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Full Length Research Paper

Shoot regeneration and micropropagation of *Panax vietnamensis* Ha et Grushv. from *ex vitro* leaf-derived callus

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The methods for leaf-derived callus induction, callus proliferation, adventitious shoot induction and plant regeneration of Vietnamese ginseng (*Panax vietnamensis* Ha et Grushv.) were examined. In this study, callus induction was formed on both medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) alone or in combination with thidiazuron (TDZ). The highest callus induction frequency was obtained on Murashige and Skoog (MS) medium supplemented with 1.0 mg/l 2,4-D and 0.2 mg/l TDZ. The best callus proliferation medium was Schenk and Hildebrandt (SH) supplemented with 0.2 mg/l TDZ and 1.0 mg/l 2,4-D. The maximum callus-derived shoot number (8.2) was obtained on SH medium supplemented with 50 g/l sucrose in combination with 2.0 mg/l 6-benzylaminopurine (BA). The most successful rooting of regenerated adventitious shoots was obtained on SH medium with 1.0 mg/l α-naphthalene acetic acid (NAA). Plantlets were successfully acclimatized without growth chamber facility on Ngoc Linh mountain with a survival rate of 85% after two months. On the other hand, substantial increase of root length was observed. This study describes an efficient method for *in vitro* regeneration of *P. vietnamensis*, which could be considered for large-scale multiplication and propagation of this important medicinal plant.

Key words: Acclimatization, callus, *Panax vietnamensis*, regeneration, root, shoot.

INTRODUCTION

Ginseng is one of the most widely used herbal drugs. It has various therapeutic and pharmacological activities (Bladt et al., 1990; Chuang et al., 1995). The major components contributing to its pharmacological activities are ginsenosides, a group of steroidal saponins. About 40 ginsenosides have been isolated and characterized so far, including the recently identified ginsenosides Ki and Km (Tung et al., 2009).

Researches on ginseng culture, mainly on its medium composition, have been widely done in the last few decades (Akalezi et al., 1999; Zhang and Zhong, 1997; Zhang et al., 1996). *Panax ginseng* has been successfully cultivated at a large scale in 2,000 L and 20,000 L stirred tank bioreactors to produce 500 to 700 mg/l/day of ginseng saponins (Furuya, 1988). A number of reports on ginseng cell culture were also published (Choi et al., 1994; Hibino and Ushiyama, 1999; Wu and Zhong, 1999). More recently, adventitious roots of *P. ginseng* were studied in various volumes of bubble column bioreactors to obtain ginsenosides (Yu et al., 2002; Jeong et al., 2008).

Vietnamese ginseng was first found on the Central Highland of Vietnam in 1973, and was regarded as a new species with the scientific name *Panax vietnamensis* Ha et Grushv (1985). This is the most southern distribution of *Panax* genus (Araliaceae). The Sedang ethnic group in

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; **BA, 6-**benzylaminopurine; **MS,** Murashige and Skoog, 1962; **NAA,** α-naphthalene acetic acid; **SH,** Schenk and Hildebrandt, 1972; **TDZ,** Thidiazuron.

the region has been using the plant as their miraculous, life-saving drug to treat many serious diseases and enhance body strength for long journeys in high mountains. Investigation on the saponin composition of rhizomes and roots of *P. vietnamensis* has resulted in the isolation and structural elucidation of seven new dammarane saponins named vina-ginsenosides-R3 to -R9, together with the identification of six known saponins including 20-gluco-ginsenoside-Rf, ginsenoside-Rc, notoginsenoside-R6, quinquenoside-R1, gypenoside XVII and majoroside F1 (Nguyen et al., 1994). 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 the 250 endangered species, and ranked at high risk of extinction in the Vietnam's Red Data book.

The investigation is the first report on a protocol for rapid clonal propagation through callus formation from leaf explants of *P. vietnamensis*. Callus-derived plantlets could be successfully acclimatized in their natural habitats.

MATERIALS AND METHODS

Leaves of *ex vitro P. vietnamensis* (Figure 1a) were used as starting material for callus induction. These calli served as a general source for various organogenetic programs later.

