International Journal of Medical Toxicology & Legal Medicine **Volume 27, No. 5, 2024**
e-ISSN: 0974-4614
p-ISSN: 0972-0448
https://doi.org/10.47059/ijmtlm/V27I5/038

Study on asexual propagation for Vietnamese ginseng species by tissue culture

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Received: 15.09.2024 Revised: 02.10.2024 Accepted: 09.11.2024

ABSTRACT
Plant tissue culture has been widely employed to maintain and conservemany important medicinal and endangered plant species. In this study, samples of four Vietnamese ginseng belonging to the Panax genus includingNgoc Linh, Lai Chau, Vu Diep and Tam That Hoang species were propagated by tissue culture technologies. MS medium supplemented with 05 mg/L 2.4-D was suitable for callus induction from rhizome tissue while MS supplemented with 1mg/L 2.4-D, and was suitable for callus induction from tuber tissue. The highest embryogenesis rate was exhibited on a medium containing MS + 1.0mg/L 2.4-D + 1mg/L NAA + 0.5mg/L TDZ for Ngoc Linh ginseng, Lai Chau ginseng and Vu Diep ginseng, while Tam That Hoang required to decrease the concentration of TDZ to 0.3mg/L, respectively. The optimum media for embryos that germinated into plantlets was MS + 0.5 mg/L NAA + 1.0 mg/L BA. The best rooting occurred in medium SH1/2 + 1.0 mg/L NAA + 0.2 mg/L BA + 0.2 g/L activated charcoal. In order to increase the survival rate of in vitro plantlets in the nursery, this research investigated the effect of substrate on the growth of ginseng plants propagated by tissue culture. In substrate composition, humus in mountain soils, perlite, vermiculite, and peat moss were used in different conditions (net house and natural condition).Our results demonstrated that treatment T3 (mixture of humus in mountain soils, perlite, and veclite at 2:1:3 was more favorable to the survival of ginseng seedlings after transplantation and the survival rate of the seedlings transplanted for 6 months was the highest (82.74%).Therefore, this study completed the propagation process starting from callus induction to plantlet regenerationfor tissue culture seedings of Vietnamese ginseng.

Keywords: In vitro, Plant tissue culture, Lai Chau ginseng, Ngoc Linh ginseng

INTRODUCTION

Ginseng, Panax ginseng is one of the most valuable oriental herbs in the plant kingdom (Wu et al., 1999). Among its components, ginsenoside is the most valuable secondary metabolite for the treatment of several medical ailments (Lee et al., 2021, Zhou and Brown, 2006). In Vietnam, 4 species belong to Panax genus whichis naturally distributedin conservation areas, namely Vu Diep ginseng (Panax bipinatifidus), Tam ThatHoang (Panax stipuleanatus) distributed in Hoang Lien Son mountain range (Lao Cai province); Puxailaileng ginseng, also known as Lao ginseng, found in the high mountains of Phu Xai Lai Leng in the Truong Son range (Nghe An province)and Vietnamese ginseng (Panax vietnamensis Ha et Grushv.) with the common name Ngoc Linh ginseng. Vietnamese Ginseng(Panax vietnamensis Ha et Grushv.)includes3 sub-species: Ngoc Linh ginseng (Panax vietnamensis Ha et Grushv. var. vietnamensis), distributed in Kon Tum and Quang Nam; Lai Chau ginseng–(Panax vietnamensis var. fuscidiscus)distributed in Lai Chauand the novel Vietnamese ginseng (Panax vietnamensis var. Langbianensis)distributed in Lang Biangmountain (Lam Dong) (Ministry of Science and Technology, 2007; Duy et al., 2016;Long et al., 2014).

In recent years, Vietnamese ginseng species in the wild are increasingly scarceand in danger of extinction due to overexploitation.They are generally propagated through the seeds from the mother plant. This conventional method of propagation is very inefficient and time-consuming because of the slow growth cycle of the plant (Uchendu et al., 2011; Nhung et al., 2024).Hence, the rapid propagation of elite clones and the production of healthy and disease-free plants are urgently needed in Vietnamese ginseng improvement programs. To address this, the tissue culture technique is a practical approach to accelerate plant production. There are only a few

reports on the in vitro cultivation of ginseng in Vietnam.Some recent studies have shown the success of using the technique of somatic embryosvia the callus stage to quickly propagate Ngoc Linh ginseng, Lai Chau ginseng and Vu Diep ginseng (Hien et al., 2016, Mai et al., 2013; Linh et al., 2017a, 2017b; Luong et al., 2019).This study aimed to create an in vitro protocol for some Panax genera in Vietnam. This is the first report carrying outthe process of clonal propagation of ginseng varieties (Ngoc Linh, Lai Chau, Vu Diep and Tam That Hoang) from callus induction to complete plantlets in the nursery stage.

