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HIGH EFFICIENT MICROPROPAGATION OF *Panax vietnamensis* Ha et Grushv. FROM IMMATURE ZYGOTIC EMBRYOS

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Abstract

Despite its significant economic benefit as medicinal plant, cultivation of Ngoc Linh ginseng (*Panax vietnamensis* Ha et Grushv.) has not been enlarged due to limited source of planting material. Even though considerable research efforts to develop tissue culture systems have been made, micropropagation of Ngoc Linh ginseng is still considered difficult. Here, a micropropagation protocol was established for Ngoc Linh ginseng through somatic embryogenesis from immature zygotic embryos. Direct somatic embryogenesis from immature zygotic embryos was obtained at a rate of 44.33 % on Schenk and Hildebrandt (SH) basal medium with 7 % sucrose. Secondary somatic embryogenesis with proliferation coefficient of 26.71 was induced in liquid-shake culture for 4 week in $^1/_2$ SH medium with 0.1 mg/l kinetin and 0.1 mg/l thidiazuron. The SH medium supplemented with 1 mg/l α-naphthalenacetic acid, 0.5 mg/l benzyladenine and 5 mg/l gibberellic acid stimulated germination of somatic embryos and early formation of microrhizomes. On two next 10-week cultures on SH medium with 0.5 mg/l α-naphthalenacetic acid, 1 mg/l benzyladenine and 4 % sucrose, micropropagated plantlets were produced with well-developed root system and thickened microrhizome with a dormant bud. The acclimatization of micropropagated plantlets were successfully accomplished with survival rate of 91.67 % when using the soil mixing forest humus with sand in a ratio of 1:2. An efficient protocol developed for Ngoc Linh ginseng micropropagation from immature zygotic embryos comprised five steps, including embryogenesis induction, proliferation of somatic embryos, germination of somatic embryos, development of plantlets, and acclimatization of plantlets.

Keywords: *Panax vietnamensis* Ha et Grushv., Vietnamese ginseng, Ngoc Linh ginseng, in vitro culture, immature zygotic embryo, somatic embryogenesis, somatic embryo germination, microrhizome, micropropagation

Vietnamese ginseng (*Panax vietnamensis* Ha et Grushv.) belonging to the ginseng family *Araliaceae* is a valuable and endemic medicinal plant. It is thus so-called Ngoc Linh ginseng. In nature, Ngoc Linh ginseng grows only above 1.200 m of altitude and under the canopy of tropical moist forests with daytime temperature, ranging from 20 to 25 °C and at night from 15 to 18 °C. Ngoc Linh ginseng is a slow-growing, long-lived herbaceous perennial plant with a height from 40 to 100 cm. Its bamboo-like rhizomes are characterized by permanent scars caused by the annual loss of aerial stem. The rhizome of Ngoc Linh ginseng contains 52 saponins, among which 24 are not found in other ginsengs [1]. Saponin content

in the rhizome of Ngoc Linh ginseng is higher than that in other *Panax* species [2, 3].

Despite high economic value, cultivation of Ngoc Linh ginseng is difficult to expand to a large scale. Since 1983, many studies have aimed to develop Ngoc Linh ginseng as a medicinal plant [4].

Based on successful micropropagation of Korean ginseng (*Panax ginseng* C.A. Meyer) and other ginseng species, there are a number of studies on Ngoc Linh ginseng tissue culture, such as organogenesis [5], artificial seed formation [6], rhizome formation [7], somatic embryogenesis [8-12]. Some studies have focused on secondary and adventitious root culture [13] for biomass production of Ngoc Linh ginseng for production of ginsenosides [14-18]. Among different methods of micropropagation, somatic embryogenesis has been most studied in Ngoc Linh ginseng due to large quantity of somatic embryos produced and high frequency of plant regeneration. Truong et al. [11, 12] reported a potential of somatic embryogenesis and shoot or root morphogenesis from cell suspension culture of leaf explant-derived calli. In the study of Nhut et al. [9], a high-frequency somatic embryogenesis was also induced from the thin cell layers of main roots. However, since survival rate of plantlets after transferred to the nursery was quiet low, *in vitro* propagation of Ngoc Linh ginseng is still considered fractious

To date, no studies have been reported on the use of immature seeds of Ngoc Linh ginseng as an explant source for getting immature zygotic embryos (IZE) for in vitro culture.

