African Journal of Biotechnology Vol. 11(5), pp. 1084-1091, 16 January, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3186
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Effects of spermidine, proline and carbohydrate sources on somatic embryogenesis from main root transverse thin cell layers of Vietnamese ginseng (*Panax vietnamensis* Ha et. Grushv.)

Nhut, D. T. [1*], Vinh, B. V. T. [2], Hien, T. T. [1], Huy, N. P. [1], Nam, N. B. [1] and Chien, H. X.[1]

[1]Tay Nguyen Institute of Biology, Vietnam Academy of Science and Technology, 116 Xo Viet Nghe Tinh, Dalat, Lam Dong, Vietnam.
[2]Ho Chi Minh City University of Technology, 144/24 Dien Bien Phu, Ho Chi Minh, Vietnam.

Accepted 16 December, 2011

The *in vitro* main roots transverse thin cell layers of Vietnamese ginseng (*Panax vietnamensis* Ha et. Grushv.) were cultured on Murashige and Skoog (MS) medium supplemented with 1.0 mg.l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2 mg.l^{-1} kinetin and 0.5 mg.l^{-1} naphthaleneacetic acid (NAA), 30 g.l^{-1} sucrose and 8.5 g.l^{-1} agar to establish embryogenic culture. The effects of exogenous spermidine and proline on enhancement of somatic embryogenesis was investigated. The results show that spermidine (0.1 mM) and proline (300 mg.l^{-1}) resulted in a high frequency of somatic embryogenesis (93.3% and 86.7%, respectively). To further optimize a culture medium for induction of embryo formation of *P. vietnamensis*, three carbohydrate sources (sucrose, glucose and fructose) at 10 to 60 g.l^{-1} were tested. Among them, glucose and fructose were not suitable for somatic embryogenesis in this species while sucrose at 50 g.l^{-1} produced the highest embryogenic frequency (86.7%) number of embryos per responding explant (167). This study confirmed the importance of spermidine, proline and osmotic potential provided by sucrose in enhancement of somatic embryogenesis.

Key words: *Panax vietnamensis*, somatic embryos, spermidine, proline, carbohydrate.

INTRODUCTION

The word “ginseng” derives from the Chinese term, based on the roots of several distinct species of plants, mainly Korean or Asian ginseng (*Panax ginseng*), Siberian ginseng (*Eleutherococcus senticosus*), and American ginseng (*Panax quinquefolius*). Vietnamese ginseng (*Panax vietnamensis* Ha et Grushv.), which is native to Vietnam, was a new species belong to the genus *Panax*. All of these species belong to the Araliaceae family and have been well known for their various effects on human health.

Chemical studies of the constituents of Vietnamese ginseng have identified 49 saponins, including 25 compounds common to other *Panax* species such as protopanaxadiol and protopanaxatriol saponins, and 24 new compounds named vina-ginsenosid R_1 to R_{24}. In addition, an extremely high concentration of ocotillol saponins, that is, majonoside-R2 (5.3% of the dried rhizome) has been identified (Duc et al., 1999). The main active components of *P. vietnamensis* are ginsenosides (Yamasaki, 2000), which have been shown to have a variety of beneficial effects, including antioxidant (Huong et al., 1998), anticancer (Konoshima et al., 1999) and suppressive effects of psychological stress (Yobimoto et

*Corresponding author. E-mail: duongtannhut@gmail.com. Tel: 84-63-3831056. Fax: 84-63-3831028.

Abbreviations: MS, Murashige and Skoog medium; **NAA,** naphthaleneacetic acid; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **BA,** 6-benzyladenine.

al., 2000).

The demand for market values of *P. vietnamensis* have increased dramatically because of long-term conventional production cycle (5 to 7 years), narrow distribution range and deficiency of plant breeding stock. One of the most practical and efficient ways to solve this problem is to produce large-scale plantlets by vegetative propagation. However, our previous report showed that *in vitro* propagation of this species is still limited due to the complicated transplantation process and low survival percentage 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, especially in ginseng (Monteiro et al., 2002). A number of researches into *P. ginseng* propagation by plant tissue culture and particularly by somatic embryogenesis have been investigated in the early 1980s (Chang and Hsing, 1980; Tirajoh et al., 1998; Choi and Soh, 1994, 1996; Shoyama et al., 1997; Kevers et al., 2000). However, to the best of our knowledge, no report on somatic embryogenesis of *P. vietnamensis* has been established up to now.

In general, somatic embryogenesis is thought to occur in response to modifications of different exogenous and endogenous factors including growth regulators (Steward et al., 1964). Apart from conventional plant hormones, polyamines, mainly spermidine, spermine and putrescine are also involved in enhancing somatic embryogenesis in a variety of plant species, such as *Araucaria angustifolia* (Silveira et al., 2006; Steiner et al., 2007), *Picea rubens* Sarg. (Minocha et al, 2004), *Gossypium hirsutum* L. (Sakhanokho et al., 2005), *Momordica charantia* L. (Paul et al., 2009) and *Panax ginseng* CA Meyer (Monteiro et al., 2002; Parvin et al., 2010). Somatic embryogenesis has also been found to be associated with increases in endogenous levels of amino acids, that is, proline, serine, threonine (Thorpe, 1993). In addition, there was a considerable amount of data on the effect of different exogenous carbohydrate supplies on somatic embryo development (Blanc et al., 1999; Helena and Hana, 2004; Hong et al., 2008).

