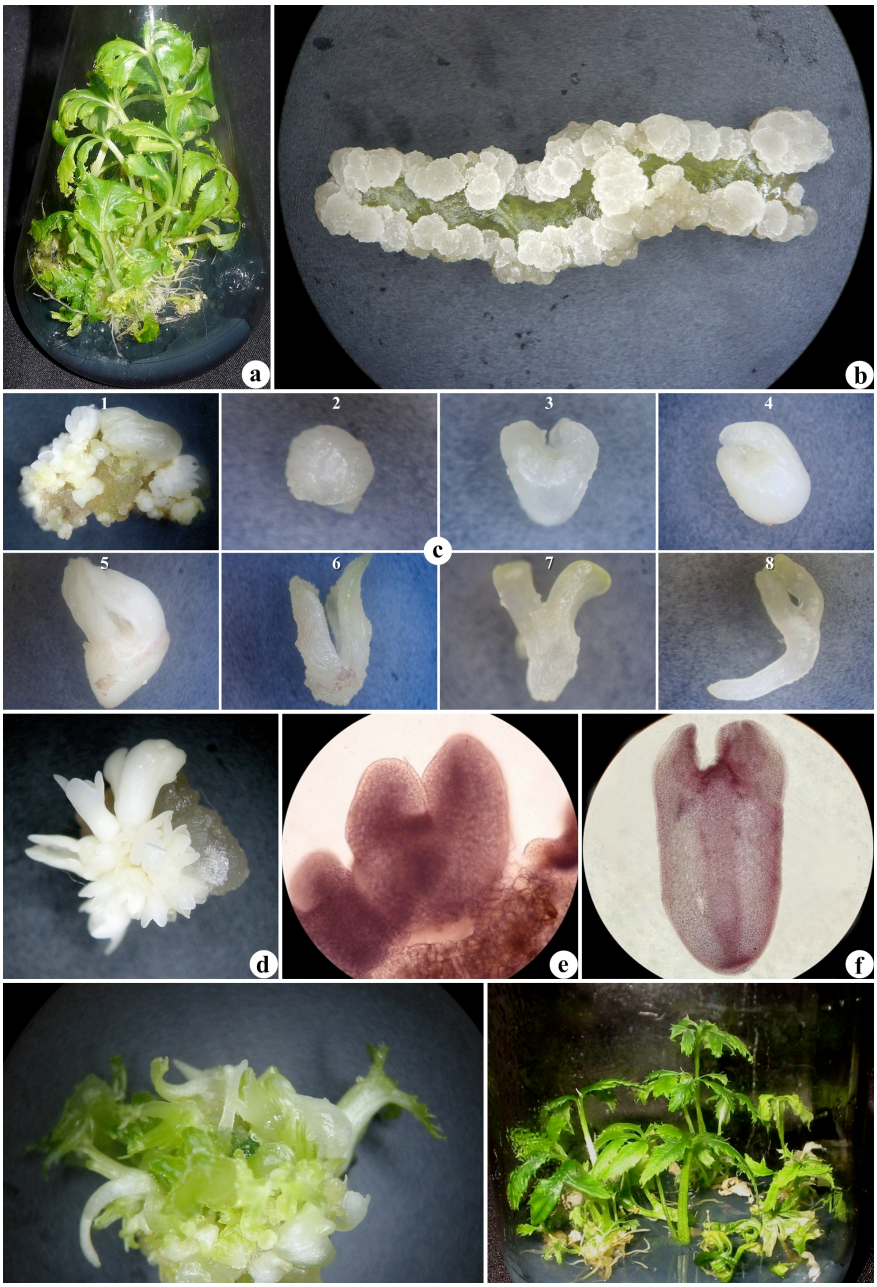

Figure 1. Somatic embryogenesis from leaf tTCLs derived-callus of *P. vietnamensis*. **a** 3-month-old *in vitro* plantlets, **b** Callus formation, **c** Somatic embryogenesis (1 Embryo cluster, 2 Global shape, 3, 4, 5 Heart shape, 6 Cotyledonary, 7, 8 Embryos with roots), **d** Embryo cluster, **e**, **f** Embryo structure, **g**, **h** Embryo germinating.

Embryogenesis

PGRs are required for induction of embryogenesis; and the most commonly-used PGRs for this purpose are 2,4-D, dicamba and picloram (Roostika, Mariska, 2003). Investigations on somatic embryogenesis of *Panax* species showed that synthetic auxins added to the culture media had an important role. Among all the growth regulators

evaluated, 2,4-D gave the highest frequency of callus and somatic embryo formation in *Panax ginseng* (Arya *et al*., 1993; Chang, Hsing 1980; Shoyama *et al*., 1987; Zhong, Zhong 1992).