In this study, we optimized a protocol for micropropagation of Ngoc Linh ginseng through somatic embryogenesis from IZEs. This protocol comprises five steps, including somatic embryogenesis induction, proliferation of somatic embryos, germination of somatic embryos, development of plantlets, and acclimatization of plantlets. The protocol will be useful for large-scale propagation of Ngoc Linh ginseng

Materials and methods. Immature seeds of Ngoc Linh ginseng were collected from fresh fruits, which were harvested from a research field in Ngoc Linh mountain (14°58′34″N and 107°54′41″E). The seeds were removed from capsules, and then sterilized by 1% NaClO for 15 min, treated with 500 mg/L streptomycin for 10 min followed by a brief immersion in 70% ethanol. Finally, the seeds thoroughly rinsed with sterile distilled water. After sterilization, IZEs were collected by removal of seed coat and $^2/_3$ endoderm that were used as explants for *in vitro* culture.

The collected IZEs were incubated on culture medium for induction of primary somatic embryos (SE). SH [19] and MS [20] basal media of different strength (full, half) supplemented with 7% sucrose were screened in combination with or without one of such plant growth regulators as 2,4-dichlorophenoxyacetic acid (2,4-D) (0.2 and 0.5 mg/l), thidiazuron (TDZ) (0.2 and 0.5 mg/l), 1 mg/l α-napththaleneacetic acid (NAA) and 3 mg/l indole-3-butyric acid (IBA). The rate of explants induced primary SEs was scored after 10 weeks of culture.

First, the formed primary SEs were separated from embryogenic tissue masses. For that, embryogenic tissue masses were transferred to half-strength SH liquid medium supplemented with 3% sucrose followed by a ratio from $^1/_5$ to $^1/_{10}$ (mass/volume). The separated primary SEs were used for proliferation of secondary SEs. We investigated the effect of two type of culture media (liquid and solid media) containing half-strength SH medium with 3% sucrose and various combination of kinetin (0.1 mg/l) and TDZ (0, 0.05, 0.1 and 0.2 mg/l). The solid medium was supplemented with 7 g/l agar, and the liquid medium was prepared at a 1:20 explant:medium ratio with shaking at 100 rpm. The proliferation coefficient

was evaluated after 4 weeks of culture, and calculated as the ratio of fresh weight of secondary SEs after 4 weeks of culture to fresh weight of inoculated primary SEs.

For germination of the secondary SEs, solid SH medium supplemented with 1 mg/l NAA and 0.5 mg/l benzyladenine (BA) in combination with different concentrations of gibberellic acid (GA_3) (0, 1, 3, 5 and 7 mg/l) was tested. After a 10-week culture, the number of germinated SEs was recorded to compute the germination frequency.

After germination, the obtained small plantlets with roots and shoots were transferred to SH medium with 0.5 mg/l NAA, 1 mg/l BA and different concentrations of sucrose (2, 3, 4, 5 and 6%) for induction of microrhizomes. After 10 weeks, the plantlets were transferred to the same medium for an additional 10 weeks for initiation of dormant buds on the microrhizomes. After a total of about 5 months of culture, the plant biomass and length of shoots were measured. The presence of dormant buds was also observed.

Well-developed plantlets with microrhizomes and dormant buds were transferred to a research field at an altitude of 1,800 m. A two-level experiment was conducted. Before transplanting, the plantlets were soaked in 3% chitosan solution for 10 min. After that, the plantlets were transplanted to plastic bags containing various types of soil mixtures: (1) forest humus, (2) $^2/_3$ forest humus + $^1/_3$ sand, (3) $^1/_3$ forest humus + $^2/_3$ sand, and (4) $^1/_5$ forest humus + $^4/_5$ sand, and (5) $^1/_3$ forest humus + $^1/_3$ coconut fiber + $^1/_3$ perlite. After 3 weeks of planting, we scored percentage of plantlets sprouted, and the survival rate and different growth parameters of plants were assessed after 3 months of planting.

All experiments were conducted with 3 replicates and 20 explants for each treatment. All cultures were maintained in culture room at 23 °C with a 14 h photoperiod.

For comparison of the results, Tukey's multiple range test was applied using Statgraphics Centurion XVI (https://www.statgraphics.com/download-statgraphics-centurion-xvi).