MATERIALS AND METHODS

Materials

Ngoc Linh ginseng samples(P. vietnamensis var. vietnamensis) were collected in nature conservation areas in Quang Nam and Kon Tum; Lai Chau ginseng samples Chau (P. vietnamensis var. fuscidiscus)were collected in Lai Chau; Vu Diep ginseng samples (Panax bipinatifidus)and Tam That Hoang ginseng samples(Panax stipuleanatus) were collected in Lao Cai province.

Methods

Sterilization and sample preparation

Explants were pre-washed under running water for 10 min, then sterilized with 0.7% methyl thiophanate solution and 0.1% streptomycin solution for 20 m, 1.5% sodium hypochlorite solution for 10 m, and 70% ethylalcohol solution for 1 minute, respectively.After each treatment witha sterile solution, the material samples were washed with sterile distilled water and then drained.

Culture medium and growing conditions

Murashige and Skoog (MS), Schenk and Hildebrandt (SH) basic nutrient medium supplemented with 7 g/L agar, 30 g/L sucrose and growth regulators including2.4-D (2.4Dichlorophenoxy acetic acid), NAA (Acid acetic naphthalene), TDZ (Thidiazuron), BA (Benzylaminopurine) and GA3 (Gibberellin) were used for experiments.The medium was autoclaved at 121^{o}C for 20 minutes, pH 5.8. The cultures were placed under growth room conditions at 21-23^{o}C with a 12-14 h photoperiod (1800 – 2000 lux).

Callus induction

Thin slices of root cells, rhizomes, and leaf tissues of samples were cultured on MS basal medium with different concentrations of 2.4-D (0.3, 0.5, and 1.0 mg/L). MS medium without growth regulators was used as the control. The experiment was performed with three replicates, five flasks per replicate, and five samples per flask.

Embryogenic callus generation

Callus was initially cultured on MS medium supplemented with 0.1 mg/L 2.4-D and 70 g/L sucrose. The culture was incubatedin the dark for 7-10 days, followed by a 12-h photoperiod for 4 weeks. The experiment was performed in 3 replicates, with 5 flasksand 5 samples per flask.

Somatic inductionand somatic embryogenesis

Well-developed embryogenic callus were transferred to MS medium containing 30g/L sucrose and supplemented with 2.4-D growth regulator (0.5 - 1.0 mg/L) simultaneously or separately, NAA (0.5 - 1.0 mg/L) and TDZ with the different concentrations (0.1, 0.3, 0.5 and 1.0 mg/L). The somatic embryo formation was evaluatedafter 2 - 3 months. The experiment was performed with at least 3 replicates with 5 flasks, and 5 samples per flask.

Somatic embryo germination and complete plantlet development

Somatic embryos were cultured on MS basic mediums with optimal sugar concentration including MS + 0.5 mg/L GA3; MS + 1.0 mg/L GA3; MS + 3.0 mg/L GA3; MS + 5.0 mg/L GA3; MS + 1.0 mg/L NAA + 0.5 mg/L BA; MS + 1.0 mg/L NAA + 1.0 mg/L BA; and MS + 0.5 mg/L NAA + 1.0 mg/L BA.MS medium without a growth regulator was used as the control.The experiment was carried out in 3 replicates, with 5 flasks, and 5 samples per flask. The number of germinated embryoswas evaluated after 6 weeks.

Suitable media for the growth of complete plantlets

To investigate the effects of BA and NAA on the nutrient medium (MS and SH) on the complete plantlet growth: Plantlets with micro tubers were cultured into MS and SH medium with concentrations of 1 mg/l NAA and different concentrations of BA (0.2, 0.5 mg/l). The experiment was carried out with 3 replicates, and each with 5 flasks(25 plants in total), monitoring and evaluation after 90 days of culture.

Toinvestigate the effects of activated charcoal content on the growth and rooting ability to create complete plantlets: After determining the optimal medium for root development, plantlets with auniform height of 2.5 - 3 cm witha tuberous root diameter of 0.3 - 0.4 cm and with true leaves were used for the study of the effects of

activated charcoal at concentrations (0.2, 0.5, 1 mg/L) on the growth and rooting of complete plantlets. The experiment was carried out with 3 replicates, and each with 5 flasks (25 plants in total), monitoring and evaluation after 90 days of culture.

Toinvestigate the effects of basic nutrient content in the optimal medium on in vitroplant growth: Complete plantlets with a uniform height of 2.5 - 3 cm, tuberous root diameter 0.3 - 0.4 cm, with true leaves were cultured in an optimal nutrient medium with adequate basic nutrient content, reduced by 1/2 and 1/3 of the content in order to evaluatein vitro plant growth. The experiment was carried out with 3 replicates, and each with 5 flasks (25 plants in total), monitoring and evaluation after 90 days of culture.

