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Effect of spermidine, glutamine, and proline on somatic embryogenesis and silver nanoparticles supplied culture improved rhizome formation of *Panax vietnamensis* var. *langbianensis*

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A B S T R A C T

Lang Bian ginseng (*Panax vietnamensis* var. *langbianensis*) is a rare and traditional herbal medicine with many pharmacological activities. It was first discovered by Duy et al. (2016) in Lam Dong province, Vietnam. Until now, there has been only one publication on Lang Bian ginseng micropropagation via somatic embryogenesis (Anh et al., 2022). However, this study still had limitations, including the small number of somatic embryos and the time-consuming. This study investigated the effect of spermidine, glutamine, and proline on the enhancement of somatic embryogenesis (SE) of Lang Bian ginseng using thin cell layer culture technology. The results showed that the optimal SE rate and the number of somatic embryos per explant were achieved on MS medium supplemented with 0.01 mM spermidine in leaf explants (93.32 % and 54.20 embryos, respectively) and petiole explants (96.66 % and 68.80 embryos, respectively) and higher compared to these in other treatments, including control, glutamine, and proline treatments. Besides, adding 0.01 mM spermidine to the culture medium also improved the quality of somatic embryos, mainly cotyledonary stage, through an enhanced synthesis of antioxidant enzymes [catalase (CAT) and superoxide dismutase (SOD), and ascorbate peroxidase (APX)]. In addition, the fluctuations of endogenous hormones during initiation and maturation of SE derived from 0.01 mM spermidine treatment were recorded. Endogenous CKs (ZEA, 2iP, KIN, mT), IAA, and GA_3 concentrations were the highest during the induction stage in both explants. Meanwhile, the remaining endogenous hormones (MEL, ABA, and SA) exhibited no inevitable fluctuation trends. In addition, IAA was only detected at the induction stage of SE in both explants. Moreover, cotyledonary somatic embryos derived from 0.01 mM spermidine treatment grew well in MS medium supplemented with 1.2 mg/L AgNPs.
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1. Introduction

A new variety of *Panax vietnamensis* var. *langbianensis* was published in 2016 based on the distinguishable DNA barcodes and morphological characteristics (Duy et al., 2016). Two hundred to two hundred fifty individual plants have been found in the Langbian mountain of Lac Duong District, Vietnam. Otherwise, the habitat of this region was narrow and has been influenced by human interventional activities (Trieu et al., 2019). Since being discovered by Duy et al. (2016), only one publication has been made on the micropropagation process of Lang Bian ginseng through the thin layer culture technique to receive secondary somatic embryos, thereby regenerating into plantlets (Anh et al., 2022). In addition, Anh et al. (2022) also isolated some valuable saponin from this ginseng. However, there were still limitations to this study, such as the small number of somatic embryos and the time-consuming. Somatic embryos are mainly obtained in globular shape, so more stages are needed for the embryos to mature (Anh et al., 2022). Therefore, it is

Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; 2iP, N6-isopentenyladenine; ABA, Abscisic acid; AgNPs, Silver nanoparticles solution; APX, Ascorbate peroxidase; CAT, Catalase; CKs, Cytokinins; GA_3, Gibberellin A3; HPLC, High-performance liquid chromatography; IAA, Indole-3-acetic acid; KIN, Kinetin; MEL, N-acetyl-5-methoxytryptamine; MS, Murashige and Skoog (1962); mT, 6-(3-hydroxybenzylamino) purine; NAA, Naphthalene acetic acid; ROS, Reactive oxygen species; SA, Salicylic acid; SE, Somatic embryogenesis; SOD, Superoxide dismutase; ZEA, Trans-zeatin

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necessary to carry out further studies to overcome the above problems. This study aimed to increase the embryo number and quality by supplementing the suitable type and concentration of glutamine, proline, and spermidine.

Somatic embryogenesis (SE) is a vital plant micropropagation method and a helpful tool in various plant breeding and improvement strategies (Talapatra et al., 2014). SE is an *in vitro* propagation technique of individual cells or groups of capable embryogenic somatic cells, like in zygotic embryonic development. Compared with other *in vitro* culture techniques, SE has many advantages, such as obtaining large numbers of somatic embryos, minimizing costs for the production process, and producing genetically stable somatic embryos (Maruyama and Hosoi, 2019). In SE, many factors affect the efficiency of the culture process, such as amino acids and polyamines.

Somatic embryogenesis includes cell division, differentiation, and physiological changes (Dodeman et al., 1997; Jariteh et al., 2015). External factors greatly influence somatic embryogenesis, such as explant damages (wounding stress), surface sterilization (oxidative stress), and culture medium (Dehydration/osmotic stress) (Nolan et al., 2006). Moreover, stress signaling is crucial in cell differentiation (Zhang et al., 2009). Stress causes reactive oxygen species (ROS) production. It has been demonstrated that there is a relationship between increased ROS production and improved somatic embryogenesis in many plants (Pasternak et al., 2007; Zavattieri et al., 2010; Rose, 2019). However, a moderate level of ROS can induce somatic cells to change morphology and differentiate (Prudente et al., 2020). In contrast, the excessive ROS content disrupts membrane permeability and changes irreversible essential components such as lipids, proteins, and nucleic acids, leading to loss of totipotency and even cell death (Varghese et al., 2012; Orłowska and Kępczyńska, 2020). In order to address the ROS disadvantages limitation, plants have mechanisms to control it by biosynthesizing the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX). These enzymes play an essential role in somatic embryogenesis, partly due to their ability to remove ROS generated by external stress conditions imposed on plants (Elhiti et al., 2013; Jariteh et al., 2015; Ighodaro and Akinloye, 2018).

