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THE EFFECTS OF SOME FACTORS ON *IN VITRO* BIOMASS PRODUCTION OF VIETNAMESE GINSENG (*PANAX VIETNAMENSIS* HA ET GRUSHV.) AND PRELIMINARY ANALYSIS OF SAPONIN CONTENT

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SUMMARY

Panax vietnamensis Ha et Grushv., a rare *Panax* genus of Vietnam, is a well known Vietnamese ginseng (Ngoc Linh Ginseng) for its rich pharmaceutical compositions, most importantly saponin. In order to obtain a stable and saponin-rich biomass of *P. vietnamensis*, a tissue culture procedure was established. A TLC analysis of saponin composition was also conducted to investigate the presence of saponin in callus, shoot and root biomass. Successful callus induction from leaf and petiole explants was obtained from MS medium supplemented with 1.0 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid), 0.2 mg/l TDZ (Thidiazuron) under a photoperiod of 16 h. In the following steps, the optimal auxin and its concentration, appropriate photoperiod condition as well as callus size that were the best for callus proliferation were investigated. Among the auxins, including 2,4-D, IBA (Indole-3-butyric acid) and NAA (α-Naphthaleneacetic acid), 2,4-D at 1.0 mg/l was found to be the most effective for callus growth. Callus at the size of 0.5 × 0.5 cm grew the best as compared to bigger ones, such as 0.7 × 0.7 cm and 1.0 × 1.0 cm. The effects of phytohormones, sucrose and activated charcoal (AC) on shoot regeneration from callus and shoot proliferation have also been studied. Calli cultured on MS medium supplemented with 1.0 mg/l BA and 1.0 mg/l NAA regenerated more shoots. The suitable medium for shoot proliferation was MS½ medium, supplemented with 1.0 mg/l BA, 0.5 mg/l NAA, 50 g/l sucrose and 2.0 mg/l AC. Callus was grown on MS½ medium supplemented with 3.0 mg/l NAA to regenerate roots. Root proliferation was obtained on MS½ medium containing 5.0 mg/l NAA. In saponin analysis experiment, thin layer chromatograms show that obtained calli, shoots and roots from the above experiments had ginsenoside-Rg1 and majonoside-R2, two main ginsenosides of Vietnamese Ginseng but only roots have ginsenoside-Rb1. These results indicate that Vietnamese Ginseng biomass can be used as a new source for saponin isolation for pharmaceutical and cosmetic industry.

Keywords: *Panax vietnamensis, callus, regeneration, shoot, root, saponin*

INTRODUCTION

Ngoc Linh Ginseng, with the scientific name *Panax vietnamensis* Ha et Grushv, is a famous Vietnamese Ginseng. Ngoc Linh Ginseng had not only typical medical effects but also specific physical actions like anti-stress, anti-depression, *in vitro* and *in vivo* antioxidation, etc. and saponin triterpenoic compounds are the main effective group. Ngoc Linh Ginseng 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 (Dong *et al.*, 2007).

At present, Ngoc Linh Ginseng supply is very limited because it is grown mainly in Ngoc Linh mountain area and takes long time to grow. Due to excessively harvesting, the gingseng is among 250 endangered species, at high risk of extinction (Vietnam's Red Data book).

Dung (1995) performed some research in order to improve Ngoc Linh Ginseng culture medium. In 2006, Nhut *et al.* (2006) had some studies on rapid multiplication of Ngoc Linh Ginseng secondary roots. Jacques *et al.* (2007) investigated optimum conditions to increase ginseng biomass in bioreactor. Recently, Duong *et al.* (2008) have initially performed HPLC in order to quantify ginsenoside-Rg1, -Rg2, -Rd in cell extract from ginseng biomass.

Apparently, the collection of biomass and examination of saponin component presented in collected biomass are essential in considering the effectiveness of *in vitro* growth.

In the current research, we investigated the effects of medium compositions, culture conditions, as well as culture explant's size on *in vitro* multiplication of Ngoc Linh Ginseng from callus induction to root and shoot regeneration stage, together with qualification of saponin in collected biomass, therefore initially assess the effectiveness of *in vitro* culturing.

MATERIALS AND METHODS

Materials

Explants: leaves and petioles of Ngoc Linh Ginseng, grown at Tay Nguyen Institute of Biology, were used as starting material for the induction of calli. Collected materials were gently washed with Javel water (Sodium chloride), and continuously washed with water in 2 hours. Explants were shaken in 70% alcohol in 30 seconds and continuously rinsed 4 - 5 times with water, then in 0.1% $HgCl_2$ contained a few drop of Tweens-20 in 5 minutes. Explants were then washed with distilled water 5 - 6 times. The leaves were cut into pieces with size 1.0 × 10 cm, while the petioles were vertically cleft and cut into 1.0 cm parts.

Collected calli with different sizes were used for different subsequent experiments.

Culture medium: MS basal medium (Murashige, Skoog, 1962), modified ½MS (originated essential minerals and half of microminerals) and modified MS½ (half essential and microminerals) media supplemented with 30 g/l sucrose, 8.0 g/l agar and pH 5.7. During examining the effects of sucrose or active charcoal, the other components' concentration could be changed depending on experimental conditions.

Culture conditions: callus induction and development, shoot regeneration and proliferation were carried out at average temperature 25 ± 2°C, lighting intensity 2.500 - 3.000 lux, average humidity 75 - 80%. To investigate the effects of lighting condition, explants were cultured and kept in dark room or lighting room 16 hours/day. Root regeneration and proliferation experiments were carried out in the dark.

Experiment designs

Effect of auxin type and concentration on callus induction from leaf and petiole

Disinfected leaf and petiole explants were cultured on MS medium supplemented 0.2 mg/l TDZ and auxins such as 2,4-D, IBA and NAA with different concentrations (0.5, 1.0, 2.0, 3.0 mg/l).

Effect of lighting condition on callus induction from leaf and petiole

The most suitable medium for initial callus formation from leaf and petiole explants was used for investigating lighting conditions. Explants were kept in dark room or lighting condition in 16 hours/day.

Effect of auxin types and their concentration on callus multiplication

Calli formed in induction stage were cultured in MS medium supplemented 0.2 mg/l TDZ and auxins such as 2,4-D, IBA and NAA with different concentrations varied from 0.5; 1.0; 2.0; 3.0; 5.0 mg/l in lighting condition 16 hours/day.