Effect of different substrates on survival and growth of transplanted in-vitroin nethouse and natural environment

The experiment was designed according to Randomized Complete Block Design (RCBD) with one factor and replicated thrice. The required number of plantlets is 240 plantlets (10 plantlets/block x 4 treatments x 3 times x 2 conditions) consisting 4 blocks:

T1: 100% of humus in mountain soils(Control)

T2: Mixture of humus in mountain soils and perlite in 1:3 ratios

T3: Mixture of humus in mountain soils, perlite, veclite in 2:1:3 ratios

T4: Mixture of humus in mountain soils, vermiculite, and peat mossin 2:1:1

Nethouse condition: The experiments were performed at Tu Mo Rong Pharmaceutical Co-operative, Tu Mo Rong district, Kon Tum province. The average day and night temperature varies from 9 – 23.60C. The monthly average of 193 sunshine hours, humidity 76.8-80% (outdoors) and 80-85% (forest canopy), the cover over 85% (using black mesh)

Natural conditions: The nursery was located 1700 m above sea level, under the canopy of a natural forest with a thick layer of decayed vegetation and high humidity. Temperature 16-18℃, humidity 85-90%, 2000-2500 mm rainfall/year, light intensity 7-15 µmol m-2s-1 were established.The plantlets were monitored for 6 months.

Time and experiment site

The study was carried out at the Department of Molecular Biology, Agricultural Genetics Institute and Tu Mo Rong Pharmaceutical co-operative, Kontum (14°58′22″N 108°0′2″E)from January 2017 to December 2020.

Data analysis

All experiments were in triplicate, and each replicated with 5 explants in 5 culture flasks per replicate and under the environment. All data were subjected to analysis by using Excel 2010 and IRRISTAT 5.0.

RESULTS AND DISCUSSION

Callus induction

Callus induction was observed within 5 weeks, after inoculating the explants on MS medium containing different concentrations of 2.4-D. Although in all concentrations of 2.4-D (0.3; 0.5; and 1.0 mg/L) the callus induction was triggered, however, more profuse callus induction was observed at 1.0 mg/L of 2.4-D with full potential of callus regeneration from the leaf and tuber tissue samples of the cultivated varieties. In this media composition, the explants produced light yellow callus. The percentage of callus induction was 80-100 % (Table 1). However, 0.5 mg/L of 2, 4-D produced the highest percentage of callus induction from rhizome tissue in a variety of Ngoc Linh ginseng, Tam That Hoang and Lai Chau ginseng.

These results suggest that callus induction from leaf segments and tube tissues required higher concentrations of plant growth regulators than callus induction from rhizome segments.

Table 1. Effects of 2.4-D on the formation of callus in Ngoc Linh ginseng

Medium	Avg. rate of callus formation (%)		Characteristics
	Tubers	Rhizome	
MS	0.0	0.0	-
MS + 0.3 mg/L 2.4-D	62.5	90.0	Light yellow,
MS + 0.5 mg/L 2.4-D	75.0	100.0	friable
MS + 1.0 mg/L 2.4-D	87.5	80.0	
$LSD_{(0.05)}$	3.1	2.0	-
CV $_{(\%)}$	1.5	1.2	-

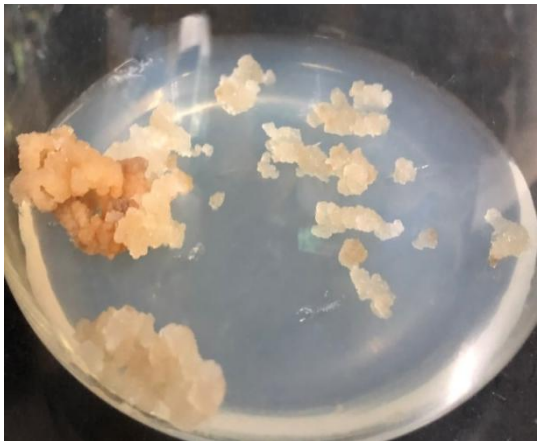

Figure 1. Ngoc Linh ginseng callus formed from rhizomeon MS medium + 0.5mg/L 2.4-D.

Embryogenic callus generation

In this study, the initial callus was culturedin MS medium with high sugar content (70 g/L) and a reduced concentration of 2.4-D to 0.1 mg/L. After 7 - 10 days of culture in the dark and 4 weeks in the light condition for 12 - 14 hours/day, callus was generated inembryos (Figure 2).