Polyamines (PAs) are low-molecule diamines crucial in regulating fundamental plant metabolism. They regulate cellular functions, including molecular signaling, cell division and differentiation, totipotency, growth and development processes, oxidative damage protection, and environmental stress responses (Mustafavi et al., 2018; Jangra et al., 2023). Many studies have demonstrated that polyamines (PAs) can engage in synergistic or antagonistic interactions with diverse plant hormones, suggesting crosstalk between hormones and PAs (Chen et al., 2019; Tyagi et al., 2023). In addition, polyamine protects cells from the damage of reactive oxygen groups and free radicals by maintaining cell turgor and osmosis (Tang and Newton, 2004), eliminating oxygen species, protecting DNA and cell membranes from damage, and reducing H_2O_2 and superoxide synthesis under stress conditions (Medeiros et al., 2014; Prudente et al., 2020). Therefore, polyamine added to the culture medium promotes embryogenesis by adjusting activated oxygen content and plant growth hormone. Many studies have successfully supplemented culture media with the suitable type and concentration of amino acids and polyamines to increase the efficiency of SE. Ageel and Elmeer (2011) reported that *Phoenix dactylifera* L. cultured on a medium supplemented with organic nitrogen, especially glutamine at high concentrations, improved callus formation, growth rate and induction of SE. Proline added to the culture medium at optimal concentrations also improved SE in some plants, such as *Panax vietnamensis* (Nhut et al., 2012) and strawberry (Martínez et al., 2017). The SE rate was enhanced by supplementing spermidine into a cultured medium of *Saccharum* spp. at the concentration of 15 mg/L (Sathish et al., 2020); *Oryza sativa* L. at 1 mM (Sundararajan et al., 2021); *P. ginseng* at 1 mM (Kevers et al., 2000), *P. vietnamensis* at 0.1 mM (Nhut et al., 2012); *Cunninghamia lanceolate* at 2–4 mg/L (Wang et al., 2020). Furthermore, polyamine also helped to maintain the growth and development of *Pinus sylvestris* L., particularly in adverse or stress conditions under stress conditions (Rakesh et al., 2021). Thus, the presence of amino acids and polyamines in appropriate concentrations will significantly improve SE performance. However, the impact of glutamine, proline, and spermidine varies depending on the specific plant species. Furthermore, even within the same plant, the optimal concentrations of these substances can differ. For instance, in the micropropagation of Ngoc Linh ginseng via somatic embryogenesis, the most effective proline concentration was found to be 2.6 mM, resulting in the highest embryogenesis rate and somatic embryo number (86.7 % and 167 embryos per explant, respectively). In contrast, it was demonstrated that spermidine concentrations as low as 0.1 mM were highly efficient for somatic embryogenesis, achieving a remarkable embryogenesis rate of 93.3 % and 353 embryos per explant. However, both of these parameters decreased when the spermidine concentration was raised to 0.2 mM (Nhut et al., 2012). In summary, both spermidine and proline are effective inducers of somatic embryogenesis, but the proline concentration used was 26 times higher than that of spermidine.

Recently, literature revealed that silver nanoparticles (AgNPs) exhibit effectiveness in various aspects of plant biology, including explant disinfection, morphogenesis, proliferation, and the accumulation of secondary metabolites (Parzymies, 2021; Abdelkawy et al., 2023; Pérez Caselles et al., 2023). AgNPs are absorbed into plant cells, stimulating the synthesis of reactive oxygen species (ROS) and initiating cell signaling pathways that contribute to improved plant growth and development (Sewelam et al., 2016; Lala, 2021). The stimulating influence of AgNPs on cell, tissue growth, and development has been verified in various plant species, including *Panax vietnamensis* (Cuong et al., 2021), *Stevia rebaudiana* Bert. (Laha et al., 2023) and *Gaillardia pulchella* Foug cv. 'Torch Yellow' (Manokari et al., 2023).

Hence, studying the role of amino acids and spermidine on the SE of Lang Bian ginseng has great significance in increasing the efficiency of SE and improving the quality of the somatic embryo, thereby creating a source of materials for the micropropagation of this precious ginseng. In addition, some physiological-biochemical parameters of this ginseng have not yet been recorded, so the effects of amino acids and polyamines added to the culture medium on antioxidant enzymes and the fluctuations of endogenous hormones during SE were also noted and clarified in this study.

2. Materials and methods

2.1. Plant material

Leaf and petiole explants of 3-month-old Lang Bian ginseng plantlets *in vitro* derived from *ex vitro* rhizome explants treated with 0.15 % AgNPs disinfection solution for 30 min (Anh et al., 2022) were used as explants for SE experiments. Plantlets were cultured in MS medium supplemented with 0.5 mg/L BA, 0.5 mg/L NAA, 30 g/L sucrose, 8.0 g/L agar, and 1.0 g/L activated charcoal. The plantlets were placed at a temperature of 22 ± 2 °C in a 16h/8h photoperiod with an average humidity of 55–60 %.

2.2. Silver nanoparticles solution

AgNPs solution (average size <20 nm) was synthesized by the aqueous solution reduction method at a concentration of 1 g/L, which was provided by The Institute of Environmental Technology (VAST, Hanoi, Vietnam). Carboxymethyl cellulose (CMC) was a stabilizer, and sodium borohydride ($NaBH_4$) was a reducing agent. $AgNO_3$, $NaBH_4$, and CMC were purchased from Merck Chemical Reagent Co. The process of nanosilver formation occurred within a homogeneous solution containing $AgNO_3$. This process was carried out with

continuous stirring at room temperature while the reducing agent was added drop by drop. UV spectroscopy, scanning, and transmission electron microscopy methods characterized the silver nanoparticle size and properties. The smallest particle size was achieved when the sodium borohydride solution was added to the reaction mixture at a rate of 10–12 drops per minute, and a mole ratio of $[NaBH_4]/[AgNO_3]$ at 1:4 (Chau et al., 2008).

2.3. Effects of proline, glutamine and spermidine on somatic embryogenesis of Lang Bian ginseng leaf and petiole explants

Leaf and petiole explants of *in vitro* Lang Bian ginseng derived from *ex vitro* rhizome explants treated with 0.15 % AgNPs disinfection solution for 30 min (Anh et al., 2022) were cut in sizes of 5 × 5 mm (length × width) and 10 × 0.5 mm (length × thickness), respectively. Subsequently, the explants were placed on MS medium (Murashige and Skoog, 1962), added 7 mg/L naphthalene acetic acid (NAA; Sigma-Aldrich®, USA) (for leaf explants) or 1 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D; Sigma-Aldrich®, USA) (for petiole explants) (Anh et al., 2022), together with proline (0.87; 1.74; 2.6; 3.47 mM) or glutamine (1; 3; 5; 7 mM) or spermidine (0.01; 0.05; 0.1 and 0.2 mM). The culture medium's pH was controlled within the range of 5.7–5.8. Explants were cultured in a 6 cm diameter petri dish (Sigma-Aldrich®, USA), containing 5 mL of culture medium. The explants were then placed at a temperature of 22 ± 2 °C in the dark with an average humidity of 55–60 %. After 12 weeks of culture, the SE formation rate (%), number of somatic embryos per explant and somatic embryo regeneration coefficient were recorded. Somatic embryo regeneration coefficient of each treatment was also quantified *via* the growth correction factor value as described by Teixeira da Silva and Dobránszki (2014).