Results. The effect of various basal media and different plant growth regulators on SE induction was observed. Somatic embryogenesis started initiating on IZEs on all examined media after 5 weeks of culture (Fig. 1, A). After 10 weeks of culture, of 10 media examined, SH medium with 7% sucrose exhibited maximal rate of SE initiation (44.33%), which was significantly higher than other basal media (i.e. $^1/_2$ MS, $^1/_2$ SH, and MS) and the media with plant growth regulators (i.e. 2,4-D, TDZ, NAA and IBA). After 10 weeks of culture on SH medium with 7% sucrose, we observed formation of somatic embryos at several developmental stages (Figs. 1, B-D). In addition, generation of abnormal somatic embryos were also observed, with high frequency on MS media.

After a brief shake of embryogenic tissue masses in liquid medium, SEs were definitely isolated from the parent explants which were then removed (Fig. 1, E). For proliferation of the obtained SEs, the solid and liquid $^1/_2$ SH media supplemented with 0.1 mg/l kinetin and different concentrations of TDZ were tested. After 3 weeks of culture, new embryos started generating surrounding the base of the primary SEs, then formed clusters of 4-12 single embryos which were observed at different developmental stages. The induced embryos were compact and light yellow within 4 weeks of culture (Figs. 1, F, G), and became greenish yellow when continuing the culture. The lowest efficiency of secondary embryogenesis was observed on $^1/_2$ SH medium with single addition of kinetin (0.1 mg/l) in both solid and liquid-shake cultures, with proliferation coefficient of 8.63 and 8.27, respectively. On the solid culture, coefficient of SE proliferation increased

gradually from 8.63 to 13.9 with the presence and increasing concentration of TDZ, especially, at 0.2 mg/l TDZ. Interestingly, secondary embryogenesis was significantly improved in the liquid-shake culture in comparison with the solid culture of the same medium composition. The highest proliferation coefficient (26.71) was achieved in liquid 1/2 SH medium with 0.1 mg/l kinetin and 0.1 mg/l TDZ.

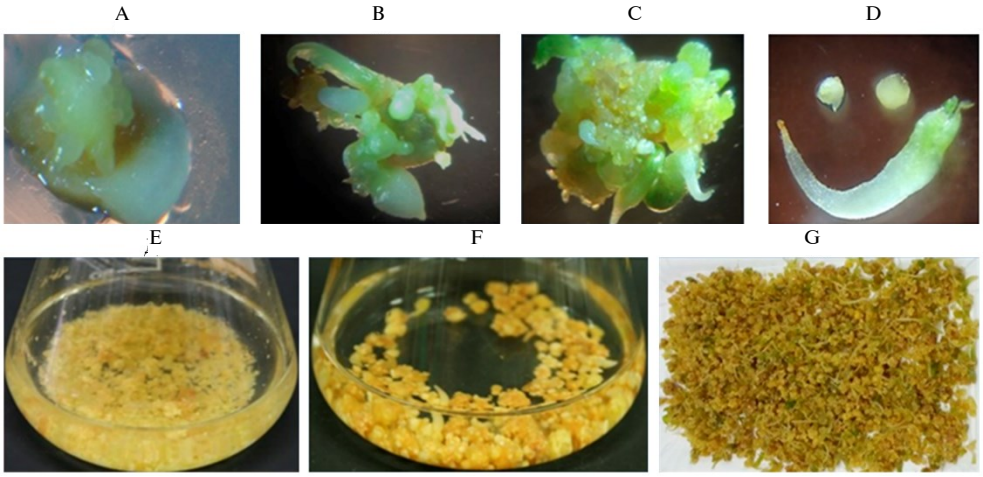

Fig. 1. Direct somatic embryogenesis in in vitro culture of immature zygotic embryos (A, B, C, D) **and proliferation of secondary somatic embryos** (E, F, G) **of Ngoc Linh ginseng (*Panax vietnamensis* Ha et Grushv.):** A — somatic embryogenesis after 5 weeks of culture; B — somatic embryogenesis after 8 weeks of culture; C — somatic embryogenesis after 10 weeks of culture; D — primary somatic embryos at globular and bipolar stages; E — after isolation of primary somatic embryos; F — after 2 weeks of somatic embryo proliferation; G — after 4 weeks of somatic embryo proliferation

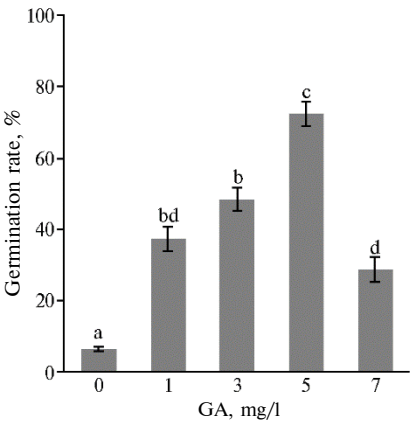

Fig. 2. Effect of GA_3 concentration on germination of somatic embryos of Ngoc Linh ginseng (*Panax vietnamensis* Ha et Grushv.) in in vitro culture. Differences between values marked with different letters are statistically significant according to Tukey's multiple range test at $p < 0.05$.