The objective of this study was to establish an *in vitro* induction of somatic embryos of *P. vietnamensis*, an important medicinal herbal plant of Vietnam. Further investigation on the effects of polyamines and carbohydrate sources on somatic embryogenesis in *P. vietnamensis* was also carried out.

MATERIALS AND METHODS

Callus formation

Main roots of three-month old *in vitro P. vietnamensis* plants cultured on MS (Murashige and Skoog, 1962) medium supplemented with 2.0 mg.l^{-1} BA and 1.0 mg.l^{-1} NAA (Chien et al., 2011) were cut into small, 1-mm thick transverse thin cell layers (tTCLs) and cultured on MS medium supplemented with 1.0 mg.l^{-1} 2,4-D 30 g.l^{-1} sucrose and 8 g.l^{-1} agar (Nhut et al., 2009). After eight weeks of culture, the white calli were used as primary explants to establish embryogenic cultures.

Establishment of embryogenic cultures

Calli derived from *in vitro* main root were cut into small pieces (1.0 x 1.0 cm dimension) and placed on MS medium containing 1.0 mg.l^{-1} 2,4-D in combination with NAA and/or kinetin at various concentrations (0.1, 0.2, 0.5 or 1.0 mg.l^{-1}). After 12 weeks of culture, the frequency of explants producing somatic embryo, number of embryos at different stages, and fresh weight of embryo mass were scored. The optimal embryogenic medium (EM) was used as basal medium for subsequent experiments.

Effects of spermidine, proline, and carbohydrate sources on enhancing the frequency of somatic embryogenesis

For investigating the stimulated effects of exogenously supplied acid amine (proline) or polyamine (spermidine) on somatic embryogenesis of *P. vietnamensis*, the initial calli were cultured on EM media supplemented separately with spermidine (0.01, 0.05, 0.1 or 0.2 mM) or proline (100, 200, 300 or 400 mg.l^{-1}). Proline and spermidine were filter-sterilized and added to the culture media after autoclaving. Different sources of carbohydrate (sucrose, glucose and fructose) at various concentrations (10, 20, 30, 40, 50 or 60 g.l^{-1}) were supplied to EM media to examine the role of carbohydrates on enhancing the frequency of somatic embryogenesis. After 12 weeks of culture, embryo masses were weighed and number of produced somatic embryos was determined.

Culture condition and data analysis

All cultures were incubated at 22 ± 1°C with a 16-h photoperiod at a light intensity of 40 µmol.s^{-1}.m^{-2} fluorescent light. The experiments were triplicated with nine explants per replication. Data were analyzed for significance by analysis of variance with mean separation by Duncan's multiple range test (Duncan, 1995) using Statgraphics Centurion XV (StatPoint Technologies Inc., Warrenton, VA, USA).

Histological studies

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

RESULTS AND DISCUSSION

Effects of plant growth regulators on somatic embryogenesis

Most of previous researches on *Panax* species focused on *P. ginseng*. Several sources of explants were tested for callus and embryos formation, that is roots (Chang

Table 1. Effects of 2,4-D in combination with NAA or kinetin on somatic embryogenesis of *P. vietnamensis.*

Growth regulator concentration (mg.l^{-1})			Embryogenesis (%)	Number of embryos/explant
2,4-D	NAA	Kinetin		
-	-	-	-	-
1.0	0.1	-	26.7 c*	22 cd
1.0	0.2	-	40.0 b	38 b
1.0	0.5	-	60.0 a	52 a
1.0	1.0	-	40.0 b	13 d
1.0	-	0.1	-	-
1.0	-	0.2	30.3 bc	28 c
1.0	-	0.5	13.3 d	15 d
1.0	-	1.0	6.7 d	11 d

*Means followed by the same letter within a column are not significantly different at $P<0.05$ according to Duncan multiple range test..

and Hsing, 1980; Yang, 1992), zygotic embryos (Arya et al., 1993; Lee et al., 1989; Zhong and Zhong, 1992), cotyledons (Choi and Soh, 1994; 1996), leaves (Cellárová et al., 1992) and flower buds (Shoyama et al., 1995). Among all the sources of explants, roots have received the greatest attention because they are easier to obtain and form large callus tissues rapidly. In this work, we used the *in vitro* main roots of *P. vietnamensis* to obtain white calli to be used as primary explants to establish embryogenic cultures.