Effect of explant size on callus development

Calli were sliced (prepared) into 3 sizes: 0.5 × 0.5 cm (KT I); 0.7 × 0.7 cm (KT II) and 1.0 × 1.0 cm (KT III). Callus slides with specific size were cultured in optimal medium for multiplication. Calli after multiplication were used for shoot and adventitious root regeneration.

Effect of BA and NAA on shoot regeneration from callus

Callus derived from rapid multiplication were collected and subcultured into shoot regenerative medium supplemented with BA (0.5; 1.0; 2.0; 4.0 mg/l) and NAA (0.5; 1.0; 2.0; 4.0 mg/l).

Effect of BA on shoot development

The best shoots from above experiment were collected and transferred into ½MS supplemented with 1.0 g/l charcoal, 30 g/l sucrose, 0.5 mg/l NAA and BA (0.5; 1.0; 2.0; 4.0 mg/l).

Effect of sucrose concentration on in vitro shoot development

The best shoots from callus-derived regeneration experiment were collected and subcultured in ½MS medium supplemented with 0.5 mg/l NAA, 1.0 mg/l BA, pH = 5.7 and sugar with different concentrations

(10, 20, 30, 40, 50, and 60 g/l).

Effect of activated charcoal on in vitro shoot development

The best shoots from callus-derived regeneration experiment were collected and subcultured in ½MS medium supplemented with 0.5 mg/l NAA, 1.0 mg/l BA and pH = 5.7 with different charcoal concentrations (0, 1.0, 2.0, 3.0, 40 g/l).

Effect of IAA, IBA and NAA on adventitious root formation from callus

Calli were cultured into root induction medium contained auxins (NAA, IBA, and IAA) with different concentrations (1.0, 3.0, 5.0, 7.0 mg/l).

Effect of IBA and NAA on adventitious root multiplication

Callus-derived adventitious roots were collected and subcultured into root multiplication medium supplemented with auxins (NAA and IBA) at different concentrations (1.0, 3.0, 5.0 mg/l).

Saponin isolation from callus, shoot and root biomass of Panax vietnamensis by TCL method

Thin layer chromatography was employed in order to (clarify) qualify saponins with following steps.

Solvent preparation: two solvent systems were used with chemical ratio as followed: Solvent system 1: $CHCl_3$ - MeOH - H_2O (65 : 35 : 10, lower layer); solvent system 2: n-BuOH - AcOH - H_2O (4 : 1 : 5, upper layer).

Thin layer and sample loading solution preparation: Silica gel plate (Merck) was prepared with suitable size. Sample from Namba extraction method was diluted with several drops of methanol and loaded to the plate.

Sample loading and color detection: samples were loaded with same horizontal position and the bands on a plate were distributed as followed: 1 standard Korean Ginseng, 1 standard Ngoc Linh Ginseng, 3 standard ginsenosides including majonoside R2 provided by Ho Chi Minh City Ginseng and Medical Institute, ginsenoside-Rb1 (Rb1) and ginsenoside-Rg1 (Rg1) provided by Nacalai tesque (Japan); 1 sample (calli, shoots and roots) and 1 sample contained all 3 above standard ginsenosides. After running, the plate was sprayed with 10% sulfuric acid (H_2SO_4) in alcohol, dried at 110°C in 5 minutes for color detection. Thin layer was then photographed and stored.

Retention factor (Rf) calculation: Rf value and color visualization with different detection agents compared with those on color chart were critical factors to determine the present of saponins in analyzing solutions. Rf value of compound A is defined as the distance traveled by compound A (I_A) divided by the distance traveled by the solvent (I_o).

RESULTS AND DISCUSSION

Effect of auxin types and concentration on callus induction from leaf and petiole

Research on other species belonging to *Panax* genus showed that callus induction stage usually required the combination between cytokinins and auxins. In case of Korean Ginseng, if seed is used, induction medium should be MS supplemented 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 1.0 mg/l 2,4-D and 0.1 mg/l kinetin (Lim, Lee, 1997). In callus induction and multiplication experiments, we used TDZ as cytokinin with constant concentration (0.2 mg/l) to investigate the effects of auxin types and concentrations.

Table 1. Effect of auxin types on callus induction from leaf and petiole.

Auxin	Concentration (mg/l)	Callus induction (%)	
		Petiole	Leaf
2,4-D	0.5	100	20
	1.0	100	90
	2.0	100	90
	3.0	100	80
IBA	0.5	0	0
	1.0	0	0
	2.0	0	0
	3.0	0	0
NAA	0.5	0	0
	1.0	0	0
	2.0	0	0
	3.0	0	0

Table 1 shows our records after 8 weeks cultured. Among 3 auxins added to induction medium, 2,4-D exhibited the ability to stimulate

callus formation from leaf and petiole. In medium supplemented 1.0 mg/l 2,4-D, cultured explants gave the highest ratio of callus formation (90% for leaf explants and 100% for petiole), with a high number of rigid structure and bright yellow calli. At 3.0 mg/l 2,4-D, calli started to form crystalline. According to Rakhakrishana *et al.* (2001), the cells can only utilize a limit amount of auxin and over-use of auxins at any level can lead to cell development inhibition. Therefore, above 3.0 mg/l of 2,4-D is not suitable for callus induction from Ngoc Linh Ginseng leaves.

Effect of lighting condition on callus induction from leaf and petiole

Depending on explants, lighting can be used or not during callus induction period. In case of leaf explants, callus formation is would rather carried out in the dark. However, in some cases, culture explants can produce better calli in lighting conditions. Data in table 2 show that callus formation ratio is almost the same between leaf and petiole explants either in dark or lighting condition. Nevertheless, in the dark, the number and quality of calli are lower than in lighting condition due to crystalline formation, especially in medium supplemented with 3.0 mg/l 2,4-D (Fig 1. a_1, a_2). These results are consistent with those from Lim and Lee (1997) on Korean Ginseng. Therefore, the lighting period of 16 hours/day is able to stimulate callus formation from Ngoc Linh Ginseng leaves similarly to those in dark condition.

Table 2. Effect of lighting condition on callus induction from leaf and petiole.

2,4-D (mg/l)	Explant	Callus induction (%)	
		Light (16 hours/day)	Dark
0.5	Leaf	20	30
1.0		90	80
2.0		90	90
3.0		80	80
0.5	Petiole	100	100
1.0		100	100
2.0		100	100
3.0		100	100

Effect of auxin type and concentration on callus multiplication

Table 3 showed that after multiplication stage, calli cultured in medium supplemented 0.5 mg/l IBA had the highest dry weight (9.62%) but highest increase ratio of dry callus weight was observed at medium contained 1.0 mg/l 2,4-D.