Germination of SEs occurred at very low frequency (6.33%) when inoculated on the GA_3-free medium (Fig 2). With GA_3 treatments, SEs rapidly germinated with an increase of frequency up to 72.28% on the medium with 5 mg/l GA_3. However, addition of higher concentration of GA_3 (7 mg/l) reduced the germination frequency of SEs by 28.67%.

The obtained results showed significant effect of sucrose on plantlet growth and microrhizome thickening (Table 1). Although on the medium with 2% sucrose, microrhizomes was thickened, but dormant bud was not produced. In higher concentrations of sucrose, the growth of plantlets was more intensive with greater plant biomass and plant height. Nevertheless, monitoring the experiment, we observed that, on media with 5% and 6% sucrose, leaf margins turned dry and whitish after 3 first days of culture; in next days, plants grew slowly; after 10 weeks of culture, induction of somatic embryos on microrhizomes was detected. The optimal sucrose concentration for growth of plantlets was 4%, on which plantlets was reached on average 1.60 g in biomass and 7.44 cm in height with visible microrhizome (about 5 mm) and dormant bud after approximately 5 months of culture.

1. Effect of sucrose concentration on the growth of Ngoc Linh ginseng (*Panax vietnamensis* Ha et Grushv.) plantlets in in vitro culture (*M*±SEM)

Medium	Sucrose concentration, %	Plantlet biomass, g	Plantlet height, cm	Presence of dormant bud
SH + 0.5 mg/l NAA + 1 mg/l BA	2	0.58±0.01[a]	4.35±0.03[a]	No
SH + 0.5 mg/l NAA + 1 mg/l BA	3	0.80±0.01[b]	6.12±0.06[b]	Yes
SH + 0.5 mg/l NAA + 1 mg/l BA	4	1.60±0.02[c]	7.44±0.09[c]	Yes
SH + 0.5 mg/l NAA + 1 mg/l BA	5	1.14±0.01[d]	6.24±0.06[b]	Yes
SH + 0.5 mg/l NAA + 1 mg/l BA	6	1.33±0.00[e]	5.62±0.05[d]	Yes

N o t e. SH — Schenk-Hildebrandt medium, NAA — α-napththaleneacetic acid, BA — benzyladenine.
[abcde] Values followed by different letters are significantly different according to Tukey's multiple range test at $p < 0,05$.

2. Effect of soil mixtures on acclimatization of micropropagated Ngoc Linh ginseng (*Panax vietnamensis* Ha et Grushv.) plantlets (*M*±SEM)

Soil mixture	After 3 weeks of planting	After 3 months of planting		
	percentage of planlets sprouted, %	roots		survival rate, %
		number	length, cm	
Forest humus	10.00±4.08[a]	0.92±0.12[a]	0.49±0.00[a]	71.67±1.67[a]
$^2/_3$ forest humus + $^1/_3$ sand	38.33±6.39[bc]	2.58±0.14[b]	1.04±0.01[b]	73.33±1.67[a]
$^1/_3$ forest humus + $^2/_3$ sand	61.67±7.26[c]	5.05±0.20[c]	1.90±0.03[c]	91.67±1.67[b]
$^1/_5$ forest humus + $^4/_5$ sand	36.67±8.04[abc]	2.52±0.12[bd]	0.88±0.01[d]	80.00±5.77[ab]
$^1/_3$ forest humus + $^1/_3$ coconut fiber + $^1/_3$ perlite	20.00±6.07[ab]	2.00±0.10[d]	0.55±0.01[e]	70.00±2.89[a]

[abcd] Values followed by different letters are significantly different according to Tukey's multiple range test at $p < 0,05$.

Within 3 weeks of planting, the dormant buds started to sprout in the range from 10.00 to 61.67% depending on the types of soil mixture (Table 2).