Establishment of aseptic cultures

The leaves were surface sterilized with 70% (v/v) ethyl alcohol for 30 s and 0.1% (w/v) $HgCl_2$ for 5 min followed by repeated rinses with sterile distilled water. These leaves were horizontally cut into pieces with 1 mm in width for callus induction. Collected calli were used for subsequent experiments. Culture media, including MS basal medium (Murashige and Skoog, 1962), modified ½MS (full-strength essential minerals but half-strength micro-minerals) and SH (Schenk and Hildebrandt, 1972), were supplemented with 8 g/l agar. Sucrose and/or plant growth regulators, including 2,4-D, TDZ, NAA (α-naphthalene acetic acid), and BA (6-benzylaminopurine), were added separately or in combination to the media at different concentrations to evaluate their effects on callus and shoot induction. For all the experiments, pH of the media was adjusted to 5.7 ± 0.1 prior to autoclaving. The culture conditions for callus induction, callus proliferation, shoot regeneration and root formation were maintained at 25 ± 2°C, 10 h photoperiod using cool white florescent light (2,500 to 3,000 lux) and 75 to 80% relative humidity. Data were analyzed after eight weeks of culture.

Acclimatization

For the purpose of hardening and acclimatization to the field conditions, 200 plantlets (three-month-old) with well-developed roots were taken out of the culture vessels, washed gently under running tap water, and transferred to soil in the designated area at 2,000 m elevation on Ngoc Linh mountain (Te Xang Commune, Tu Mo Rong District, Kontum province). The natural conditions of the regions were at 15 to 18°C, 90% relative humidity, and 300 to 750 lux light intensity. After two months, survival rate, root length, shoot height and the number of two-shoot plants were determined.

Statistical analysis

The experiments were triplicated with 30 explants per replication. Data of callus induction, callus proliferation, shoot regeneration and root formation were analyzed using Duncan's test ($p < 0.05$).

RESULTS AND DISCUSSION

Effect of 2,4-D alone and in combination with TDZ on callus induction from leaf explants

Research on other species belonging to the *Panax* genus showed that callus induction stage usually required a combination of cytokinins and auxins. In case of Korean ginseng, if seed is used, induction medium should be MS supplemented with 1.0 mg/l 2,4-D and 0.01 mg/l kinetin (Arya et al., 1993); if leaf and the other explants are used, induction medium should be MS supplemented with 1.0 mg/l 2,4-D and 0.1 mg/l kinetin (Lim et al., 1997). In our callus induction experiments, the leaf explants were cultured on SH medium containing different concentrations of 2,4-D alone and in combination with TDZ to investigate the effects of these regulators on the effectiveness of callus induction.

After eight weeks of culture, data showed that the percentage of explants induced to develop callus, callus color and degree of callus formation varied with culture media formulations. Among different concentrations of 2,4-D, the highest callus induction rate from leaf explants of *P. vietnamensis* was 40% in SH medium containing 1.0 mg/l 2,4-D (Table 1). At 2.0 mg/l 2,4-D, crystalline calli were observed.

According to Radhakrishnan et al. (2001), cells can only ultilize a limited amout of auxin and over-use of auxins at any level can lead to cell development inhibition. In our study, 2,4-D at 2.0 mg/l was not suitable for callus induction from *P. vietnamensis* leaf explants. When 2.4-D and TDZ were used in combination in SH media, the highest callus induction rate of 80% was observed in the medium containing 1.0 mg/l 2,4-D and 0.2 mg/l TDZ (Table 2). High number of rigid structure and bright yellow calli were also observed (Figure 1b).

Effect of mineral salt formulations on callus proliferation

One of the major differences between MS or ½MS and SH salts media is the nitrogen concentration. In SH medium, both the ammonium ion and the nitrate ion concentration were lower than those in MS and ½MS mineral salt formulations and the NO_3/NH_4 or NO_3/NO_3+NH_4 ratio was higher in SH medium (Samson et al., 2006; George et al., 2008).

The difference in mineral salt formulation influenced the callus proliferation in this study. The highest callus production was obtained on SH media (Table 3; Figure 1c, 1d). This culture medium allowed a rapid production of callus from leaf explants of *P. vietnamensis* after eight