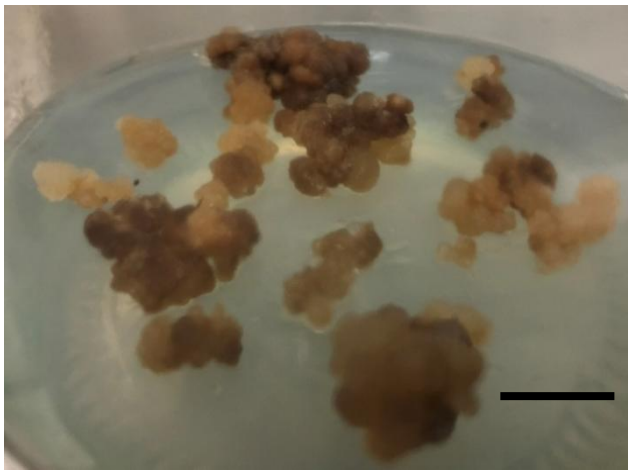

Figure2. Callus tissue generated Ngoc Linh ginseng embryos

Somatic induction and somatic embryogenesis

The embryogenic callus masses were subdivided and transferred into the medium for somatic embryogenesis induction. After 8-12 weeks, the number of somatic embryogenesis was calculated. MS medium supplemented with 1.0 mg/L 2.4-D + 1.0 mg/L NAA + 0.5 mg/L TDZ yielded the highest conversion rate of somatic embryogenesis in Ngoc Linh ginseng, Lai Chau ginseng and Vu Diep ginseng, while MS + 1.0 mg/L 2.4-D + 1.0 mg/L NAA + 0.3 mg/L TDZ was the best for Tam that hoang somatic induction. According to Nhut et al. (2012), the medium supplemented with 2 mg/L NAA was suitable for generating Ngoc Linh ginseng somatic embryos after 8 weeks of incubation in the dark. Mai Truong et al. (2013) reported that Ngoc Linh ginseng also induced somatic embryos (embryo tissue) in MS medium supplemented with 1.0 mg/L 2.4-D, 1 mg/L NAA, 0.2 mg/L Kinetin and 10% coconut water. Besides, Zhang et al. (2014) found that 2.4-D at a concentration of 0.5 mg/L was suitable for inducing embryogenesis in Panax notoginseng and Korean ginseng Panax ginseng. This suggested that different species resulted in different abilities to induce somatic embryogenesis.

Investigations on the regeneration of Panax species through somatic embryogenesis have been carried out by many workers (Tirajoh et al., 1998; Choi and Jeong, 2002; Zhou and Brown, 2006). In this study, somatic embryos in Ngoc Linh ginseng, Lai Chau ginseng and Vu Diep ginseng began to form after 8-12 weeks. Medium supplemented with only 2.4-D (0.5 mg/L), NAA (0.5 mg/L) or with TDZ (0.1 - 1.0 mg/L) in combination with 0.5 mg/L 2.4-D were not able to induce clonal embryo formation but only provide a strong growth of callus, especially on MS+0.5 mg/L 2.4-D + medium 0.3 mg/L TDZ.

The results in Table 2 show that somatic embryoswere formed in the medium supplemented with 0.5 mg/L NAA and TDZ at the same time, or 1.0 mg/L 2.4-D and 1.0 mg/L NAA, or 1.0 mg/L 2.4-D, 1.0 mg/L NAA and TDZ. However, the time and rate of somatic embryos formed differed among culture media.

Table 2. Effects of 2.4-D, NAA and TDZ on somatic embryogenesis from callus of Ngoc Linh ginseng

Auxin \ Cytokinin	TDZ, mg/L	Proportion of somatic embryoformed (%)	Avg. number of somatic embryos/callus
0.5 mg/L NAA	0	0	0
	0.1	45.0	12.6
	0.3	80.0	18.5
	0.5	50.0	16.3
	1.0	20.0	12.1
1.0 mg/L 2.4-D + 1.0 mg/L NAA	0	35.0	12.2
	0.1	55.0	13.6
	0.3	85.0	17.5
	0.5	95.0	20.1
	1.0	45.0	10.7
$LSD_{(0.05)}$		1.8	0.5
$CV_{(\%)}$		1.8	2.0

For the culture medium supplemented with only 1 mg/L 2.4-D and 1.0 mg/L NAA, embryogenesis was relatively slow (12 weeks) with a high percentage of soma of 35% and the average number of embryos per explant was 12.2. Meanwhile, in the medium supplemented with TDZ, embryogenesis took place earlier (8 weeks) and the embryo formation rate, as well as the number of embryos per explant, were higher. The medium was supplemented with 1.0 mg/L 2.4-D simultaneously, 1.0 mg/L NAA and 0.5 mg/L TDZ had the highest embryogenesis rate, reaching 95.0%.