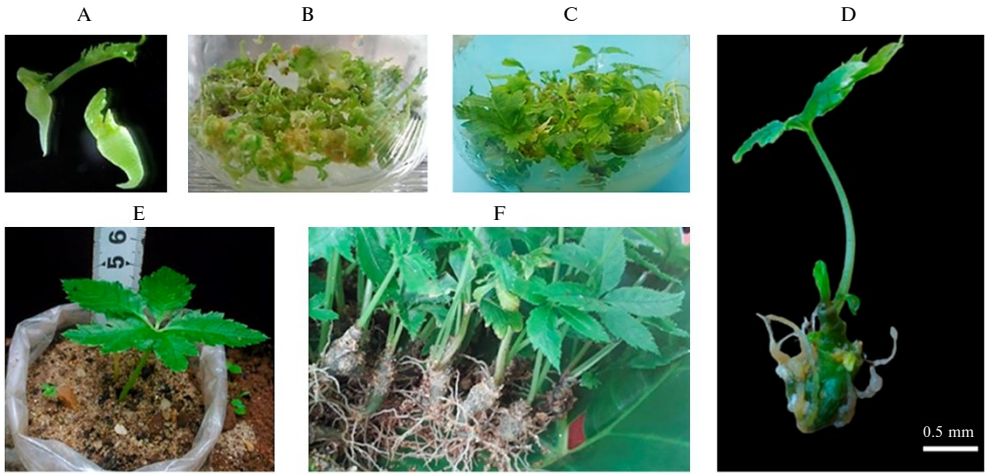

Fig. 3. Germination of somatic embryos and plantlet development in invitro micropropagation of Ngoc Linh ginseng (Panax vietnamensis Ha et Grushv.): A — germinating somatic embryos; B — after 4 weeks of culture on germination medium; C — after 10 weeks of culture on germination medium; D — plant with microrhizome thickened after culture on germination medium; E — plantlets after 8 weeks of planting; F — plantlets after 3 months of planting.

In next weeks, the sprouting continued to occur and was an indicator for predicting survival of micropropagated ginseng plantlets (see Table 2). As a result, the survival rate evaluated after 3 months of planting was directly proportional to the sprouting percentage at 3 weeks of planting. The tested soil mixtures were found to definitely influence the survival and growth of micropropagated plantlets. Plantlets were restarted in forest humus, in which sprouting percentage of plantlets was quiet low (10%) at 3 weeks of planting and reached 71.67% after 3 months with 0.92 roots of 0.49 cm length. Higher number of roots formed was obtained in the soil mixture containing $^1/_3$ forest humus + $^1/_3$ coconut fiber + $^1/_3$ perlite

(2.00 roots), followed by the soil mixture of $^1/_5$ forest humus + $^4/_5$ sand (2.52 roots) and the soil mixture of $^2/_3$ forest humus + $^1/_3$ sand (2.58 roots) (see Table 2). The maximal root growth (5.05 roots with an average length of 1.9 cm) as well as the maximal survival rate (91.67%) were recorded in the soil mixture containing $^1/_3$ forest humus + $^2/_3$ sand (Figs. 3, E, F).

The present report is the first tissue culture study using IZEs as initial explants to generate SEs from Ngoc Linh ginseng. In this study, IZEs were not completely separated from the endoderm but were cut together with a part of endoderm. It is because that IZE of Ngoc Linh ginseng and ginseng in general, are tiny [21, 22], and get injured easily when cutting off from the endoderm. After 10 weeks of culture, the best result for direct somatic embryogenesis was observed on SH medium with 7% sucrose without any plant growth regulators. Use of high concentration of sucrose (5%) for SE induction was also reported by [21-23] on cotyledons excised from immature or mature zygotic embryos of Korean ginseng. Similarly to [24, 25], addition of plant growth regulators resulted to indirect somatic embryogenesis. Interestingly, the highest rate (44.33%) of explants induced SEs in this study was recorded on SH basal medium, not on MS basal medium as identified in [22]. Moreover, SH basal medium induced formation of friable embryogenesis tissue masses, on which single somatic embryos were generated, meanwhile large number of somatic embryos emerged on MS basal medium was abnormal or conjoined, thus, leading to production of defective plantlets. In ginseng tissue culture, many studies have also reported high production of abnormal somatic embryos [23, 24, 26-28]. In in vitro plant propagation, it is important to produce morphologically normal plants, particularly from single somatic embryos in the case of this study. As that, we conducted harvesting of single primary SEs from formed embryogenesis tissue masses before proliferation of secondary SEs. The single primary SEs were easily isolated from the parent explants by a brief shaking in liquid culture. Types of culture and plant growth regulators both affected on proliferation coefficient of SEs that showed the best result in liquid culture on $^1/_2$ SH media supplemented with 0.1 mg/l kinetin and 0.1 mg/l TDZ. After 4 weeks of culture, primary SEs generated numerous new embryos, considered as secondary embryogenesis (see Figs. 1, E-G). Since formation of new embryos occurred asynchronous, the embryos obtained after 4 weeks of culture were at different stages of development, from globular to cotyledonary stages.