Somatic embryogenesis could be further improved when other PGRs were added to medium containing 2,4-D, such as KIN (Choi *et al*., 1984; Furuya *et al*., 1986; Lee *et al*., 1989) or NAA (Wang *et al*., 1999). In the present study, the combinations of 2,4-D (1.0 mg l^{-1}), NAA (0.5 mg l^{-1}) and TDZ at various concentrations were tested. Table 10 summarizes the response, which shows that 0.2 mg l^{-1} TDZ in combination with 1.0 mg.l^{-1} 2,4-D and 0.5 mg.l^{-1} NAA had a maximum effect on somatic embryogenesis of *P. vietnamensis*. On this medium, small globular, glossy somatic embryos started to appear from the upper surface of callus mass (Fig. 1c, 1d, 1e, 1f) and these embryos developed into normal plantlets on PGR-free MS medium (Fig. 1g, 1h).

Table 10. Effect of 1.0 mg l^{-1} 2,4-D in combination with 0.5 mg l^{-1} NAA and various concentration of TDZ on somatic embryogenesis of *P. vietnamensis*.

TDZ (mg l^{-1})	Embryogenesis (%)	Number of embryos/explant	Stages of embryos development
-	17.7^{c*}	12^{d}	Globular
0.01	21.0^{c}	16^{c}	Globular
0.1	40.0^{b}	21^{b}	Globular, heart shape
0.2	53.3^{a}	35^{a}	Globular, heart shape
0.5	46.7^{ab}	17^{c}	Globular

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

CONCLUSION

In summary, the present study outlines a protocol for somatic embryogenesis of *P. vietnamensis* Ha *et* Grushv. from leaf tTCL explants. Our results showed that calli were successfully induced from the leaf tTCL explants cultured on medium supplemented with either 2,4-D and BA or 2,4-D and TDZ. Callogenesis was observed under both light and dark conditions. The best results were obtained with MS media supplemented with 1.0 mg l^{-1} 2,4-D and 0.1 mg l^{-1} TDZ under total darkness. Callus proliferation could be obtained on MS media containing 1.0 mg l^{-1} 2,4-D and 0.2 mg l^{-1} TDZ. These calli were sub-cultured onto MS media supplemented with 1.0 mg l^{-1} 2,4-D, 0.5 mg l^{-1} NAA and 0.2 mg l^{-1} TDZ to induce somatic embryogenesis. This technique could be used as a tool for large scale micropropagation of *P. vietnamensis*.

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SỰ HÌNH THÀNH PHÔI VÔ TÍNH TỪ MÔ SẸO CÓ NGUỒN GỐC TỪ LỚP MỎNG TẾ BÀO LÁ CẮT NGANG Ở SÂM VIỆT NAM (*PANAX VIETNAMENSIS* HA *ET* GRUSHV.)

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TÓM TẮT

Đến nay, chưa có báo cáo nào công bố về sự tái sinh cây thông qua quá trình phát sinh phôi vô tính ở sâm Việt Nam. Trong nghiên cứu này, khả năng phát sinh phôi vô tính gián tiếp qua sự hình thành mô sẹo từ việc nuôi cấy mẫu lớp mỏng tế bào cắt ngang của lá (tTCL) ở sâm Việt Nam (*Panax vietnamensis* Ha *et* Grushv.) đã được tiến hành nghiên cứu. Các chất điều hòa sinh trưởng hực vật α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BA) và thidiazuron (TDZ) đã được bổ sung ở dạng riêng lẻ hoặc kết hợp vào môi trường nuôi cấy. Mẫu cấy bị hoại tử hoặc tỷ lệ tạo mô sẹo thấp đã được ghi nhận khi các mẫu tTCL với độ rộng 1 mm được nuôi cấy trên môi trường có bổ sung chỉ một chất điều hòa sinh trưởng thực vật. Trong khi, việc cảm ứng hình thành mô sẹo thành công từ các mẫu được nuôi cấy trên môi trường có bổ sung 2,4-D và BA hoặc 2,4-D kết hợp với TDZ. Sự hình thành mô sẹo đã được ghi nhận cả dưới điều kiện chiếu sáng và trong điều kiện tối. Tỷ lệ hình thành mô sẹo cao nhất (100%) thu được trên môi trường Murashige và Skoog (MS) có bổ sung 1,0 mg l^{-1} 2,4-D kết hợp với 0,1 mg l^{-1} TDZ trong điều kiện tối sau 8 tuần. Mô sẹo màu trắng được cắt thành những mẫu nhỏ (1,0 x 1,0 cm) và cấy lên môi trường MS có bổ sung 1,0 mg l^{-1} 2,4-D, 0,5 mg l^{-1} NAA và TDZ ở các nồng độ khác nhau (0,01; 0,1; 0,2 và 0,5 mg l^{-1}) và sự tăng sinh mô sẹo tốt nhất đã được ghi nhận trên môi trường có bổ sung 1,0 mg l^{-1} 2,4-D và 0,2 mg l^{-1} TDZ. Sự phát sinh phôi vô tính thành công với tỷ lệ tạo phôi 53,3% và 35 phôi/mẫu cấy đã thu được khi mô sẹo được nuôi cấy trên môi trường MS có bổ sung 1,0 mg l^{-1} 2,4-D, 0,5 mg l^{-1} NAA and 0,2 mg l^{-1} TDZ.

Từ khóa: *Mô sẹo, Panax vietnamensis, phôi vô tính, lớp mỏng tế bào*

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