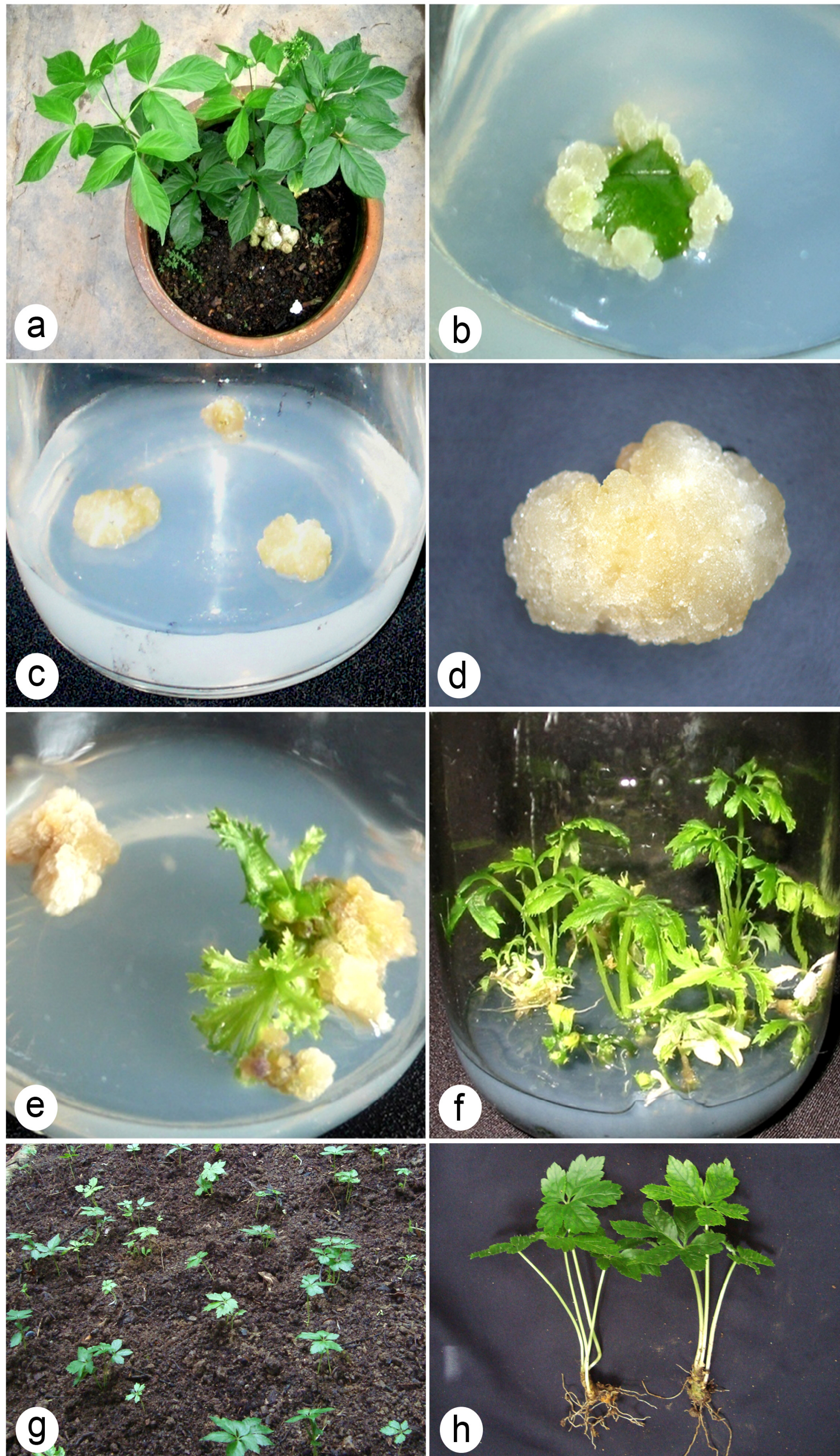

Figure 1. Stages of *P. vietnamensis* plant regeneration from leaf-derived calli. (a) Original plant; (b) callus formation; (c and d) callus proliferation; (e) shoot regeneration; (f) root formation; (g and h) *in vitro P. vietnamensis* plantlets growing on Ngoc Linh mountain, Te Xang Commune, Tu Mo Rong District, Kontum province after two months of acclimatization.

Table 1. Effect of 2,4-D on callus induction from *Panax vietnamensis* leaf explants.

2,4-D (mg/l)	Callus induction (%)
0.1	0.0[e]
0.2	6.7[d]
0.5	13.3[c]
1.0	40.0[a]
2.0	23.3[b]

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

Table 2. Effect of 2,4-D in combination with TDZ on callus induction from *Panax vietnamensis* leaf explants.

TDZ (mg/l)	2,4-D (mg/l)	Callus induction (%)
0.02	1.0	11[c]
0.05	1.0	19[c]
0.1	1.0	65[b]
0.2	1.0	80[a]
0.5	1.0	70[ab]

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

Table 3. Effect of mineral salt formulations on *Panax vietnamensis* callus proliferation.

Mineral salt formulation	Callus fresh weight (mg)
MS	223[b]
½MS	228[b]
SH	264[a]

Initial fresh weight was 63 ± 2 mg. Different letters in the same column indicate significantly different means using Duncan's test ($p < 0.05$).

Table 4. Effect of BA on shoot regeneration from *Panax vietnamensis* callus.

BA (mg/l)	Number of shoot/explant	Shoot fresh weight (mg)
0.0	0.0[d]	0[e]
0.5	2.7[c]	78[d]
1.0	4.4[b]	103[c]
2.0	5.0[a]	144[a]
4.0	4.3[b]	121[b]

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

Table 5. Effect of sucrose concentration on shoot regeneration from *Panax vietnamensis* callus.