Somatic embryo formation rate:

$$\text{SE rate}(\%) = \frac{\text{Number of explants forming somatic embyos}}{\text{The total number of explants}} \times 100\%$$

The growth correction factor:

$$\text{GCF} = \text{SE rate} \times \text{the total number of explants} \times \text{the number of somatic embryos per explant}$$

2.4. Subsequent growth of Lang Bian ginseng somatic embryos

The cotyledonary somatic embryos (1.5 mm in length) obtained from the best treatment were cultured on MS medium supplemented with 0.5 mg/L BA, 0.5 mg/L NAA, 30 g/L sucrose, 8.0 g/L agar, 1.2 mg/L AgNPs, and 1.0 g/L activated charcoal (Cuong et al., 2021). As a control, cotyledonary somatic embryos were cultured on the same medium without the addition of AgNPs. The rhizome formation rate (%), rhizome diameter (cm), and rhizome length (cm) were recorded after 20 weeks of culture.

2.5. Determination of antioxidant enzyme activity by ultraviolet-visible (UV–vis) spectroscopy

For the extraction of the enzymes (SOD, CAT, and APX), 0.3 g of somatic embryos were frozen and powdered using liquid nitrogen and then homogenized by ultrasonic scattering in 2 mL of 0.1 M phosphate buffer (pH 7.4) with 0.1 mM EDTA. Every homogenate was put into centrifuge tubes, where it was spun at 15,000 g for 20 min at 4 °C. To measure the activity of the antioxidant enzymes, the supernatants were collected and kept on ice.

The procedure described by Marklund and Marklund (1974) was used to evaluate the SOD activity. Pyrogallol is produced in an alkaline environment by oxidation in the presence of oxygen in the air, with the resultant product having a maximum absorption wavelength of 320 nm. SOD in the sample catalyzes the breakdown of peroxide radicals (-O-O-), halting the self-oxidation of pyrogallol. The superoxide dismutase activity in the sample is shown by the inhibition ratio. A change in absorbance at 320 nm is used to calculate an enzyme activity unit (U), which is equal to a 50 % inhibition of pyrogallol's self-oxidation. This is calculated using the formula: Enzyme unit (U/g prot) = (% inhibited/50) dilution ratio.

The test sample's CAT activity was assessed by allowing it to react for 2 min with 100 μL of 65 mM H_2O_2 before the residual H_2O_2 was mixed with 100 μL of ammonium molybdate $[(NH_4)_6Mo_7O_{24}]$, which creates a stable yellowish complex that is most absorbent at 405 nm. One active catalase unit (U/g prot) is equal to 1 mol of H_2O_2 hydrolysis for 1 min (Goth, 1991).

The maximal ascorbate absorbance (at 290 nm) during the course of 3 min in the presence of 0.5 mM H_2O_2 is used to compute the APX unit of activity (U). An absorption value of 2.8 mM/cm is used to directly compute the quantity of oxidized ascorbate. When experimental, an enzyme activity unit (U/g prot) is needed to oxidize 1 μm ascorbate for 1 min (Nakano and Asada, 1981).

2.6. Determination of endogenous hormone content by HPLC

Bieleski solution - $CHCl_3$:MeOH:HCOOH:H_2O (25:60:5:10, v/v/v) was used to powder fresh leaf, petiole, and somatic embryo samples (induction phage, embryogenesis callus, globular embryo, and cotyledon embryo) produced from the 0.01 mM spermidine treatment (0.1 g explant:1 mL solution). After being reextracted in 4 mL of 80 % methanol for 1 h at 4 °C, the residue was centrifuged once more. The supernatants were combined and put into Sep-Pak C18 cartridges after being adjusted with 1 mL each of 100 % and 80 % methanol. 500 μL of 80 % methanol were used to rinse the cartridge. The solvent was removed from the pure extract by drying it in a vacuum evaporator at 50 °C, and it was then reconstituted in 1–2 mL of water with a pH of 2 (adjusted with formic acid). Prior to injecting the solution into the HPLC system, a 0.45 μm membrane was used as a filter. The hormones (2iP, ZEA, KIN, mT, IAA, GA3, SA, ABA, and MEL) were separated using a Thermo-Ultimate 3000 HPLC system (Thermo Scientific, USA) equipped with a BDS Hypersil C18 column (with a 25 mm × 4.6 mm) particle size of 0.5 m and connected to a UV detector monitored at 280 nm. In particular, acetonitrile and Milli-Q water that had been acidified with 0.5 % formic acid were employed as a binary solvent system. Separations were performed using segmented gradients of 0–10 min A from 100 % to 75 %, 11–17 min A from 75 % to 50 %, and finally 18–25 min A from 50 % to 75 % with a flow rate of 0.7 mL/min. Based on calibration curves of particular standards, the hormone concentrations were determined (Khai et al., 2021). Corresponding standards were purchased from Sigma-Aldrich®, USA.

2.7. Statistical analysis

All experiments were performed randomly with 3 replicates, 30 explants per treatment and 1 explant per petri dish. The data were analyzed using Microsoft Excel 2010 and SPSS 16.0 statistical analysis software, based on Duncan's multiple range test at a significance level of $p < 0.05$ (Duncan, 1955).

3. Results and discussion

3.1. Results

3.1.1. Effects of glutamine, proline and spermidine on somatic embryogenesis of the *in vitro* leaf explant

The effects of glutamine, proline, and spermidine on SE from leaf explants after 12 weeks were recorded in Table 1. The results showed that the addition of 1 mM glutamine to MS medium provided notably incensement in both SE rate and number of somatic embryos per explant (83.32 % and 52.20 respectively) compared to the control

Table 1
Somatic embryogenesis from leaf explants on MS medium supplemented with glutamine, proline or spermidine after 12 weeks of culture.