Table 3. Effect of auxin types on callus development.

Auxin	Concentration (mg/l)	Initial fresh weight (mg)	Biomass (after 4 weeks culture)		
			Fresh weight (mg)	Dry weight (mg)	Dry weight (%)
2,4-D	0.5	203 ± 16	584 ± 34	43.3 ± 2.5	7.42
	1.0	212 ± 14	809 ± 37	66.2 ± 3.0	8.18
	2.0	204 ± 17	711 ± 32	52.4 ± 2.4	7.37
	3.0	205 ± 9	508 ± 24	36.6 ± 2.2	7.21
	5.0	201 ± 13	493 ± 38	34.6 ± 1.7	7.01
IBA	0.5	197 ± 18	474 ± 23	45.6 ± 2.2	9.62
	1.0	203 ± 19	532 ± 29	48.6 ± 2.7	9.14
	2.0	207 ± 13	631 ± 32	49.5 ± 2.5	7.84
	3.0	203 ± 15	552 ± 26	41.1 ± 1.9	7.45
	5.0	209 ± 12	531 ± 23	35.3 ± 1.5	6.66
NAA	0.5	218 ± 8	485 ± 13	41.2 ± 1.1	8.49
	1.0	212 ± 14	548 ± 21	45.0 ± 1.8	8.22
	2.0	206 ± 15	588 ± 18	46.6 ± 1.4	7.92
	3.0	199 ± 7	602 ± 32	45.7 ± 2.4	7.60
	5.0	205 ± 14	720 ± 48	51.6 ± 3.4	7.20

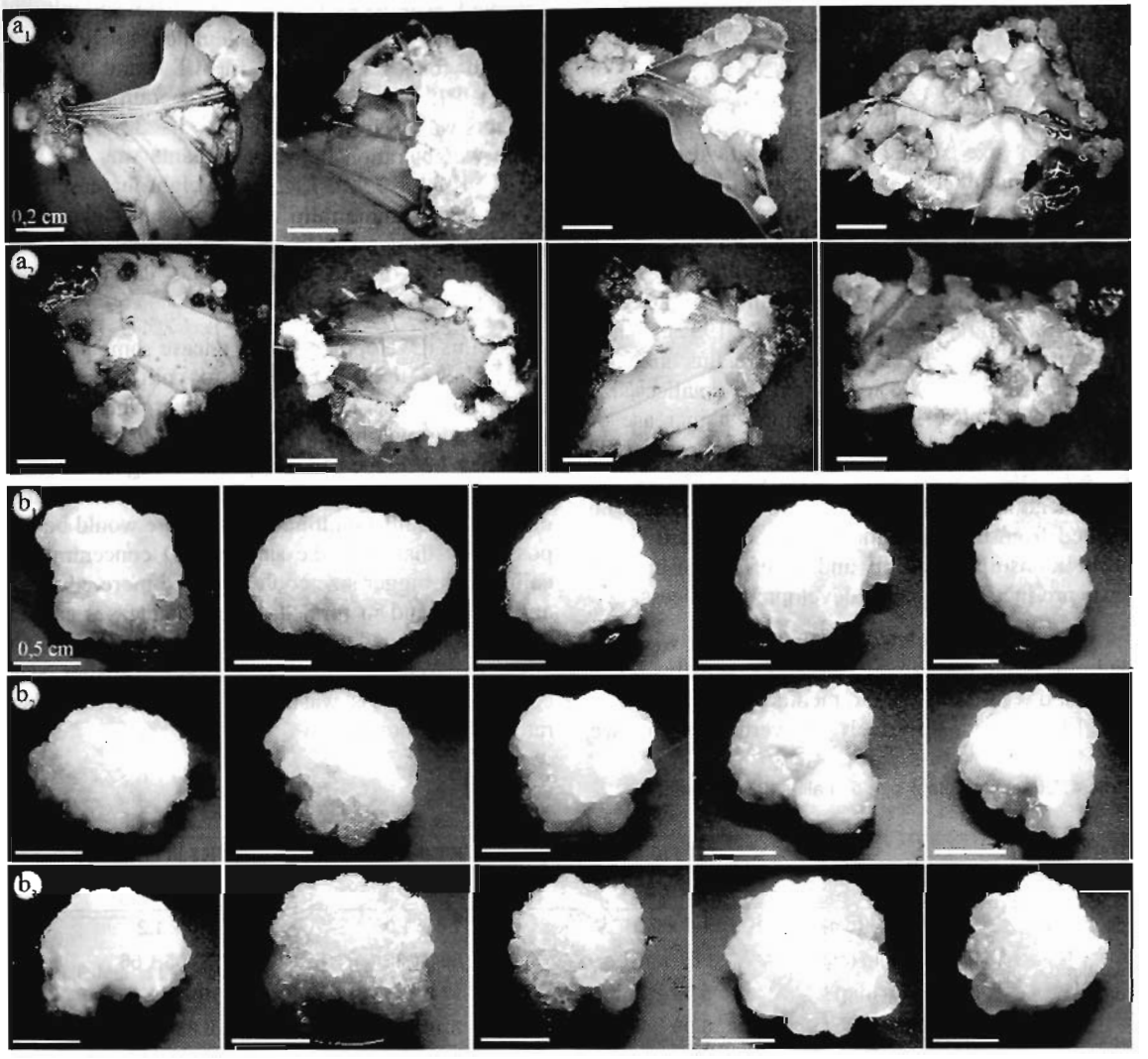

Figure 1. Callus formation and multiplication of Ngoc Linh Ginseng. From the left to the right, respectively. a_1. callus formation from leaves on medium containing 0.5; 1.0; 2.0; 3.0 mg/l 2,4-D in lighting condition 16 hours/day; a_2. callus formation from ginseng leaves on medium containing 0.5; 1.0; 2.0; 3.0 mg/l 2,4-D in dark condition. b_1. callus multiplication on medium containing 0.5; 1.0; 2.0; 3.0 mg/l 2,4-D; b_2. callus multiplication on medium containing 0.5; 1.0; 2.0; 3.0 and 5.0 mg/l IBA; b_3. callus multiplication on medium containing 0.5; 1.0; 2.0; 3.0 and 5.0 mg/l NAA.