Investigations on regeneration of *Panax* species through somatic embryogenesis showed that synthetic auxins added to the culture medium had an important role. Among all the growth regulators evaluated, 2,4-D gave the highest frequency of callus and somatic embryo formation in *P. ginseng* (Arya et al., 1993; Chang and Hsing, 1980; Zhong and Zhong, 1992). Somatic embryogenesis could be further improved when kinetin was added to medium containing 2,4-D (Choi et al., 1984; Lee et al., 1989; 1990). Wang et al. (1999) investigated the effects of auxins on somatic embryogenesis of American ginseng (*P. quinquefolius*). Their results showed that the combined effect of 2,4-D and NAA in culture medium gave a better result compared to medium containing only 2,4-D. Moreover, there were no somatic embryo formation when calli were cultured on media supplemented with 2,4-D or NAA alone in *P. vietnamensis* Ha et Grushv. (Nhut et al., 2009). In the present study, the combinations of 2,4-D (1.0 mg.l^{-1}) and NAA or kinetin at various concentrations were tested. Table 1 summarizes the response, which shows that 1.0 mg.l^{-1} 2,4-D plus 0.5 mg.l^{-1} NAA had a maximum favourable effect on somatic embryogenesis.

2,4-D in low concentration is an auxin that especially promotes cell division and dedifferentiation of plant tissues (Borkird et al., 1986). In the present work, the combined effect of 2,4-D and kinetin resulted in a lower percentage of somatic embryogenesis (Table 1). When using 1.0 mg.l^{-1} 2,4-D in combination with NAA at various concentrations (0.2 to 1.0 mg.l^{-1}), high frequency of somatic embryogenesis (40 to 60%) was recorded. The highest number of embryos (52 embryos/explant) was obtained on MS medium supplemented with 1.0 mg.l^{-1} 2,4-D and 0.5 mg.l^{-1} NAA. Similar results were obtained by Zhou and Brown (2006) when studying the effects of auxins on somatic embryogenesis of *P. quinquefolius*. Their results showed that the combination of 1.0 mg.l^{-1} 2,4-D and 1.0 mg.l^{-1} NAA resulted in a higher percentage of explants with embryos compared to 2,4-D alone. Wang et al. (1999) used higher concentrations of 2,4-D and NAA to induce somatic embryogenesis in *P. quinquefolius* and concluded that the optimal concentrations were 1.1 mg.l^{-1} 2,4-D or 2.8 mg.l^{-1} NAA, and when used together, the effect of 2,4-D was independent of the concentrations of NAA.

For further investigating the effects of 2,4-D in combination with NAA and kinetin on somatic embryogenesis of *P. vietnamensis*, we fixed the concentrations of 2,4-D and kinetin at 1.0 mg.l^{-1} and 0.2 mg.l^{-1}, respectively, and combined with NAA at various concentrations. Among all the media evaluated, MS medium supplemented with 1.0 mg.l^{-1} 2,4-D plus 0.2 mg.l^{-1} kinetin and 0.5 mg.l^{-1} NAA resulted in the highest frequency of somatic embryogenesis (80%) as well as the number of embryos induced per explant (117 embryos per explant) (Table 2).

Effects of spermidine on enhancing the frequency of somatic embryogenesis

When spermidine was added to the embryogenic media at high concentrations (0.1 to 0.2 mM), the frequency of embryogenesis as well as number of somatic embryos increased significantly. Among treatments with spermidine, the concentration of 0.1 mM was found to be the most effective in increasing somatic embryogenesis of *P. vietnamensis* (Table 3). However, exogenous spermidine

Table 2. Effects of 2,4-D in combination with NAA and kinetin on somatic embryogenesis of *P. vietnamensis.*

Growth regulator concentration (mg.l^{-1})			Embryogenesis (%)	Number of embryos/explant
2,4-D	NAA	kinetin		
1.0	0.1	0.2	33.3^{c*}	35^{d}
1.0	0.2	0.2	53.3^{bc}	68^{c}
1.0	0.5	0.2	80.0^{a}	117^{a}
1.0	1.0	0.2	60.0^{b}	96^{b}

*Means followed by the same letter within a column are not significantly different at $P<0.05$ according to Duncan multiple range test.

Table 3. Effects of spermidine on somatic embryogenesis of *P. vietnamensis*.

Spermidine (mM)	Embryogenesis (%)	Number of embryo/explant	Fresh weight (g)
0.01	33.3 d*	86 d	29.15 d
0.05	53.3 c	132 c	31.36 c
0.1	93.3 a	353 a	36.75 a
0.2	86.7 b	283 b	33.65 b

*Means followed by the same letter within a column are not significantly different at $P<0.05$ according to Duncan multiple range test.

Table 4. Effects of proline on somatic embryogenesis of *P. vietnamensis*.

Proline (mg.l^{-1})	Embryogenesis (%)	Number of embryos/explant	Fresh weight (g)
100	40.0^{c*}	65^{c}	27.45^{c}
200	66.7^{b}	97^{bc}	29.15^{bc}
300	86.7^{a}	167^{a}	40.55^{a}
400	66.7^{b}	83^{bc}	30.90^{bc}

*Means followed by the same letter within a column are not significantly different at $P<0.05$ according to Duncan multiple range test.

at highest concentration of the experiment (0.2 mM) reduced the capacity of somatic embryogenesis.