The results of soma embryogenesis induction from Tam ThatHoang callus showed a positive effect of TDZ on embryo induction rate and number of embryos/sample (Figure 3). However, when the concentration of TDZ added to the medium was greater than 0.3 mg/L, this ratio began to decrease. The best medium for induction of somaticembryogenesis from Tam That Hoang callus was MS + 1.0 mg/L 2.4-D + 1.0 mg/L NAA + 0.3 mg/L TDZ. This result is different from the results when studying on thesamples of Ngoc Linh ginseng,Vu Diep ginseng and Lai Chau ginseng (the optimal medium was 1.0 mg/L 2.4-D + 1.0 mg/L NAA + 0.5 mg/L TDZ). This further confirms, for different species the embryo induction medium used is different.

Figure3. Soma of Tam That Hoang from embryogenic callus

Germination of somatic embryosand development of complete plantlets development

To optimize the development of embryos into complete plantlets, the experiment was conducted to study the effects of GA3, NAA, and BA on the ability of embryos to develop into plantletsin vitro with micro tubers (mini tubers). The germination rate and development of embryos into plantlets after 6 weeks of culture were evaluated.The obtained results are given in Table 3.

In Table 3 all culture media supplemented with growth regulators GA3, NAA and BA gave a higher percentage of embryos germinating than control (no growth regulators). However, the growth characteristics of in vitroplantlets in these media depended on the type and concentration of growth regulators used.

Table 3. Effects of GA3, NAA and BA on the germination of embryos into plantlets and tubers in vitro of Ngoc Linh ginseng

Culture medium	The percentage of embryos germinated into in vitro plantlets after 6 weeks of culture (%)	Characteristics of plantlets in vitro
MS	26.5	Slow growth, no micro tubers produced
MS + 0.5 mg/L GA3	31.0	Foliage development, no micro tubers produced
MS + 1.0 mg/L GA3	38.5	Slow growth, no micro tubers produced
MS + 3.0 mg/L GA3	51.5	Foliage development, no micro tubers produced
MS + 5.0 mg/L GA3	88.0	Foliage development, no micro tubers produced
MS + 1.0 mg/L NAA + 0.5 mg/L BA	60.5	Regular plant growth, micro tubers produced
MS + 1.0 mg/L NAA + 1.0 mg/L BA	45.5	Regular plant growth, micro tubers produced
MS + 0.5 mg/L NAA + 1.0 mg/L BA	89.0	Good plant growth, micro tubers produced
$LSD_{(0.05)}$	0.7	-
CV $_{(\%)}$	0.7	-

In Table 3, all the formulas containing GA3 gave somas germinated into plantlets with strong leaf stem but no micro tubers were produced. That proves, for Ngoc Linh ginseng, the growth regulator GA3 when used alone in the culture medium only has the effect of stimulating the germination of somas. This result is similar to the research results of Zhang et al. (2014) on Korean ginseng. Besides, in the remaining experimental treatments (Medium6, Medium7 and Medium8), embryos germinated into well-developed, growing plantlets with micro tubers formed (Figure 4), especially in Medium8 (MS + 1.0 mg/L BA and 0.5 mg/L NAA) had the highest percentage of somas germinating into plantlets with micro tubers reaching 89%. This is a very important feature that determines the growth of tubers at the next stage as well as the survival rate of in vitro plants at the nursery stage. Thus, in this case, growth regulators BA and NAA at a certain concentration not only have the effect of stimulating embryo germination but also help plantlets to develop microtubes (Figure 4).

The germination rate and embryonic development into plantlets after 6 weeks of the culture of Vu Diep ginseng, Tam That Hoang ginseng and Lai Chau ginseng were evaluated.The results obtained were similar to those of Ngoc Linh ginseng. The study results confirmed that the combination of BA and NAA at the ratio of 1 mg/L:0.5 mg/L achieved the highest efficiency for embryo germination and micro tuber formation. Specifically, MS medium + 1.0 mg/L BA and 0.5 mg/L NAA, the percentage of embryos that germinated into plantletswith micro tubers was the highest at 88.5% for Vu Diep ginseng, 89.5% for the Tam ThatHoang ginseng and 90% for Lai Chau ginseng.