According to Medina *et al.* (1998), carbohydrate was responsible for most of callus dry weight. The main carbon source in medium was sucrose, and sugar utilization of callus depends on types of medium and sample sources (Medina *et al.*, 1998). Therefore, probably the utilization of both auxin and cytokinin could help calli improving their sugar and other nutrients absorption from culture medium, which caused the development of callus, especially dry biomass. According to our results, IBA could be more effectively used with TDZ, than it did with NAA and 2,4-D, in order to enhance nutrition utilization. Dry weight ratio of calli in 0.5 mg/l IBA-containing medium was highest among 3 treatments using 3 auxins (Table 3). Although dry weight of calli in IBA-containing medium was highest, 2,4-D offered the highest dry weight increase ratio and relative high level of weight in calli. Alternatively,

calli had the best conditions and were the high-regenerative calli, in 2,4-D-containing medium (Fig. $1b_1$).

Effect of explant size on callus development

Explant size is one of the most critical factors in *in vitro* multiplication. The initial difference of explant size can lead to a significant difference in cell density in multiplying process, which causes a mass change in the other factors in culture medium; therefore, cell metabolization could be affected directly or indirectly (Akalezi *et al.*, 1999). When investigating the effects of initial callus size on callus development, we learnt that the smallest size (0.5 x 0.5 cm, KT I) gave the highest increase of fresh and dry weight, while the remaining size (KT II, KT III) did not show any difference in multiplication (Table 4). This increase could be resulted from the correlation between explant size - nutrition absorption ability and the effects of internal waste products from callus development process.

Callus size is related to contact area with the medium. In the experiment, the area that callus KT I, KT II and KT III exposed to medium were 0.25, 0.49 and 1.00 cm^2, respectively. Nevertheless, as we observed, average real contact area between cultured explant and medium was calculated as followed: 0.24 cm^2 for KT I, 0.40 cm^2 for KT II and 0.84 cm^2 for KT III. Therefore, contact area ration between explants was: KT III : KT II : KT I = 3.5 : 1.67 : 1; while weight ration between explants was KT III : KT II : KT I = 3.92 : 1.82 : 1. Weight increase ratio higher than volume ratio would prevent callus from absorbing nutrients from culture medium, and this may be one of the main reasons causing lower development of bigger callus size.

Callus development could release some products which have toxic feedbacks to calli themselves. Garcia and Einset (1983) realized that when tobacco calli were grown in the present of 2,4-D at 0.5 to 25 mg, they could be able to produce higher internal ethane and ethylene into the medium, which later decelerated callus multiplication. There would be the possibility that with the same 2,4-D concentration, callus with bigger size could produce more ethylene and ethane and in turn, these internal gases caused toxic and inhibited callus development of Ngoc Linh Ginseng. And therefore, callus with smaller size could produce less wasting gases and have higher rate of development.

Table 4. Effect of explant size on callus development.

Data collection		KT I (0.5 x 0.5 cm)	KT II (0.7 x 0.7 cm)	KT III (1.0 x 1.0 cm)
Initial fresh weight (mg)		147 ± 6	267 ± 18	576 ± 24
Biomass after 4 weeks culture	Explant size (cm)	1.1 x 0,9	1.4 x 1.0	1.6 x 1.2
	Fresh weight (mg)	667 ± 45	804 ± 35	1.505 ± 66
	Dry weight (mg)	53.9 ± 3.6	57.8 ± 2.5	102.8 ± 4.5
	Dry weight (%)	8.08	7.19	6.83
Dry weight biomass increase rate		5.46	3.22	2.65

Effect of BA and NAA on shoot regeneration from callus

The ratio between auxins and cytokinins plays an important role in shoot regeneration. Cytokinins usually promote shoot formation, which can be stimulated with a low concentration of auxins. In our experiment, BA and NAA were simultaneously used in order to investigate the effects of this combination on shoot regeneration ability from Ngoc Linh Ginseng callus. The results showed that among different combining ratios between BA and NAA, 1.0 mg/l BA and 1.0 mg/l NAA gave the highest shoot number (6.3 shoots/explant) and the average weight of 0.185 g.

Effect of BA on shoot development

In used BA concentrations, 1.0 mg/l BA together with 0.5 mg/l NAA showed the best shoot regeneration with fresh weight of new shoot 0.87 g and height 6.16 cm (Table 6, Figure 3a). Therefore, medium supplemented 1.0 mg/l BA and 0.5 mg/l NAA offered the optimal conditions for shoot regeneration for Ngoc Linh Ginseng callus.

Table 5. Shoot regeneration from callus on MS medium containing BA and NAA.

BA (mg/l)	NAA (mg/l)	No of shoots/ explant	Shoot fresh weight (g)
0.5	0.5	5.0	0.106
	1.0	6.1	0.141
	1.5	4.6	0.193
	2.0	3.3	0.197
	2.5	3.0	0.094
1.0	0.5	5.5	0.163
	1.0	6.3	0.185
	1.5	5.9	0.158
	2.0	3.9	0.148
	2.5	3.7	0.157
2.0	0.5	4.2	0.152
	1.0	5.5	0.141
	1.5	2.9	0.144
	2.0	2.8	0.112
	2.5	2.7	0.108
4.0	0.5	3.3	0.154
	1.0	3.0	0.122
	1.5	2.6	0.122
	2.0	0.8	0.108
	2.5	0	0

Table 6. Effect of BA on shoot development.

BA (mg/l)	Shoot fresh weight (g)	Shoot height (cm)	No of leaves/ shoot
0.5	0.61	5.66	3.0
1.0	0.87	6.16	3.3
2.0	0.72	4.11	4.0
4.0	0.71	4.33	3.9

Effect of sucrose concentration on shoot development

Research on shoot regeneration showed that sucrose was the preferred dissolving carbohydrate and the concentrations are usually 30 - 120 g/l. Experiments on Ngoc Linh Ginseng shoot development showed that adding sucrose into culture medium had positive effects on shoot growth. The increase of sucrose concentration in culture medium not only stimulated shoot development but also effectively increase their weight. Sucrose concentration of 50 g/l showed the best results on weight, height and leaf number (Table 7, Figure 3b).

Table 7. Effect of sucrose concentration on shoot development.