Polyamines have been recognized as a new class of growth substances (Bagni and Torrigiani, 1992). Polyamines were able to interact with negatively charged macromolecules as DNA, RNA, proteins and phospholipids, thereby activating and stabilizing these molecules because of their polycationic nature at physiological pH (Evans and Malmberg, 1989). High concentrations of polyamines were commonly observed in tissues undergoing somatic embryogenesis (Santanen and Simola, 1992; Kevers et al., 2000). Spermidine is a small aliphatic polyamine that are ubiquitous in all living cells. Previous works with wild carrot have shown that spermidine can restore embryogenesis in cultures treated with polyamine biosynthesis inhibitors, indicating a direct role of spermidine in somatic embryogenesis (Montague et al., 1978). Since then, a number of studies conducted on various plants have provided evidence showing that spermidine was putatively involved in somatic embryogenesis (Cvikrová et al., 1999; Kevers et al., 2000; Bertoldi et al., 2004).

Related studies with *P. ginseng* have shown that the production of somatic embryos could be increased by adding spermidine (10^{-4} M) to the initiation medium (Kevers et al., 2000). Monteiro et al. (2002) confirmed that among the polyamines, spermidine was the most effective for somatic embryogenesis in *P. ginseng.* They demonstrated a correlation between increased endogenous spermidine and embryogenic capacity.

Effects of proline on enhancing the frequency of somatic embryogenesis

Amino acids played an important role in raising the levels of reduced nitrogen which stimulates the development of somatic embryogenesis in several species (George, 1993). Proline is one of the amino acids shown to have a stimulatory effect on embryogenesis and osmotic tolerance (Shimizu et al., 1997; Hita et al., 2003).

In this work, further experiments were carried out in an attempt to optimize somatic embryos formation by adding proline (100 to 400 mg.l^{-1}) to embryogenic media. Experiments on different concentrations of proline revealed that the highest frequency of somatic embryogenesis occurred at 300 mg.l^{-1} (86.7%). The maximum number of somatic embryos (167 embryos per explant) were also produced at 300 mg.l^{-1} proline (Table 4).

Santos et al. (1996) found that the addition of proline

Table 5. Effects of carbohydrates on somatic embryogenesis of *P. vietnamensis*.

Sources of carbohydrate	Concentration (g.l^{-1})	Embryogenesis (%)	Number of embryos/explant	Fresh weight (g)
Sucrose	10	20.0^{de*}	8^{e}	24.65^{cd}
	20	60.0^{bc}	25de	26.36^{cd}
	30	80.0^{ab}	120^{b}	35.35^{ab}
	40	80.0^{ab}	143^{ab}	37.63^{ab}
	50	86.7^{a}	167^{a}	45.15^{a}
	60	73.3^{b}	96^{bc}	30.76^{abc}
Glucose	10	13.3^{e}	2^{e}	12.55^{d}
	20	13.3^{e}	11^{e}	13.15^{d}
	30	40.0^{c}	26de	14.86^{cde}
	40	53.3^{bc}	53^{cd}	15.05^{cde}
	50	66.7^{bc}	75^{c}	17.86^{cd}
	60	40.0^{bcd}	63^{c}	20.68^{cd}
Fructose	10	-	-	5.74^{e}
	20	13.3^{e}	7^{e}	7.74^{e}
	30	13.3^{e}	14^{e}	6.12^{e}
	40	33.3^{cd}	17de	8.26^{de}
	50	40.0^{c}	35^{d}	9.74^{de}
	60	26.7^{d}	26de	10.53^{de}

*Means followed by the same letter within a column are not significantly different at $P<0.05$ according to Duncan multiple range test.

had a positive effect on the total protein content of embryogenic callus. They thought that the presence of proline in the culture medium seems to produce a required stress condition, decreasing water potential level in plant cell culture medium, increasing the accumulation of nutritional elements in the cells, and finally enhancing somatic embryogenesis (Santos et al., 1996). In previous study, proline was found to give optimum response of both primary and secondary somatic embryogenesis in rose (Marchant et al., 1996). Other publications have mentioned a positive role of proline on embryo formation in maize (Suprasanna et al., 1994), and kodo millet (Vikrant and Rashid, 2002).

Effects of carbohydrate sources on enhancing the frequency of somatic embryogenesis

The influence of three different carbohydrates (sucrose, fructose and glucose) at various concentrations was tested in MS basal medium containing 1.0 mg.l^{-1} 2,4-D, 0.2 mg.l^{-1} kinetin and 0.5 mg.l^{-1} NAA for somatic embryogenesis in *P. vietnamensis*.