Treatment	Concentration (mM)	Somatic embryogenesis rate (%)	No. of embryos/ explant	Somatic embryo morphology
Control	0	$76.68 \pm 14.88^{b*}$	33.40 ± 4.67^{c}	Globular
Glutamine	1	83.34 ± 16.65^{ab}	52.20 ± 5.26^{a}	Globular, heart, torpedo
	3	83.32 ± 11.77^{ab}	22.60 ± 4.67^{d}	
	5	60.02 ± 9.15^{c}	12.60 ± 2.07^{ef}	
	7	56.68 ± 9.15^{cd}	10.40 ± 2.07^{ef}	
Proline	0.87	53.34 ± 7.47^{cd}	21.00 ± 4.06^{d}	
	1.74	29.98 ± 7.42^{e}	14.60 ± 3.65^{e}	
	2.60	0.00 ± 0.00^{f}	0.00 ± 0.00^{g}	-
	3.47	0.00 ± 0.00^{f}	0.00 ± 0.00^{g}	-
Spermidine	0.01	93.32 ± 9.15^{a}	54.20 ± 4.44^{a}	Globular, heart, torpedo, and cotyledon
	0.05	80.00 ± 13.92^{ab}	38.60 ± 3.43^{b}	
	0.1	60.02 ± 9.15^{c}	8.60 ± 1.14^{f}	
	0.2	43.32 ± 14.94^{de}	3.40 ± 0.89^{g}	

* Data are presented in form of Mean±SD and different letters (a, b, ...) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's multiple range test)(Duncan, 1955).

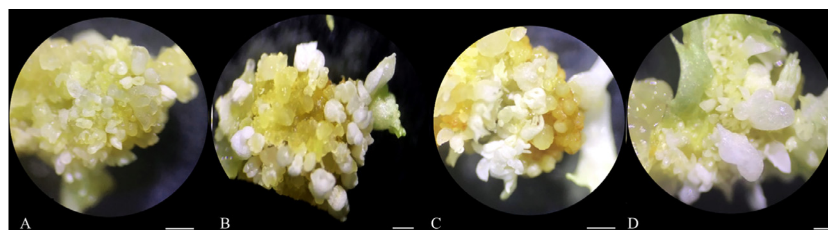

Fig. 1. Somatic embryogenesis from leaf explant of Lang Bian ginseng after 12 weeks of culture (*Bar.* 1 mm)
A. Control; B. 1 mM glutamine; C. 0.87 mM proline; D. 0.01 mM spermidine.

treatment (76.68 % and 33.40 respectively) and other treatments. However, higher concentrations of glutamine (5 and 7 mM) gradually reduced the efficiency of SE. Besides, in the control treatment, only embryo-inducted callus and globular embryos were recorded; meanwhile, the torpedo-shaped embryos, which are essential sources of materials for studies on secondary SE, were formed on glutamine added medium (Fig. 1A, B).

On proline treatments, proline had no significant improvement on SE from leaf explants; the highest rate of SE as well as the number of embryos were only 53.34 % and 21.00 embryos/explant respectively at 0.87 mM concentration, lower than the control (76.68 % and 33.40 embryos/explant, respectively). Proline at a concentration of 2.60 mM and 3.47 mM showed no somatic embryo induction (Table 1). However, the addition of proline at 0.87–1.74 mM induced the formation of torpedo embryos (Fig. 1C); meanwhile, in the control treatment, only embryonic callus and globular embryo were observed (Table 1).

After 12 weeks of culturing on MS medium supplemented with spermidine, the results showed that spermidine at low concentrations (0.01–0.05 mM) posed positive effects on SE whereas this polyamine ceased somatic embryo formation when applying at higher concentrations (Table 1). Moreover, somatic embryos were observed in globular, heart, torpedo, and cotyledon shapes in all spermidine treatments (Figs. 1D and 3) with the highest outcome of SE rate and somatic embryos/explant were 93.32 % and 54.20, respectively, in the 0.01 mM treatment. The somatic embryo regeneration coefficient gave the same result, the highest GCF was obtained at 0.01 mM spermidine (Fig. 4).

3.1.2. Effect of glutamine, proline and spermidine on somatic embryogenesis of Lang Bian ginseng in vitro petiole explant

For petiole explants, the addition of glutamine and proline to MS medium did not stimulate the explants' SE rate, and the number of somatic embryos per explant was also lower than that of the control. The SE rate and the number of somatic embryos per explant were only 43.32 % and 18.20, respectively, when the glutamine concentration was increased to 7 mM (Table 2 and Fig. 2). Although the SE rate was 79.98 % and 42.00 somatic embryos/explant were obtained with

Table 2
Somatic embryogenesis from petiole explants on MS medium supplemented with glutamine, proline or spermidine after 12 weeks of culture.

Treatment	Concentration (mM)	Somatic embryogenesis rate (%)	No. of embryos/ explant	Somatic embryo morphology
Control	0	$93.32 \pm 9.15^{a*}$	54.20 ± 4.27^{b}	Globular
Glutamine	1	73.34 ± 9.09^{c}	35.00 ± 5.48^{d}	Globular, heart, torpedo
	3	63.36 ± 7.47^{cd}	34.60 ± 4.22^{d}	
	5	53.34 ± 7.47^{de}	23.00 ± 8.37^{e}	Globular
	7	43.32 ± 9.15^{e}	18.20 ± 5.07^{ef}	
Proline	0.87	79.98 ± 7.42^{b}	42.00 ± 8.60^{c}	Globular, and heart
	1.74	73.34 ± 9.09^{c}	29.80 ± 6.14^{d}	Globular
	2.60	60.02 ± 9.15^{d}	19.60 ± 3.78^{ef}	
	3.47	56.68 ± 9.15^{d}	13.40 ± 4.22^{f}	
Spermidine	0.01	96.66 ± 7.47^{a}	68.80 ± 2.95^{a}	Globular, heart, torpedo, and cotyledon
	0.05	93.32 ± 9.15^{a}	60.40 ± 5.13^{b}	
	0.1	79.98 ± 7.42^{b}	46.40 ± 2.97^{c}	
	0.2	76.66 ± 9.09^{b}	15.20 ± 3.11^{f}	

* Data are presented in form of Mean ± SD and different letters (a, b, ...) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's multiple range test) (Duncan, 1955).