Sucrose (g/l)	Shoot fresh weight (g)	Shoot height (cm)	No of leaves/ shoot
10	0.49	4.4	2.2
20	0.55	5.4	2.5
30	0.68	5.7	2.6
40	1.06	5.8	3.2
50	1.46	6.1	3.5
60	1.28	6.1	3.2

Effect of AC on shoot development

Active charcoal (AC) is not a plant growth regulator, but it can change the medium compositions. AC adjusts medium pH and absorbs chemicals preventing the development of tissues. Moreover, according to George and Sherington (1984), the presence of AC in medium showed some benefits for shoot development, increase of shoot fresh weight. Our results indicated that increase of AC concentration could lead to a considerable change in either shoot weight or height, but not the number of leaves. Shoot weight was highest at 2.0 g/l AC (1.01 g/shoot), increase 1.9 fold in compared with control. (Table 8, Fig. 3c). Thus 2.0 g/l AC is the optimal concentration for Ngoc Linh Ginseng shoot development.

Table 8. Effect of AC on shoot development.

AC (g/l)	Shoot fresh weight (g)	Shoot height (cm)	No of leaves/ shoot
0	0.53	3.3	3.6
1.0	0.61	4.6	3.7
2.0	1.01	5.3	3.3
3.0	0.97	6.8	2.7
4.0	0.94	8.5	3.1

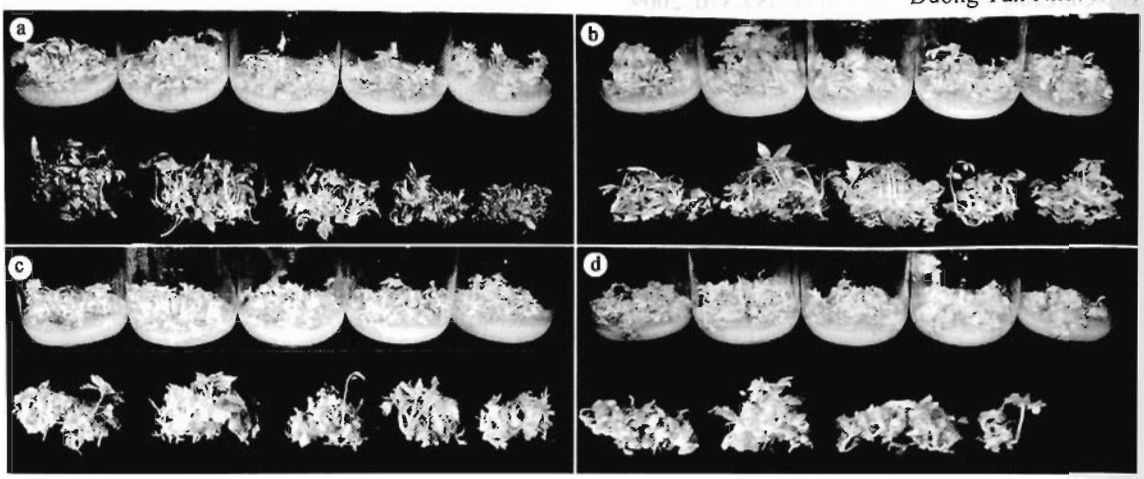

Figure 2. Shoot regeneration from Ngoc Linh Ginseng callus. From left to right, respectively. a. shoot regeneration on medium containing 0.5 mg/l BA and NAA (0.5, 1.0, 1.5, 2.0, 2.5 mg/l); b. shoot regeneration on medium containing 1.0 mg/l BA, NAA (0.5, 1.0, 1.5, 2.0, 2.5 mg/l); c. shoot regeneration on medium containing 2.0 mg/l BA, NAA (0.5, 1.0, 1.5, 2.0, 2.5 mg/l).

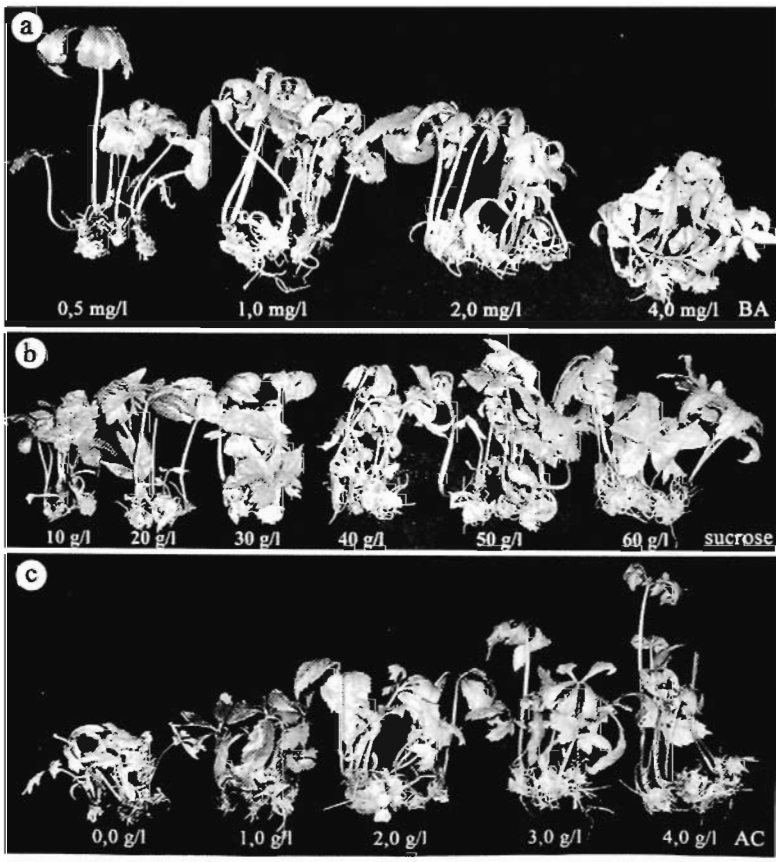

Figure 3. Effect of BA, sucrose and AC on Ngoc Linh Ginseng shoot development. a. shoot development on medium containing 0.5, 1.0, 2.0 and 4.0 mg/l BA; b. shoot development on medium containing 10, 20, 30, 40, 50, and 60 g/l; c. shoot development on medium containing 0, 1.0, 2.0, 3.0, and 4.0 g/l AC.