Significant differences for embryogenic response were found among three examined carbohydrates. The results show that glucose and fructose in the culture medium were not suitable for somatic embryogenesis in *P. vietnamensis*. Especially, at low concentration of fructose (10 g.l^{-1}), no embryos were obtained after 12 weeks of culture (Table 5). Our results are similar to reports of Sandra et al. (2000). They studied the effects of different carbohydrate sources in the medium on somatic embryogenesis in five *Coffea canephora* Pierre genotypes and found that glucose inhibited embryogenesis initiation for N128 and did not significantly improve embryo production for N91 and N75.

Most studies on using carbohydrate *in vitro* as a supplied energy source have indicated that sucrose is the best carbon source for optimal cell growth (Sandra et al., 2000). Swedlund and Locy (1993) concluded that specific carbohydrates had differential effects on morphogenesis *in vitro* and the osmotic potential provided by these sugars might support embryogenesis.

In the present work, differences were also found for somatic embryogenesis capacity when using sucrose at various concentrations. Low concentrations of sucrose (10 to 20 g.l^{-1}) had been shown to reduce somatic embryogenesis in *P. vietnamensis*. The frequency of embryogenesis and the number of embryos increased with increasing sucrose concentration to a maximum at 50 g.l^{-1} sucrose (Figure 1a) and some embryos were observed under a light microscope (Figure 1b). A further increase to 60 g.l^{-1} or higher concentrations of sucrose had no enhancing effect on embryo formation. Similar results were reported for *Picea abies* (Jain et al., 1988), *P. rubens* (Tremblay and Tremblay, 1991). When embryogenic tissue sections containing groups of 10 to 20 somatic embryos (Figure 1a) were transferred to the free-plant growth regulator MS medium, some embryos germinated within a few days (Figures 1c and d) and complete plantlets were obtained (350 plantlets) (Figures 1e, f, g and h). The well rooted *in vitro* plantlets were