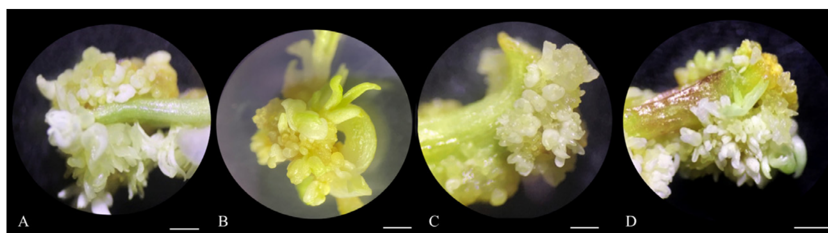

Fig. 2. Somatic embryogenesis from petiole explant of Lang Bian ginseng after 12 weeks of culture (*Bar.* 1 mm)
A. Control; B. 1 mM glutamine; C. 0.87 mM proline; D. 0.01 mM spermidine.

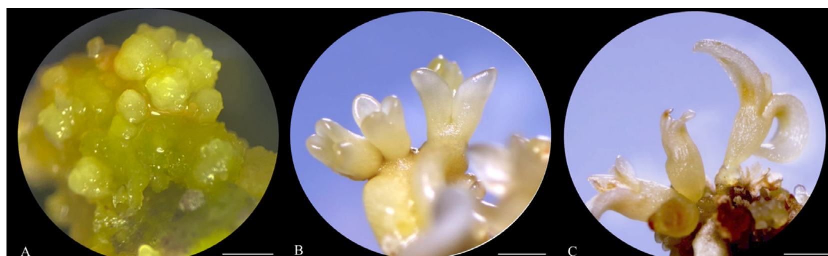

Fig. 3. Morphological observation during somatic embryogenesis of Lang Bian ginseng (*Bar. 1 mm*) A. Globular stage; B. Torpedo stage; C. Cotyledonary stage.

treatment supplemented with 0.87 mM proline, they were still lower than those of the control (93.32 % and 54.20 embryos/explant, respectively). In general, the higher the proline concentration, the lower the SE effect obtained (Table 2 and Fig. 2).

The highest value of SE rate (96.66 %), as well as the number of somatic embryos per explant (68.80) were recorded on MS medium supplemented with 0.01 mM spermidine, which is statistically significant difference from the other treatments (Table 2). Like the leaf explant, the petiole showed the lowest embryogenesis when increasing the concentration of spermidine supplement up to 0.2 mM (76.66 % and 15.20 embryos/explant). Besides, the SE coefficient also gave similar results (Fig. 4).

3.1.3. Effects of glutamine, proline and spermidine on the activities of antioxidant enzymes

The CAT, SOD, and APX activities of Lang Bian ginseng somatic embryos derived from leaf and petiole explants after 12 weeks of culture were recorded in Tables 3 and 4. For leaf- explants, the levels of

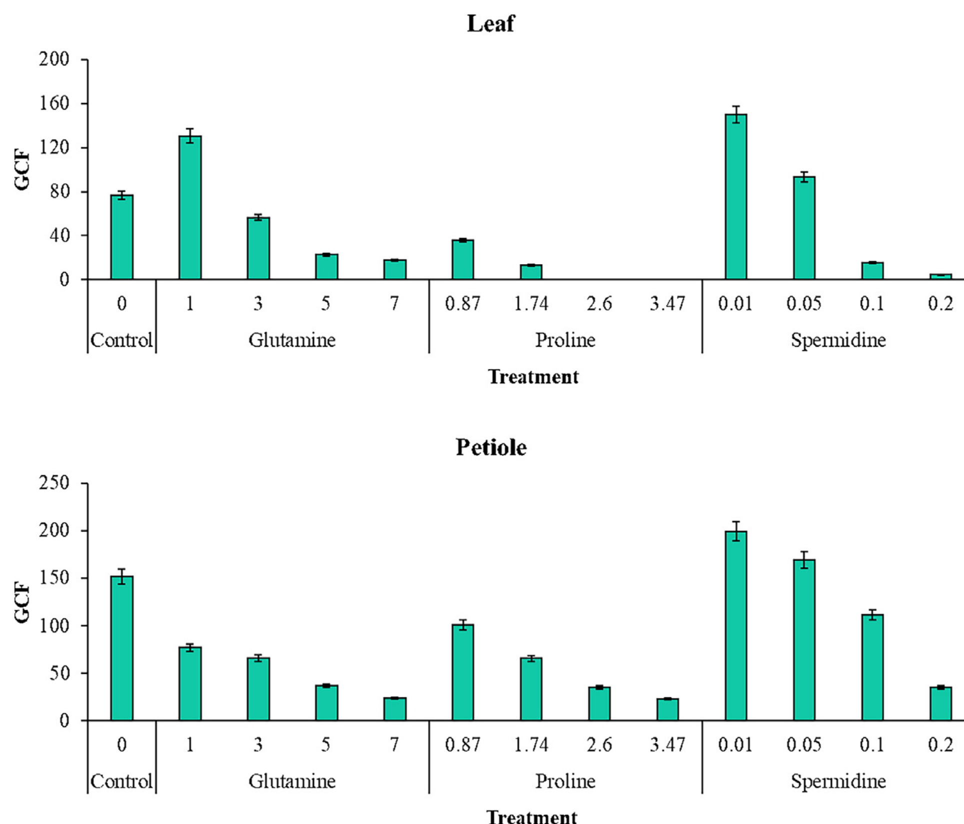

Fig. 4. Growth correction factor (GCF) from leaf and petiole explants on MS medium supplemented with glutamine, proline and spermidine after 12 weeks of culture.

Table 3
Effects of proline, glutamine and spermidine on the activities of SOD, CAT and APX in leaf explants of Lang Bian ginseng after 12 weeks of culture.

Treatment	SOD (U/g)	CAT (U/g)	APX (U/g)
Control	238.26 ± 3.68[b]*	1490.09 ± 7.35[b]	0.26 ± 0.05[d]
0.87 mM Proline	136.46 ± 1.27[d]	930.22 ± 1.28[d]	1.78 ± 0.04[c]
1 mM Glutamine	186.89 ± 1.47[c]	1145.52 ± 3.41[c]	2.65 ± 0.04[b]
0.01 mM Spermidine	355.10 ± 10.87[a]	2047.87 ± 13.76[a]	3.18 ± 0.15[a]

* Data are presented in form of Mean ± SD and different letters (a, b, …) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's multiple range test) (Duncan, 1955).