Effect of IAA, IBA and NAA on adventitious root formation from callus

When investigating effects of 3 auxins IAA, IBA, NAA, we learnt that IAA was not suitable for Ngoc Linh Ginseng root formation because this auxin did not stimulate adventitious root formation from callus. NAA and IBA could well stimulate rooting process. NAA at concentration 3.0 mg/l offered the best results with 100% of root formation; root number/biggest explant = 8.7 roots/explant; root weight/biggest explant weight = 21.88%; the longest root reached 13 mm (Table 9). IBA at the concentration 5.0 mg/l gave 100% root formation with the average root is 4.8, weight ratio is 15.81% and the longest root reached 18 mm. These phenomenon could be explained by higher activity of synthetic auxins (IBA, NAA, 2,4-D) than the natural one (IAA). IAA was not able to stimulate root formation due to its low biological activity and it sensitivity to enzyme activity. Our obtained results are consistent with other previous research. According to George and Sherington (1984), IAA, IBA and NAA were usually used for root formation, among them, IBA offered the highest effect. Moreover, auxin not only stimulated root development but also helped increase explant fresh weight; NAA and IBA were observed to be more effective than IAA (Kull, Arditti, 2002). NAA is usually used in regenerative experiments. Hence, MS½ supplemented 3.0 mg/l NAA and MS½ supplemented 5.0 mg/l IBA are the optimal medium for adventitious root formation (Figure 4a$_1$, a$_2$).

Effect of IBA and NAA on adventitious root multiplication

In order to choose the most suitable auxin for adventitious root regeneration and multiplication in Ngoc Linh ginseng, we continued to multiply adventitious roots using two auxin types, IBA and NAA. Obtaining results were showed in table 10a and table 10b.

Our obtained results indicated that explant origin had a significant effect on root multiplication. A explant showed the best result on root multiplication with all treatments (6), highest root formation ratio (60%), highest secondary root formation (9 roots). B explant showed 40% root formation (with 3 root formation treatments among 6), secondary root formation (3 roots).

Our experiments indicated that NAA was the most suitable among auxins for adventitious root multiplication at Ngoc Linh Ginseng. NAA at concentration 5.0 mg/l was optimal for root multiplication with the highest root formation ratio (60%), the highest secondary root formation (9 roots) and the highest weight increase (average fresh weight: 390 ± 20 mg, increase 3.5 folds in compared with the original one). In addition, 5 among 6 treatments which were supplemented NAA showed root formation while IBA show 4 among 6 treatments. As a result, NAA at concentration 3.0 mg/l is most suitable for root formation from callus and NAA at concentration 5.0 mg/l is most suitable for adventitious root multiplication at Ngoc Linh Ginseng (Figure 4b).

Table 9. Effect of IAA, IBA and NAA on adventitious root formation from callus.

Auxin	Concentration (mg/l)	Rooting rate (%)	No of roots/explant	Root weight rate/explant (%)	Root length (mm)
NAA	1.0	30.0	3.0 ± 0,3	5.98	18
	3.0	100.0	8.7 ± 0,1	21.88	13
	5.0	70.0	2.6 ± 0,1	6.23	9
	7.0	50.0	2.1 ± 0,1	12.21	8
IAA	1.0	0.0	–	–	–
	3.0	0.0	–	–	–
	5.0	10.0	0.2 ± 0,2	–	–
	7.0	0.0	–	–	–
IBA	1.0	70.0	1.6 ± 0, 1	7.83	16
	3.0	80.0	4.0 ± 0, 3	5.21	21
	5.0	100.0	4.8 ± 0,3	15.81	18
	7.0	60.0	3.5 ± 0,1	8.06	17

Table 10a. Effect of IBA and NAA on root multiplication with NAA-treated explants (A explant).

NAA (mg/l)	IBA (mg/l)	Rooting rate (%)	No of secondary roots	Root fresh weight (mg)
1	–	20	1	140 ± 10
3	–	30	4	290 ± 10
5	–	60	9	390 ± 20
–	1	10	1	450 ± 50
–	3	20	2	330 ± 20
–	5	30	1	280 ± 30

Table 10b. Effect of IBA and NAA on root multiplication with IBA-treated explants (B explant).

NAA (mg/l)	IBA (mg/l)	Rooting rate (%)	No of secondary roots	Root fresh weight (mg)
1	–	40	3	350 ± 10
3	–	20	1	180 ± 30
5	–	0	0	–
–	1	10	1	270 ± 10
–	3	0	0	–
–	5	0	0	–

Initial fresh weight: 40 ± 10 (mg).

Saponin detection in callus, shoot and root biomass of *in vitro* cultured Ngoc Linh ginseng by TLC method

Figure 5 and 6 showed saponin qualification results in calli, shoots and adventitious roots. Rf values of the compounds were determined by their positions and colors on the plate. The results indicated that majonoside-R2 and G-Rg1, but not ginsenoside-Rb2, were presented in calli and shoots when compared the explants color and position on the plate with the standard compounds. Especially, the color chart from root weight showed the present of three standard ginsenosides.

Ginsenoside types of callus and *in vitro* cultured biomass of Ginseng genus depended on explant sources and supplemented auxins (Bonfill *et al.*, 2002; Furuya *et al.*, 1986). The ratio of group Rb/Rg in 2-year Korean Ginseng root-stalk after 5 weeks culturing was 0.49 with the present of 2,4-D (Bonfill *et al.*, 2002). According to William (2000), Rb group amount presented in 2-year Korean Ginseng root stalk was lower than Rg (0.6% vs. 1.0%), this indicates that saponin accumulation in Korean Ginseng callus is same with natural explants. When trying on producing saponin from adventitious root of Korean Ginseng, Langhansova *et al.* (2005) learnt that total ginsenosides of cultured roots in bioreactor was about 14.48 mg/g biomass, while natural ginseng root contained 33.12 mg/g. In the collected biomass, there were about 5.02 mg/g G-Rb and 9.46 mg/g G-Rg, compared with 15.06 mg/g G-Rb and 18.06 mg/g G-Rg in roots of Korean Ginseng (Langhansova *et al.*, 2005). From these results, even in *in vitro* conditions, collected biomass was able to synthesize compounds which were presented in the original explants.