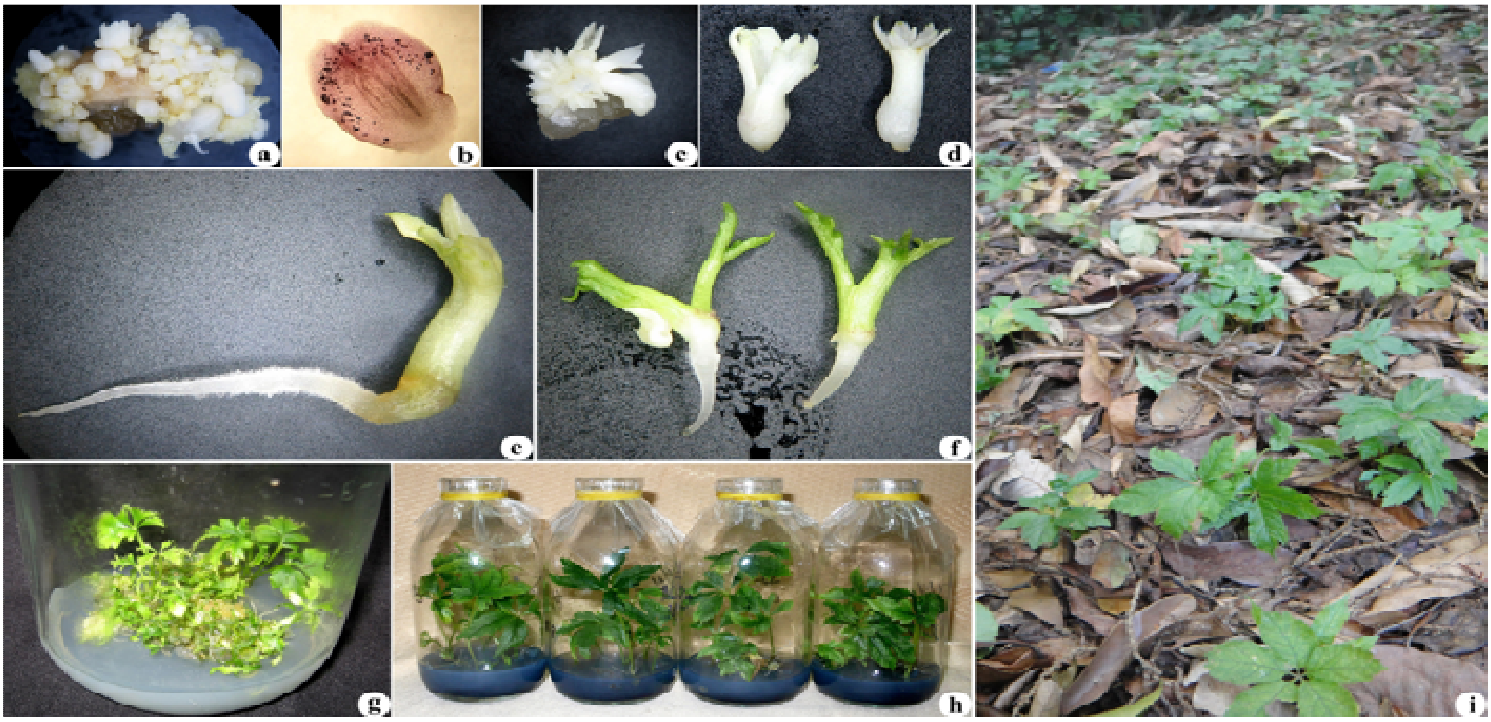

Figure 1. Somatic embryogenesis of Vietnamese ginseng (*Panax vietnamensis* Ha et Grushv.). a, Embryo cluster; b, embryo structure; c, embryo maturation; d, single embryos; e, f, embryo germinating; g, embryo-derived plantlets on MS medium supplemented with 1.0 mg.l^{-1} BA and 1.0 mg.l^{-1} NAA; h, vigorous embryo-derived plantlets after 2 months cultured on MS½ medium supplemented with 0.5 mg.l^{-1} NAA, 1.0 mg.l^{-1} BA and 2.0 mg.l^{-1} activated charcoal; I, *in vitro P. vietnamensis* plantlets after two months of acclimatization.

successfully transferred to soil and their survival rate under natural habitat was 85% (Figure 1i).

Conclusion

The results from this study have demonstrated that somatic embryogenesis in *P. vietnamensis* is controlled by several factors, including types of growth regulator combinations, sources of carbohydrates and other additives (spermidine, proline) supplemented to the medium.

ACKNOWLEDGMENT

The authors would like to thank the National Foundation for Science and Technology Development (Ministry of Science and Technology, Vietnam) for financial support.

REFERENCES

Arya S, Arya ID, Eriksson T (1993). Rapid multiplication of adventitious somatic embryos of *Panax ginseng*. Plant Cell Tiss. Org. Cult. 34: 157-162.

Bagni N, Torrigiani P (1992). Polyamines: a new class of growth substances, In; Karssen CM, van Loon LC, Vreugdenhil D (Eds) Progress in Plant Growth Regulation. Kluwer Academic Publishers, Dordrecht, pp. 264-275.

Bertoldi D, Tassoni A, Martinelli L, Bagni N (2004). Polyamines and somatic embryogenesis in two *Vitis vinifera* cultivars. Physiol. Plant, 120: 657-666.

Blanc G, Ferriere NM, Teisson C, Lardet L, Carron MP (1999). Effect of carbohydrate addition on the induction of somatic embryogenesis in *Hevea brasiliensis*. Plant Cell Tiss. Org. Cult. 59: 103-112.

Borkird C, Choi Jung H, Sung R (1986). Effect of 2,4-D on the expression of embryogenic program in carrot. Plant Physiol. 81: 1143-1146.

Cellárová E, Rychlová M, Vranová E (1992). Histological characterization of *in vitro* regenerated structures of *Panax ginseng*. Plant Cell Tiss. Org. Cult. 30: 165-170.

Chang WC, Hsing YI (1980). Plant regeneration through somatic embryogenesis in root-derived callus of ginseng (*Panax ginseng* C. A. Meyer). Theor. Appl. Genet. 57: 133-135.

Chien HX, Tai NT, Truc NB, Tinh TX, Thao LB, Luan TC, Nhut DT (2011). Effect of some factors to *in vitro* microrhizome formation (*Panax vietnamensis* Ha et Grushv.) and determination of plantlet saponin content in Ngoc Linh mountain. J. Biotechnol. Viet. 8(3B): 1211-1219.

Choi KT, Yang DC, Kim NW, Ahn IO (1984). Redifferentiation from tissue culture and isolation of viable protoplasts in *Panax ginseng C. A.* Meyer. In: Pron. 4th Int. Ginseng Syrup., Korean Ginseng and Tobacco Res. Inst., Korea, pp. 1-11.

Choi YE, Soh WY (1994). Origin of somatic embryo induced from cotyledons of zygotic embryos at various developmental stages of ginseng. J. Plant Biol. 37: 365-370.

Choi YE, Soh WY (1996). Effect of plumule and radicule on somatic embryogenesis in the cultures of ginseng zygotic embryos. Plant Cell Tiss. Org. Cult. 45: 137-143.

Cvikrová M, Binarová P, Cenklová V, Eder J, Macháčková I (1999). Reinitiation of cell division and polyamine and monoamine levels in alfalfa explants during somatic embryogenesis. Physiol. Plant. 105: 330-337.