Table 4
Effects of proline, glutamine and spermidine on the activities of SOD, CAT and APX in petiole explants of Lang Bian ginseng after 12 weeks of culture.

Treatment	SOD (U/g)	CAT (U/g)	APX (U/g)
Control	120.56 ± 1.31[d]*	1004.57 ± 2.95[c]	0.37 ± 0.02[d]
0.87 mM Proline	151.81 ± 1.09[c]	689.20 ± 6.68[d]	1.64 ± 0.06[c]
1 mM Glutamine	181.60 ± 2.73[b]	1183.11 ± 7.39[b]	2.50 ± 0.05[b]
0.01 mM Spermidine	208.19 ± 2.16[a]	1794.30 ± 16.20[a]	2.87 ± 0.03[a]

* Data are presented in form of Mean ± SD and different letters (a, b, …) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's multiple range test) (Duncan, 1955).

antioxidant enzymes, including CAT, SOD, and APX, in somatic embryos cultured on 0.01 mM spermidine medium were significantly higher than those on 0.87 mM proline and 1 mM glutamine. With 0.87 mM proline136.46 U/g; 930.22 U/g) and 1 mM glutamine (186.89 U/g, 1145.52 U/g) treatment, the SOD and CAT activities were much lower than the leaf explant (238.26 U/g, 1490.09 U/g); however, the opposite result was observed in case of APX, proline and glutamine gave higher APX activity (Table 3).

In petiole explants, adding 0.01 mM spermidine also gave the highest SOD, CAT, and APX values compared to all other treatments. However, in this type of explants, the addition of proline was able to enhance the activity of SOD and APX compared with the control (Table 4).

3.1.4. The fluctuations of endogenous hormones during somatic embryogenesis

At different SE stages of Lang Bian ginseng, there were significant differences in endogenous hormone contents (Fig. 5, S1, and S2). Endogenous CKs (ZEA, 2iP, KIN, mT), IAA, and GA3 concentrations were the highest during the induction stage in both types of explants (Fig. 5A, B, D). Meanwhile, the remaining endogenous hormones (MEL, ABA, and SA) did not exhibit any inevitable fluctuation trends (Fig. 5C, E, F). In addition, IAA was only detected at the induction stage of SE in both explants and not at any other stages (Fig. 5B). These results indicated the fluctuations of these endogenous hormones during each stage of SE. At the same time, the presence and concentration of these endogenous hormones also differed between leaf and petiole explants. However, the fluctuations of endogenous hormone content in each stage of SE in leaf and petiole samples of Lang Bian ginseng were generally similar (except for GA_3) (Fig. 5).

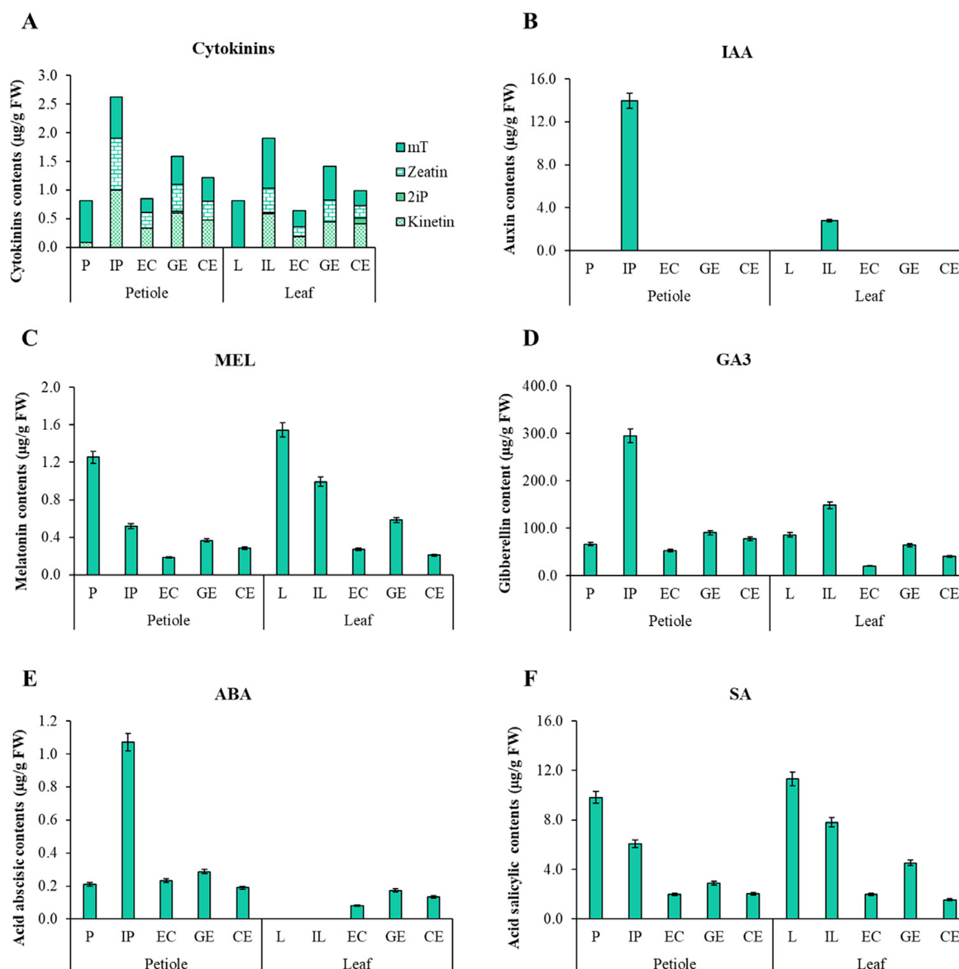

Fig. 5. Fluctuations of endogenous hormone content during somatic embryogenesis of Lang Bian ginseng. L - Leaf; P - Petiole; IP - Petiole explant in the induction phase; IL - Leaf explant in the induction phase; EC - Embryogenesis callus; GE - Globular embryo; CE - Cotyledonary embryo.