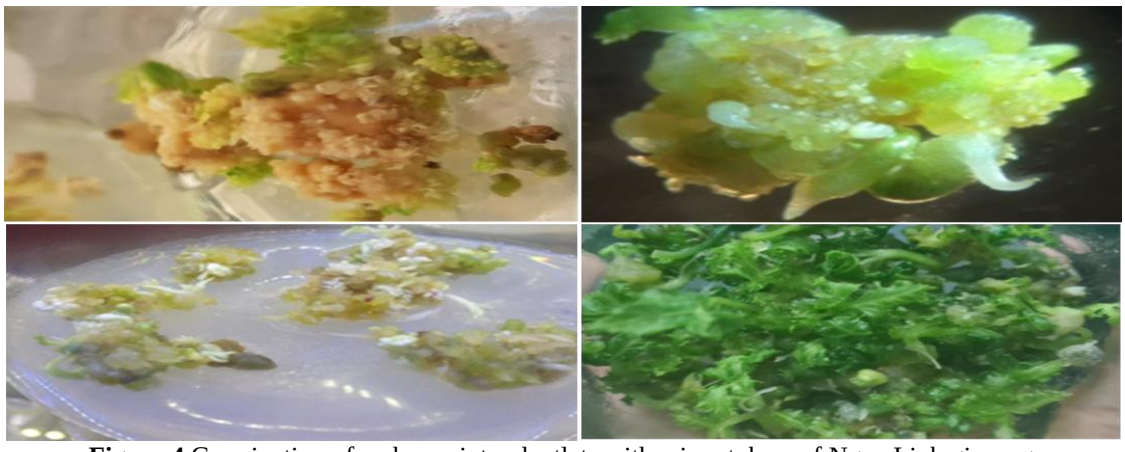

Figure 4. Germination of embryos into plantlets with micro tubers of Ngoc Linh ginseng

Research results on suitable media for the growth of complete plantlets of Vietnamese ginseng samples

To ensure high survivability when transferring in vitro ginseng plants to the nursery,ginseng plants need to have strong roots. Ngoc Linh ginseng with micro tuber was cultured on two medium MS and SH supplemented with growth regulator NAA (1.0 mg/L) alone or in combination with BA (concentrations of 0.2 and 0.5 mg/L) to evaluate their impact on the rooting ability of tubers after 90 days of culture.

The results showed that the type of nutrient medium and growth regulator used significantly affected the rooting ability of in vitroNgoc Linh ginseng (Table 4). Interms of nutrient medium, it was found that SH showed superiority in the number of roots formed compared to MS medium (Figure 5).

Table 4. Effect of nutrient medium and growth regulators on the rooting ability of plants with micro tuber of Ngoc Linh ginseng

Growth regulator concentration	Average number of roots (piece)		Average
	MS	SH	
1.0 mg/L NAA + 0.5 mg/L BA	1.2	2.6	1.9
1.0 mg/L NAA + 0.2 mg/L BA	1.6	3.8	2.7
1.0 mg/L NAA	2.0	5.5	3.3
$LSD_{0,05}$	0.8	0.4	-
CV (%)	2.2	4.5	-

The average number of roots of a plant on SH medium was 4.0 roots, while on MS medium only 1.6 roots. This result is similar to the published results on American ginseng and Korean ginseng. Research by Zhang et al. (2014) suggested that the NH_4NO_3 salt content in MS medium was 8 times higher than in SH medium, which was the cause of limiting root growth.

Figure 5. Photo of in vitro Ngoc Linh ginseng on MS and SH media

Analysis of the effect of growth regulators on the rooting ability of in vitroNgoc Linh ginseng showed that the plants had the highest number of roots when adding 1.0 mg/L NAA to the nutrient medium. (3.3 roots). When adding a combination of 1.0 mg/L NAA with BA (0.2-0.5 mg/L) to the medium, the number of formed roots decreased, and the number of roots reached the lowest in the medium supplemented with 1.0 mg/L NAA + 0.5 mg/L BA. However, the results of observing the plant's growth showed that in the medium supplemented with BA, the plants grew better than in the medium with only NAA.The summary of analytical results showed that SH medium supplemented with 1.0 mg/L NAA and 0.2 mg/L BA was suitable for the growth and rooting of plantlets with micro tubers of Ngoc Linh ginseng.

The results of the study on the effects of activated charcoal content on the growth and rooting of in vitro Ngoc Linh ginseng plants after 90 days of culture disclosed that activated charcoal significantly affected the growth of plants (Table 5).

Table 5. Effect of activated charcoal content on growth and rooting of in vitro Ngoc Linh ginseng

Culture medium	Activated charcoal content g/L	Number of roots, piece	Growth characteristics
SH + 1.0 mg/L NAA + 0.2 mg/L BA	1.0	1.2	Slow growth of the plant white edges of the leaves
	0.5	2.3	Slow-growing plant, white edges of the leaves
	0.2	4.5	Normal growth,dark green leaves, hardened plantlets
	0.0	3.8	Good growth, green leaves
$LSD_{(0.05)}$		0.2	-
CV (%)		3.3	-

At an appropriate concentration of 0.2 g/L in SH + 1.0 mg/L NAA + 0.2 mg/L BA, plants have normal growth with dark green leaves, hardened stems, firm tubers, and a maximum number of roots (4-5 roots). When the amount of activated charcoal added to the medium was from 0.5-1.0 g/L, it is observed that the plant grows very slowly, the edges of the leaves often wilt and turn to white when newly transplanted, the number of roots formed after 8-12 weeks was small (1-3 roots), especially in the medium supplemented with 1.0 g/L activated charcoal, there was still a sign of plant death.Applying the research results on the effect of activated charcoal content on the growth and rooting of Ngoc Ling plantlets with micro tuber to 3 species of Vu Diep ginseng, Tam That Hoang and Lai Chau ginseng confirmed the high efficiency of SH medium + 1.0 mg/L NAA + 0.2 mg/L BA + 0.2 g/L activated charcoal to rooting to create complete plantlets.