Duc NM, Kasai R, Yamasaki K, Nham NT, Tanaka O (1999). New

dammarane saponins from Vietnamese ginseng. Stud. Plan. S, 6: 77-82.
Duncan DB (1995). Multiple range and multiple F tests. Biometrics, 11: 1-5.
Evans PT, Malmberg RL (1989). Do polyamines have roles in plant development? Annu. Rev. Plant Physiol. Plant Mol. Biol. 40: 235-269.
George EF (1993). Plant propagation by tissue culture - Part 1. The technology, 2nd edn. Exegetics, Eddington.
Gonzalez AM, Cristóbal CL (1997). Anatomía y ontogenia de semillas de Helicteres Lhatzkyana (*Sterculiaceae*). Bonplandia, 9: 287-294.
Helena L, Hana K (2004). Somatic embryogenesis in conifers: the role of carbohydrate metabolism. *In Vitro* Cell. Dev. Biol. Plant, 40: 23-30.
Hita O, Gallego P, Villalobos N, Lanas I, Blazquez A, Martin JP, Fernandez J, Martin L, Guerra H (2003). Improvement of somatic embryogenesis in *Medicago arborea*. Plant Cell Tiss. Org. Cult. 72: 13-18.
Hong PI, Chen JT, Chang WC (2008). Promotion of direct somatic embryogenesis of *Oncidium* by adjusting carbon sources. Biol. Plant. 52(3): 597-600.
Huong NTT, Matsumoto K, Kasai R, Yamasaki K, Watanabe H (1998). *In vitro* antioxidant activity of Vietnamese ginseng saponin and its components. Biol. Pharm. Bull. 21: 978-981.
Jain SM, Newton RJ, Soltes EJ (1988). Enhancement of somatic embryogenesis in Norway spruce (*Picea abies* L.). Theor. App. Gen. 76: 501-506.
Johansen DA (1940). Plant microtechnique. New York: McGraw Hill Book Co. p. 551.
Kevers C, Le Gal N, Monteiro M, Dommes J, Gaspar TH (2000). Somatic embryogenesis of *Panax ginseng* in liquid cultures: a role for polyamines and their metabolic pathways. Plant Growth Regul. 31: 209-214.
Konoshima T, Takasaki M, Ichiishi E, Murakami T, Tokuda H, Nishino H, Duc NM, Kasai R, Yamasaki K (1999). Cancer chemopreventive activity of majonoside-R2 from Vietnamese ginseng, *Panax vietnamensis*. Cancer Lett. 147(1-2): 11-16.
Lee HS, Lee KW, Yang SG, Jeon JH, Liu JR (1989). Plant regeneration through somatic embryogenesis from mature zygotic embryos of ginseng *(Panax ginseng* C. A. Meyer) and flowering of plantlets. Kor. J. Bot. 32: 145-150.
Luque R, Sousa HC, Kraus JE (1996). Métodos de coloracao de Roeser (1972) e Kropp (1972) visando a subtituicao do azul do astra por azul de alciao 8GS ou 8GX. Acta Bot. Bras. 10: 199-212.
Marchant R, Davey MR, Lucas JA, Power JB (1996). Somatic embryogenesis and plant regeneration in Floribunda rose (*Rosa hybrida* L.) cvs. Trumpeter and Glad Tidings. Plant Sci. 120: 95-105.
Minocha R, Minocha SC, Long S (2004). Polyamines and their biosynthetic enzymes during somatic embryo development in red spruce (*Picea rubens* Sarg.). *In Vitro* Cell Dev. Biol. Plant 40: 572-580.
Montague MJ, Koppenbrink JW, Jaworski EG (1978). Polyamine metabolism in embryogenic cells of *Daucus carota*. Changes in intracellular content and rates of synthesis. Plant Physiol. 62: 430-433.
Monteiro M, Kevers C, Dommes J, Gaspar T (2002). A specific role for spermidine in the initiation phase of somatic embryogenesis in *Panax ginseng* CA Meyer. Plant Cell Tiss. Org. Cult. 68: 225-232.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 472-497.
Nhut DT, Chien HX, Truc NB, Nam NB, Tinh TX, Luan VQ, Binh NV, Hien VT, Huong TT, Nhan NCT, Thuy LNM, Nga LTM, Hien TT, Hai NT (2010). Micropropagation of *Panax vietnamensis* Ha et Grushv. J. Biotechnol. Viet. 8(3B): 1211-1219.
Nhut DT, Luan VQ, Binh NV, Phong PT, Huy BN, Ha DTN, Tam PQ, Nam NB, Hien VT, Vinh BT, Hang LTM, Ngoc DTM, Thao LB, Luan TC (2009). Effect of some factors on *in vitro* biomass of Vietnamese ginseng (*Panax vietnamensis* Ha et Grushv.) and primary analysis of saponin content. J. Biotechnol., Viet. 7(3): 365-378.
Parvin S, Kim YJ, Pulla RK, Sathiyamoorthy S, Miah MG, Kim YJ, Wasnik NG, Yang DC (2010). Identification and characterization of spermidine synthase gene from *Panax ginseng*. Mol. Biol. Rep. 37: 923-932.
Paul A, Mitter D, Raychaudhuri SS (2009). Effect of polyamines on *in vitro* somatic embryogenesis in *Momordica charantia* L. Plant Cell Tiss. Org. Cult. 97: 303-311.