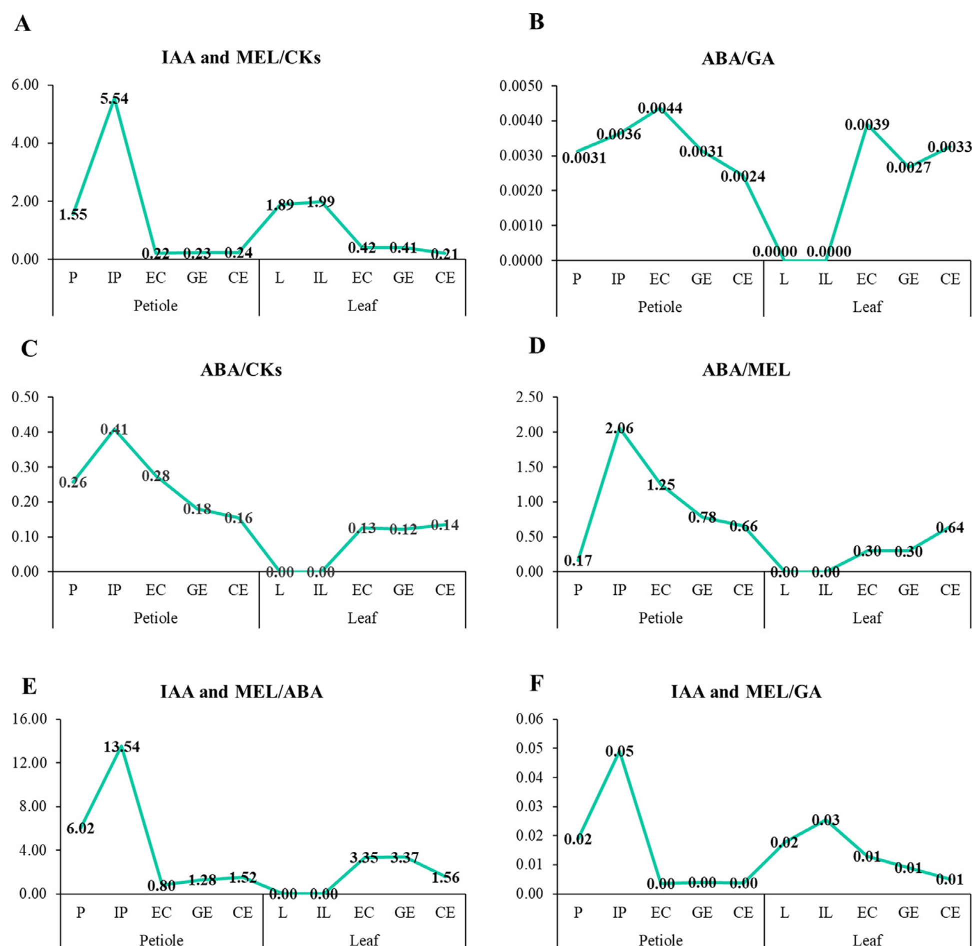

Fig. 6. The ratio of endogenous hormones in somatic embryogenesis of Lang Bian ginseng.

Depending on the type of explant, endogenous hormone ratios varied widely during SE (Fig. 6). The ratio of IAA and MEL/CKs, in both types of explants, reached the highest in the induction stage, then decreased sharply in the embryonic callus stage and remained stable (slightly increased/decreased) in the remaining SE stages (Fig. 6A). Meanwhile, the ABA/GA ratio was highest at the embryonic callus stage and decreased at the globular embryo stage (Fig. 6B). However, at the cotyledon embryo stage, this ratio continued to decrease for petioles but increased with leaf explants. In addition, in the induction phase, the ABA/GA ratio was not observed in leaf explant. The ratio of ABA/CKs, ABA/MEL, IAA and MEL/ABA and IAA and MEL/GA increased from the time of culture to the somatic embryo induction stage and gradually decreased at the subsequent SE stages for petioles. Meanwhile, ABA/CKs and ABA/MEL ratios increased during the embryogenesis callus stage and stabilized or slightly increased, and IAA and MEL/ABA and IAA and MEL/GA (Fig. 6C and D) ratios decreased in the remaining stages of SE for leaf explants (Fig. 6E and F).

3.1.5. *Subsequent growth of Lang Bian ginseng embryos*

The leaf-derived cotyledonary somatic embryos cultured on MS medium adding 0.01 mM spermidine after being transferred to MS medium with the additions of 1.2 mg/L AgNPs showed an improved rate of rhizome formation compared to the control (without the addition of AgNPs) (Table 5). In addition, adding AgNPs to the MS medium also helped increase the rhizome formation rate and improve crop quality. In the control treatment (without adding AgNPs), the plantlets had yellowing, leaf drop, and poor growth, while the plantlets on the culture MS medium supplemented with 1.2 mg/L AgNPs were dark green, and the plantlets were sturdy and developed well (Fig. 7).

3.2. *Discussion*

Glutamine is one of the amino acids commonly used as an energy source for cells, especially cells under high-rate division in *in vitro* culture (Ageel and Elmeer, 2011). Glutamine is essential in SE induction and maturation because it provides a nitrogen source for protein

Table 5
The subsequent growth of somatic embryos after 20 weeks of culture.

Treatment	Rhizome induction rate (%)	Rhizome diameter (cm)	Rhizome length (cm)
Control	$47.78 \pm 1.92^{b*}$	0.42 ± 0.03^{b}	1.20 ± 0.02^{b}
1.2 mg/L AgNPs	85.56 ± 1.93^{a}	1.14 ± 0.03^{a}	2.05 ± 0.05^{a}

[*] Data are presented in form of Mean±SD and different letters (a, b, ...) in the same column represent statistically significant differences at $p < 0.05$ (Independent - samples t-test).

Fig. 7. The subsequent growth of somatic embryos on the MS medium supplemented with or without 1.2 mg/L AgNPs after 20 weeks of culture (*Bar.* 1 cm).

and nucleic acid synthesis. Furthermore, glutamine synthesizes other amino acids and acts as a nitrogen-transporting metabolite (Corredoira et al., 2019). Moreover, glutamine plays a critical role in various aspects of plant life, including growth, development, adaptation, and response to environmental stress (Qiu et al., 2020).

Glutamine's effectiveness in inducing formation and improving embryonic maturation has also been verified in numerous plant species. According to Rai et al. (2009), the application of 0.68 mM glutamine resulted in an increased maturation rate of *Psidium guajava* somatic embryos. Vasanth and Vivier (2011) used glutamine-containing media to increase *Vitis vinifera* embryos. In addition, L-glutamine has also been shown to positively affect *Quercus suber* L. SE (Nandhakumar et al., 2018; Rahmouni et al., 2020). In banana SE, Grand Naine and Rasthali, Nandhakumar et al. (2018) suggested that glutamine (400 mg/L) significantly enhanced primary (1680 and 1850 embryos, respectively) and secondary (3597 and 3270 embryos, respectively) SE among the amino acids treatments and it was higher than the medium supplemented with proline.