The frequency of SEs germinated was much greater on the media with addition of GA_3 than on the GA_3-free medium. In this study, we observed that, on the GA_3-free medium, the process of secondary somatic embryogenesis continued, leading to inhibiting the development of embryos. Whereas, on the media with addition of GA3, the embryo development occurred normally through globular, heart-shape, bipolar and cotyledonary stages, then proceeded to germination into plantlets (see Figs. 3, A, B). The best result, which gave the germination frequency of 72.28%, was obtained with 5 mg/l GA_3 (Fig. 3, C) as reported in [29]. It indicates that GA_3 stimulated germination of somatic embryos of Ngoc Linh ginseng. This finding is consistent with the results in other ginsengs reported by [23, 28, 30, 31]. After 10 weeks cultivated on germination media, plantlets with shoots of 2-3 cm height and microrhizimes of 3 mm diameter were obtained (see Fig. 3, D). This confirmed efficiency of the used medium, in which NAA and BA were supplemented with the aim of promoting the early induction of microrhizomes.

The growth of plantlets and microrhizomes was then enhanced on the media with different sucrose concentrations (2 to 6%) for approximately 5 months. Effect of sucrose on the growth of plantlets and root thickening in ginseng has been reported [32]. In the present study, the highest success was obtained using

the medium with 4% sucrose. After approximately 5 months of culture, dormant buds were produced in cultures supplemented with 3-6% sucrose. The presence of dormant bud plays an important role in the survival of *in vitro* ginseng plantlets when transferred to soil [32]. It can be explained as that, in the natural environment, roots begin growing after the first shoot emerged.

Before planting in soil, the plantlets were treated with 3% chitosan in order to control diseases [33-35]. In our study, without chitosan treatment, the proportion of micropropagation seedlings affected by root rot reached up to 90% (data not shown). Thus, chitosan treatment was applied to the plantlets before transplanting. We have found effect of soil mixtures on acclimatization of in vitro plantlets. The soil mixing forest humus with sand in the ratio 1:2 was significantly superior to the others due to desirable soil texture for potting in vitro plantlets. On the one hand, forest humus is enriched with nutrients, on the other hand, sand provides sufficient aeration and drainage. Using this soil mixture, the micropropagated plantlets reached a survival rate of 91.67%. The old foliage turned yellow and fallen off after 5-6 weeks of planting, but the plants grew well with new leaves and roots developed (see Figs. 3, E, F).

Thus, we have optimized the protocol for micropropagation of Ngoc Linh ginseng using somatic embryogenesis in vitro culture of immature zygotic embryos. The protocol includes induction of somatic embryogenesis, proliferation of somatic embryos, germination of somatic embryos, development of seedlings and acclimatization of seedlings. The frequency of direct somatic embryogenesis on Schenk and Hildebrandt (SH) basal medium with 7% sucrose was 44.33%. Secondary somatic embryogenesis with a proliferation coefficient of 26.71 was induced in liquid culture in $^1/_2$ SH medium with kinetin (0.1 mg/l) and thidiazuron (0.1 mg/l). SH medium with α-naphthylacetic acid (NAA, 1 mg/l), 6-benzyladenine (BA, 0.5 mg/l) and gibberellic acid (GA_3, 5 mg/l) stimulated the germination of somatic embryos and early formation of microrhisomes. As a result of micropropagation on SH medium with NAA (0.5 mg/l), BA (1 mg/l) and 4% sucrose, seedlings with a well-developed root system and a thickened microrhizome with a dormant bud were obtained. When micropropagated seedlings were acclimatized on a mixture of forest humus and sand in a ratio of 1:2, the survival rate was 91.67%. The micropropagation protocol we set-up allows the propagation process to be speed up and significantly improves multiplication coefficient and survival rate in field conditions. It is can be applied for large-scale production of Ngoc Linh ginseng.

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