Sakhanokho HF, Ozias-Akins P, May OL, Chee PW (2005). Putrescine enhances somatic embryogenesis and plant regeneration in upland cotton. Plant Cell Tiss. Org. Cult. 81: 91-95.
Sandra RLF, Maria BPC, João MF, Luiz GEV (2000). The effects of silver nitrate and different carbohydrate sources on somatic embryogenesis in *Coffea canephora*. Plant Cell Tiss. Org. Cult. 60: 5-13.
Santanen A, Simola LK (1992). Changes in polyamine metabolism during somatic embryogenesis in *Picea abies*. J. Plant Physiol. 147: 145-153.
Santos MA, Camara T, Rodriguez P, Claparols I, Torné JM (1996). Influence of exogenous proline on embryogenic and organogenic maize callus subjected to salt stress. Plant Cell Tiss. Org. Cult. 47: 59-65.
Shimizu K, Nagaike H, Yabuya T, Adachi T (1997). Plant regeneration from suspension culture of *Iris germanica*. Plant Cell Tiss. Org. Cult. 50: 27-31.
Shoyama Y, Matsushita H, Zhu XX, Kishira H (1995). Somatic embryogenesis in ginseng *(Panax* species), In; Bajaj YPS (Ed) Biotechnology in agriculture and forestry. Somatic embryogenesis and synthetic seed II. Berlin: Springer-Verlag, 31: 343-356.
Shoyama Y, Zhu XX, Nakai R, Shiraishi S, Kohda H (1997). Micropropagation of *Panax notoginseng* by somatic embryogenesis and RAPD analysis of regenerated plantlets. Plant Cell Rep. 16: 450-453.
Silveira V, Santa-Catarina C, Tun NN, Scherer GFE, Handro W, Guerra MP, Floh EIS (2006). Polyamine effects on the endogenous polyamine contents, nitric oxide release, growth and differentiation of embryogenic suspension cultures of *Araucaria angustifolia* (Bert) O Ktze. Plant Sci. 171: 91-98.
Steiner N, Santa-Catarina C, Silveira V, Floh EIS, Guerra MP (2007). Polyamine effects on growth and endogenous hormones levels in *Araucaria angustifolia* embryogenic cultures. Plant Cell Tiss. Org. Cult. 89: 55-62.
Steward EC, Mapes MO, Kent AE, Holsten RD (1964). Growth and development of cultured plant cells. Biochemical and morphogenic studies with cells yield new evidence on their metabolism and totipotency. Science, 143: 20-27.
Suprasanna P, Rao KV, Reddy GM (1994). Embryogenic callus in maize: genotypic and amino acid effects. Cer. Res. Commun. 22: 79-82.
Swedlund B, Locy RD (1993). Sorbitol as the primary carbon source for the growth of embryogenic callus of maize. Plant Physiol. 103: 1339-1346.
Thorpe TA (1993). *In vitro* organogenesis and somatic embryogenesis: physiological and biochemical aspects, In; Roubelakis-Angelakis KA, Tran Thanh Van T (Eds) Morphogenesis in plants. New York: Plenum Publishing Corp.
Tirajoh A, Kyung TS, Punja ZK (1998). Somatic embryogenesis and plantlet regeneration in American ginseng (*Panax quinquefolium* L.). *In Vitro* Cell. Dev. Biol. 34: 203-211.
Tremblay L, Tremblay FM (1991). Carbohydrate requirements for the development of black spruce (*Picea mariana* (Mill.) B.S.P.) and red spruce (*P. rubens* Sarg.) somatic embryos. Plant Cell Tiss. Org. Cult. 27: 95-103.
Vikrant A, Rashid A (2002). Somatic embryogenesis from immature and mature embryos of a minor millet *Paspalum scrobiculatum* L. Plant Cell Tiss. Org. Cult. 69: 71-77.
Wang X, Proctor JTA, KrishnaRaj S, Saxena PK, Sullivan JA (1999). Rapid somatic embryogenesis and plant regeneration in American ginseng: effects of auxins and explants. J. Ginseng Res. 23: 148-163.
Yamasaki K (2000). Bioactive saponins in Vietnamese ginseng, *Panax vietnamensis*. Pharm. Bio. 38: 16-24.
Yang JS (1992). Plant regeneration from adventitious root-derived calli of ginseng *(Panax ginseng* C. A. Meyer). J. Agric. Assoc. China, 159: 41-48.
Yobimoto K, Matsumoto K, Huong NT, Kasai R, Yamasaki K, Watanabe H (2000). Suppressive effects of vietnamese ginseng saponin and its major component majonoside-R2 on psychological stress-induced enhancement of lipid peroxidation in the mouse brain. Pharmacol.

Biochem. Behav. 66(3): 661-665.
Zhong SL, Zhong SG (1992). Morphological and uhrastructural characteristics of the embryogenic callus of American ginseng. Chin. J. Bot. 4: 92-98.
Zhou S, Brown DCW (2006). High efficiency plant production of North American ginseng via somatic embryogenesis from cotyledon explants. Plant Cell Rep. 25: 166-173.