Although there is no evidence of the presenting of ginsenoside Rb and Rg groups, our results showed that there is G-Rg1, a representive of 20(*S*)-protopanaxatriol group in callus, which has a very low level in the original Ngoc Linh Ginseng leaves. Analyzing saponin components in Ngoc Linh Ginseng leaves showed that 20(*S*)-protopanaxadiol derivatives, but not G-Rg1, hold a high ratio among saponin in stems and leaves (Dong *et al.*, 2007). This can explain why G-Rb1 cannot be detected in leaf and shoot-derived callus. Moreover, there was majonoside-R2 in callus, which was not presented in saponin components from leaves. Probably auxin and cytokinin had some influences on the multiplication process, callus cells can synthesize majonoside-R2 themselves, and this saponin is critical for the specific medical impact of this ginseng.

In adventitious roots, there were all three main saponin groups in Ngoc Linh Ginseng: G-Rb1 as a representative of 20(*S*)-protopanaxadiol group, G-Rb1 as a representative of 20(*S*)-protopanaxatriol group, and majonoside-R2 as a representative of Occotillol group. Although these compounds were not quantified yet, according to the strength of visualized colors, we could infer that majonoside-R2 has the highest amount, followed with G-Rg1 and finally with G-Rb1. These results are consistent with saponin components presenting in natural Ngoc Linh Ginseng root, with 50% majonoside-R2 in total saponin in root and root stalk.

Besides chemical bands which had the same positions with standard ones, there were different bands with other colors (green, or yellow, etc) on the TLC plate. These alien bands indicated that auxin and cytokinin had stimulated the synthesis of non-saponin compounds in callus development. Types and compositions of these compounds has not

identified yet, there should be necessary identification experiments to learn about these compounds. In addition, there were some other bands which almost had the same positions with two standard samples, Korean and Ngoc Linh Ginseng. So, there may be the present of some other saponins, besides the standard ones, with low concentration, in culturing ginseng callus.

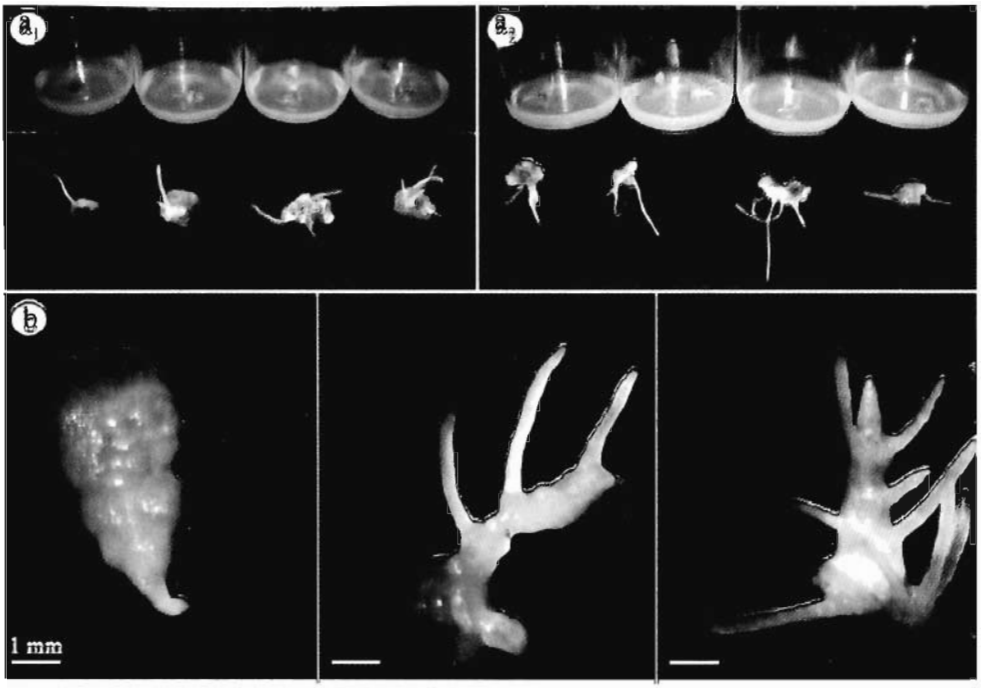

Figure 4. Effects of auxins on root formation and multiplication from Ngoc Linh Ginseng callus. From left to right, respectively. a_1. root formation from callus at 1.0, 3.0, 5.0 and 7.0 mg/l NAA; a_2. root formation from callus at 1.0, 3.0, 5.0 and 7.0 mg/l IBA. B. root multiplication on medium supplemented with 1.0, 3.0, 5.0 mg/l NAA.

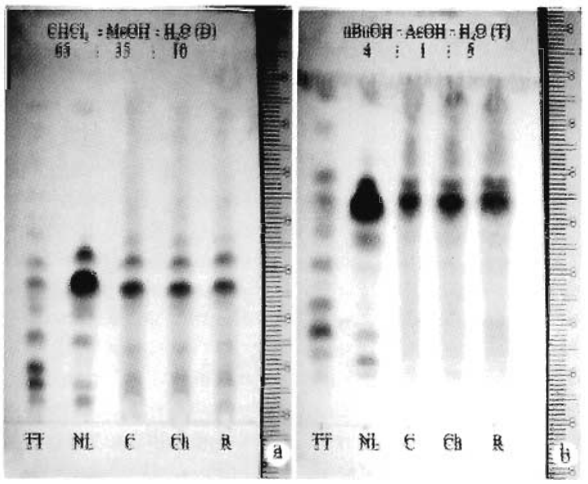

Figure 5. Saponin detection in callus, shoot and root biomass of *in vitro* cultured Ngoc Linh ginseng. a. TLC results using $CHCl_3$ - MeOH - H_2O as solvent system; b. TLC results using BuOH - AcOH - H_2O as solvent system. (TT: Korean ginseng standard, NL: Ngoc Linh standards. C, Ch, R: saponins from callus, shoot and root, respectively).