The results of study on the effect of basic nutrient content in SH medium on the growth of in vitro Ngoc Linh ginseng showed that under the same culture conditions, only the basic nutrient content (SH, SH1/2, SH1/3) had differences in growth and development of in vitro Ngoc Linh ginseng (Table 6). When the basic nutrient content in the culture medium was halved, an increase in the number of roots/plants was recorded. However, in culture media reduced to 1/3 of the basal nutrient content, the same number of roots was recorded in the medium with adequate nutrient content. In this study, the results showed that Medium 2 medium had the highest number of roots, with an average of 5.1 roots/plantlet.

Analysis of the height and growth characteristics of the plants showed that when the basic nutrient content was lowest (SH1/3), the plants grew slowly, and the height measured after 90 days of culture reached 5.2-5.3cm. Most notably, in the Medium2 (SH1/2) formula, the plants grew strong and sturdy, with no sign of the formation of secondary embryos at the roots, and had a tree height of 6.8cm.

Table 6. Effect of basic nutrients on the growth of Ngoc Linh ginseng

Formula	Medium		Number of roots/plant	Plant height (cm)	Growth characteristics
Medium 1	SH + 30g/l sucrose	+ 1,0 mg/L NAA + 0,2 mg/L BA + 0.2 g/L activated charcoal	4.4	6.5	Fast, healthy, no somatic embryos at the stem base
Medium 2	SH1/2 + 30g/l sucrose		5.1	6.8	Fast, healthy, no somatic embryos at the stem base
Medium 3	SH1/3 + 30g/l sucrose		4.5	5.2	Slow, weak, no somatic embryogenesis at the stem base
$LSD_{(0.05)}$			0.3	0.5	
CV (%)			2.5	3.4	

Complete plantlets of Vietnamese ginseng samples cultured on SH1/2 + 1.0 mg/L NAA + 0.2 mg/L BA + 0.2 g/L activated charcoalwere suitable for growth and rooting of plantlets with micro tubers (Figure 5).

Figure 5. Completein vitroNgoc Linh ginseng with roots, micro tubers, stems and leaves on optimal nutrient medium

Effect of substrates on survival and growth of in VitroNgoc Linh ginseng

The study was conducted at the beginning of the dry season (December 2020). The total of in vitro Ngoc Linh Ginseng was 300 plants. Normally, 7 days after planting, the leaves begin to drop while the tuber goes dormant. The survival, germination rate and growth were recorded after 2 months.

In Table 7, the height was assumed to be equal among treatments after 6 months in natural conditions at Tu Mo Rong Pharmaceutical co-operative. However, the survival rates were significantly different. The highest rate was observed in treatment T3 (83.25%) (Mixture of humus in mountain soils, perlite, and veclite in 2:1:3 ratios) while it was lowest in the control T1 (66.37%). In treatment T3, the germination rate was highest (83%). It was lower than the result of Duong Tan Nhut et al., 2010 and higher than the result of Phan Cong Du et al., 2019 at Tuyen Lam Lake, Lam Dong. It recorded the highest root length (8.75 cm) in treatment T3.

Figure 6. Invitro Ngoc Linh ginseng in the nethouse conditions

a. Invitro ginseng seedlings were protected by plastic cups; b. 6 months after planting