Sucrose (g/l)	Number of shoot/explant	Shoot fresh weight (mg)
10	2.0[e]	89[f]
20	4.5[d]	112[e]
30	5.6[c]	133[d]
40	6.2[c]	322[a]
50	8.2[a]	295[b]
60	6.9[b]	277[c]

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

weeks of culture.

Effect of BA and sucrose on shoot regeneration from callus

Cytokinins are generally known to promote the formation of buds in many excised and *in vitro* tissue cultured organs (Nitsch et al., 1967). In this study, effect of BA on shoot regeneration from callus was investigated. SH medium supplemented with BA was found to be effective for shoot regeneration from leaf-derived calli for *P. vietnamensis* (Table 4). Shoots were induced on media containing BA (0.5 to 4.0 mg/l), whereas there was no signal of shoot induction on the control treatment (SH medium free of plant growth regulators). Among the different concentrations, it was found that SH medium supplemented with 2.0 mg/l BA was the most suitable medium for shoot regeneration from callus. The highest number of shoots per explant (5.0) and shoot fresh weight (144 mg) were also found in this concentration.

The growth and multiplication of shoots *in vitro* are affected by many factors, one of which is the concentration and type of exogenous carbohydrate source added to the medium (Anwar et al., 2005). The carbohydrate sources serve as energy and osmotic agents to support the growth of plant tissues (Lipavska and Konradova, 2004). In addition, growth and root initiation are highly energy requiring processes that can occur at the expense of available metabolic substrates which are mainly carbohydrates (De Klerk and Calamar, 2002; Thorpe, 1982). In this study also, shoot regeneration of *P. vietnamensis* was greatly influenced by different concentrations of sucrose supplemented in the media.

Among the different concentrations, 50 g/l sucrose performed well followed by 60 and 40 g/l sucrose in terms of inducing shoot regeneration (Table 5). The maximum shoot number (8.2) together with 295 mg of shoot fresh weight was recorded at 50 g/l sucrose supplemented with SH medium containing 2.0 mg/l BA (Figure 1e). The lowest number of shoots per explant (2.0) was obtained

Table 6. Effect of NAA on *Panax vietnamensis* root formation.

NAA (mg/l)	Number of root/explant	Root length (cm)	Number of shoot/explant	Shoot height (cm)	Shoot fresh weight (mg)
0.0	1.6^{c}	0.2^{c}	1.5^{a}	3.20^{b}	440^{b}
0.5	4.1^{b}	0.9^{b}	1.5^{a}	4.40^{a}	565^{a}
1.0	5.5^{a}	1.6^{a}	1.3^{a}	3.60^{b}	451^{b}
2.0	5.0^{a}	1.1^{b}	1.0^{a}	1.53^{c}	267^{c}

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

on SH medium supplemented with 10 g/l sucrose.

Even though carbohydrates are of prime importance for cell growth, maintenance and differentiation *in vitro*, the fundamental aspects of carbon utilization and metabolism in cell and tissue cultures have to be fully understood (Romano et al., 1995; Vu et al., 1995).

Effect of NAA on root formation

The application of NAA to shoots increased root length and number of roots per explant significantly in all the concentrations as compared to the control treatment (Table 6). Auxins are well known to play a significant role in stimulating root initiation in stem cuttings of most plants (Tchoundjeu and Leakey, 1996, 2000; Tchoundjeu et al., 2002, 2004). The stimulatory effect of auxins has been shown to enhance transport of carbohydrates to the base of the cutting (Mesen et al., 1997). In this study, shoots which received 1.0 mg/l NAA gave the highest number of roots per explant and root length (Figure 1f) but the increase in NAA concentration reduced rooting after eight weeks of culture (Table 6).

Acclimatization

Plantlets were successfully acclimatized without growth chamber facility. After two months of acclimatization, a total of 170 (85%) surviving plants were obtained out of which 19 had two shoots per plant instead of one. The average root length was 2.15 cm. On the other hand, increase of shoot height (4.47 cm) was observed (Figure 1h). There was no detectable variation among the acclimatized plants with respect to morphological growth characteristics. All the micropropagated plants were free of external defects. These plantlets were successfully transferred to soil in their natural habitats (Figure 1g).

This study established an efficient method for *in vitro* shoot regeneration and micropropagation of *P. vietnamensis* through callus formation. The well rooted *in vitro* plantlets were successfully transferred to soil and their survival rate under natural environment was 85%. The results obtained could be considered for large-scale production and conservation of this important medicinal plant.

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