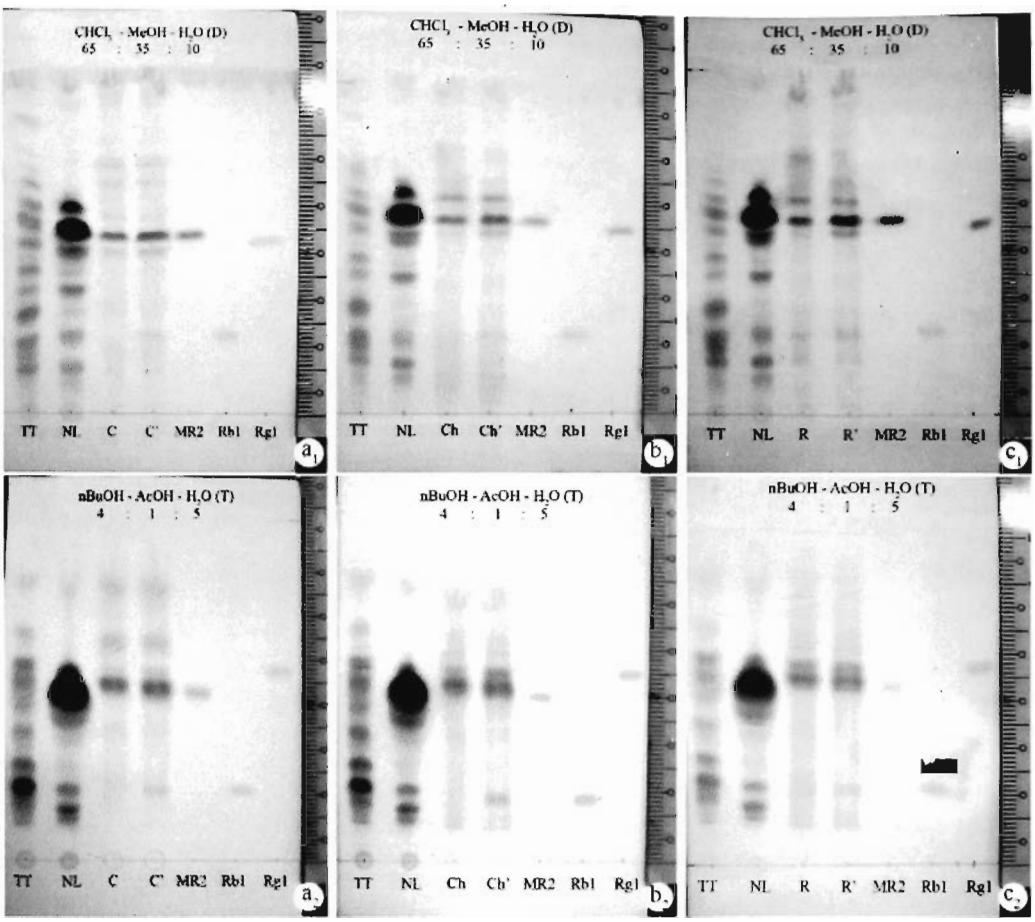

Figure 6. Saponin detection in callus, shoot and root biomass in *in vitro* Ngoc Linh ginseng using one-spot TLC. a_1, b_1, c_1. TLC color chart using $CHCl_3$ - MeOH - H_2O as solvent system; a_2, b_2, c_2. TLC results using BuOH - AcOH - H_2O as solvent system. (TT: Korean ginseng standard, NL: Ngoc Linh standards. C', Ch', R': saponins from callus, shoot and root using one-spot loading, respectively. MR2, Rb1, Rg1: majonoside-R2, ginsenoside-Rb1, ginsenoside-Rg1, respectively).

CONCLUSION

Ngoc Linh Ginseng callus was successfully induced from leaves and petiole cultured in MS medium supplemented with 1.0 mg/l 2,4-D, 0.2 mg/l TDZ in photoperiod 16 hours/day. In callus multiplication stage, MS medium supplemented with 1.0 mg/l 2,4-D, 0.3 mg/l TDZ was chosen. Among three explant sizes (0.5 x 0.5; 0.7 x 0.7; 1.0 x 1.0), the smallest callus (0.5 x 0.5) showed the best growing rate. Callus regeneration was optimal with MS medium supplemented with 1.0 mg/l BA and 1.0 mg/l NAA. Subsequently, ½MS medium containing 1.0 mg/l BA, 0.5 mg/l NAA, 50 g/l sucrose and 2.0 g/l AC offered the most suitable conditions for shoot development. In case of callus-derived root formation, the callus culture on MS½ medium containing 3.0 mg/l NAA offered the highest root formation ratio, number and fresh weight ratio. MS½ medium containing 5.0 mg/l NAA best stimulated root multiplication, gave the highest secondary root formation ratio. Saponin components qualification by TLC showed that ginsenoside-Rg1 and majonoside-R2 presented in callus, shoot and root biomass, while ginsenoside-Rb1 only presented in root biomass.

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MỘT SỐ YẾU TỐ ẢNH HƯỞNG ĐẾN SINH KHỐI CỦA CÂY SÂM NGỌC LINH (*PANAX VIETNAMENSIS* HA ET GRUSHV.) NUÔI CẤY *IN VITRO* VÀ BƯỚC ĐẦU PHÂN TÍCH HÀM LƯỢNG SAPONIN

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TÓM TẮT

Mô sẹo được cảm ứng thành công ở các mẫu lá và cuống lá trên môi trường MS bổ sung 1,0 mg/l 2,4-D và 0,2 mg/l TDZ trong điều kiện chiếu sáng 16 h/ngày và mô sẹo (0,5 × 0,5 cm) có khả năng tăng sinh nhanh trên môi trường MS có bổ sung 1,0 mg/l 2,4-D và 0,2 TDZ. Số chồi tái sinh từ mô sẹo đạt cao nhất trên môi trường MS bổ sung 1,0 mg/l BA, 1,0 mg/l NAA và 50 g/l sucrose. Trong giai đoạn tăng trưởng chồi, môi trường ½MS được bổ sung 1,0 mg/l BA và 0,5 mg/l NAA, 50 g/l sucrose, 2,0 g/l than hoạt tính là tốt nhất cho quá trình tăng

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trường chồi. Đối với quá trình ra rễ từ mô sẹo, các mẫu mô sẹo được nuôi cấy trên môi trường MS½ có bổ sung 3,0 mg/l NAA cho tỷ lệ ra rễ cao nhất, số lượng rễ nhiều nhất và tỷ lệ trọng lượng tươi của rễ/mẫu cao nhất. Môi trường MS½ có bổ sung 5,0 mg/l NAA kích thích sự nhân rễ tốt nhất, cho tỷ lệ ra rễ cao nhất và rễ phân nhánh nhiều nhất. Kết quả phân tích định tính saponin bằng phương pháp TLC cho thấy trong sinh khối mô sẹo, sinh khối chồi và sinh khối rễ nhận được đều có ginsenoside-Rg1 và majonoside-R2, riêng sinh khối rễ còn có ginsenoside-Rb1.

Từ khóa: *Cảm ứng, chồi, mô sẹo, Panax vietnamensis, rễ, saponin*