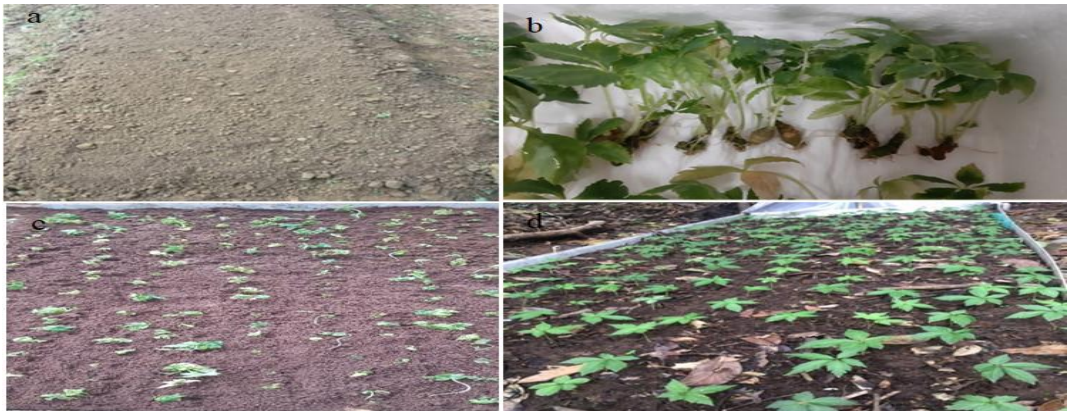

Figure 7. In vitro Ngoc Linh Ginseng at Tu Mo Rong Pharmaceutical Cooperative

a. Preparing the soil before planting in vitro Ngoc Linh ginseng; b. Standard in vitro Ngoc Linh ginseng; c. In vitro Ngoc Linh ginseng on the bed; d. 6-month Ngoc Linh ginseng after planting.
It could be concluded that the substrates affect the growth ability and survival rate of in vitro Ngoc Linh ginseng in net house and natural conditions. The mixture of humus in mountain soils, perlite, and vermiculite in 2:1:3 ratios was the best treatment. In vitro seedlings adapt quickly to the environment and have a high survival rate.

Table 7. Effect of substrates on the adaptability of invitro ginseng in net house condition after 6 months

Treatment	Survival rate (%)	Germination rate (%)	Root length (cm)	Height (cm)	Main growth characteristics
T1	66.37	80.0	7.39	8.01	The trees were deciduous after planting, tuberous in the dormant stage, and the terminal bud was actively developing and elongating.
T2	76.57	82.0	7.49	8.23	The trees were deciduous after planting, tuberous in the dormant stage, and the terminal bud was actively developing and elongating.
T3	83.25	86.0	8.75	8.58	The trees were deciduous after planting, tuberous in the dormant stage, and the terminal bud was actively developing and elongating.
T4	70.15	83.0	7.66	8.35	The trees were deciduous after planting, tuberous in the dormant stage, and the terminal bud was actively developing and elongating.
$LSD_{(0.05)}$	-	-	0.87		
$CV_{(\%)}$	-	-	5.6	-	

Table 8. Effect of substrates on the adaptability of invitro ginseng in natural conditions after 6 months

T	SR (%)	GR (%)	RL (cm)	H(cm)	Growth characteristics
T1	64.18	78.0	6,69	7.31	The trees were deciduous after planting, tuberous in the dormant stage, and the terminal bud was actively developing and elongating.
T2	74.35	80.0	6.81	7.62	The trees were deciduous after planting, tuberous in the dormant stage, and the terminal bud was actively developing and elongating.
T3	82.75	83.0	8.05	7.85	After planting, the trees were deciduous and tuberous in the dormant stage, and the terminal bud was actively developing and elongating.
T4	69.38	81.0	6.99	7.65	After planting, the trees were deciduous, and tuberous in the dormant stage, and the terminal bud was actively developing and elongatingin March.
$LSD_{(0.05)}$	-	-	0.48	-	-
$CV_{(\%)}$	-	-	3.4	-	-

Note: T: Treatment; SR: Survival rate; GR: Germination rate; RL: Root length; H: height; -: Unavailable

Figure 8. Ngoc Linh ginseng grows well in field trial

CONCLUSIONS

A highly efficient system for plant micropropagation of Vietnamese ginseng from explants was established with a simplified five-stage protocol: callus induction, somatic embryo induction, embryo germination, development of plantlets, and acclimation into the substrate.We optimized the propagation procedure so that in vitro plantlets can be efficiently produced, recovered and transplanted into the field. Plant tissue culture technology has completed the propagation process of Vietnamese ginseng varieties. The medium MS + 0.5 mg/L 2.4-D was suitable for the induction of callus from the rhizome,and MS + 1.0 mg/L 2.4-D was suitable for the induction of callus from tuberous tissue of Vietnamese ginseng samples. The percentage of somatic embryos formed was highest on MS medium + 1.0 mg/L 2.4-D + 1 mg/L NAA + 0.5 mg/L TDZ on samples ofNgoc Linh ginseng, Lai Châu ginsengandVu Diep ginseng. Particularly for samples of Tam That Hoang, the medium MS + 1.0mg/L 2.4-D + 1mg/L NAA + 0.3mg/Lachieved the highest efficiency in forming somatic embryos.The optimal medium for germinating and developing embryos into plantletswas MS + 0.5 mg/L NAA + 1.0 mg/L BA. Complete plantlets of ginseng samples cultured on SH1/2 + 1.0 mg/L NAA + 0.2 mg/L BA + 0.2 g/L activated charcoal suitable for growth and rooting of plantlets with micro tubers.

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