Rahmouni et al. (2020) studied the effects of 19 different amino acids on the formation of secondary somatic embryos in *Quercus suber* L., depending on the concentration and the type of amino acids for different embryogenesis efficiencies. Glutamine at a concentration of 3.42 mM gave the best efficiency in embryogenesis and the induction of secondary somatic embryo formation; the secondary embryogenesis rate was 79.97 %, and 11.50 new embryos were formed from a primary embryo (Rahmouni et al., 2020). In addition to increasing the efficiency of embryogenesis and embryo maturation, adding glutamine (3.42 mM) to the embryo culture medium also reduces the rate of abnormal embryos (Rahmouni et al., 2020). Similarly, Rathore et al. (2012) found that adding glutamine (15 mM) to the culture medium stimulated the maturation of *Acacia senegal* embryos and reduced the percentage of abnormal embryos. However, depending on the plant species of the study, the need for glutamine will vary.

On the other hand, the presence of amino acids in the culture medium can promote/inhibit the growth and regeneration of plantlets from somatic embryos, depending on the type and concentration used for each plant species. Pintos et al. (2010) investigated the effect of amino acids (asparagine, arginine, and glutamine) on the development of oak tree somatic embryos. The authors found that although the combination of these 3 amino acids stimulated embryonic growth, the effect was not different from that of the control. Similarly, glutamine reduced the number of embryos and secondary embryo formation rate of *Quercus ilex* (Martínez et al., 2017). Our results also showed that increasing the concentration of glutamine to 5–7 mM inhibited the SE of Lang Bian ginseng leaf and petiole explants.

Thus, glutamine plays a massive role in many plants' SE. They were used as an additional nitrogen source for explant growth. In some cases, they are also used as the sole nitrogen source in the culture medium and for their growth-stimulating effect. However, the requirements for the type and content of amino acids depend on plant variety. In this study, the somatic embryo derived from glutamine treatment had better quality than that of the control, but glutamine did not have a positive effect on SE of Lang Bian ginseng.

Proline is one of the essential sources of reducing nitrogen that is metabolized and absorbed very rapidly in the cell; it is non-toxic and facilitates an increased cell growth rate during culture (Pawar et al., 2015). When proline is added to the culture medium at an appropriate concentration, it can be used alone or in combination with other amino acids to promote cell growth and explant SE. Proline also has the function of protecting enzymes from degradation, controlling the acid concentration in the cytosol (Szabados and Savouré, 2010). In addition, proline has an active role in increasing the protein content in embryogenic cells. The presence of proline in the medium reduces the culture medium's water potential, increasing the concentration of substances accumulated in the cells, and thereby promoting SE (Ehsani Moghaddam et al., 2000). Experimental results show that proline supplementation for embryogenesis varies for each plant species. For Lang Bian ginseng, the effect of proline in SE was not high in petiole explants regarding the SE rate and the number of somatic embryos per explant. Meanwhile, in leaf explants, although the SE rate and the number of somatic embryos per explant were not high, the embryo quality was superior to the control when adding 0.87–1.74 mM proline to the culture medium.

The effect of proline on SE has been investigated in various plants such as strawberry (Martínez et al., 2017), Ngoc Linh ginseng (Nhut et al., 2012), tulip (Podwyszyńska and Marasek Ciolakowska, 2020). Gerdakaneh et al. (2011) reported that proline at a concentration of 100 mg/L had a more substantial effect than glutamine in the strawberry's induction and development of somatic embryos. The research suggested that the practical effect of proline on embryogenesis may be due to this amino acid being involved in several signaling pathways in cells (Gerdakaneh et al., 2011). Similarly, in the tulip micropropagation, Podwyszyńska and Marasek Ciolakowska (2020) reported the optimal callus proliferation and SE when explants were cultured on medium supplemented with 0.01 mg/L 2,4-D single or in combination with TDZ or BA, and 100 mg/L proline can obtain an average of 30–55 embryos/100 mg of callus. However, for some other plant species, higher proline concentration is required for SE, such as proline at a concentration of 300 mg/L suitable for Ngoc Linh ginseng (Nhut et al., 2012) and 500 mg/L is suitable for strawberry SE (Biswas et al., 2007). However, with Lang Bian ginseng, the presence of proline in the culture medium negatively affects SE.

Polyamine plays a significant role in cell division, differentiation, membrane stabilization, and DNA replication (Handa and Mattoo, 2010). Polyamines have been reported to be involved in plant developmental processes such as growth, aging, and stress response. Furthermore, polyamine also stimulates the synthesis and/or activation of endogenous hormones and protein synthesis. On the other hand, the effects of polyamine on plant growth are mediated through the regulation of gene expression, structural changes, cell membrane mobility, transcription, and translation (Dey et al., 2015). The amine cation of polyamine can bind with anions in the phosphate groups of DNA/ RNA and the carboxyl groups of proteins on the cell membrane, thus ensuring membrane flexibility (Mattoo et al., 2015).

The role of polyamines in SE in various plants has been investigated. Research by (Nhut et al., 2012) showed that the addition of

0.1 mM spermidine to the culture medium increased the SE rate, and the number of somatic embryos per explant in Ngoc Linh ginseng. Takeda et al. (2002) reported that exogenous polyamine supplementation in plants could promote embryogenesis by altering endogenous hormone concentrations and that adding a polyamine synthesis inhibitor leads to a delay or limitation in embryogenesis (Takeda et al., 2002). In addition, Kadioglu et al. (2002) suggested that it is possible to reduce undesirable ethylene concentrations and increase plant tissue growth by adding exogenous polyamine.

In studying the SE of *Agave angustifolia*, Elbl et al. (2015) suggested that the globular phase embryo development of *A. angustifolia* is marked by an increase in cell division and differentiation rate, the expression of genes involved in carbohydrate biosynthesis and oxidative stress. Following embryonic development from spherical to seedling, there are a series of physiological and biochemical changes in the cell. During embryonic induction and development, the content of several endogenous polyamines varies according to the stage of SE (Elbl et al., 2015). The obtained results showed that spermidine has a positive effect on SE in both *in vitro* leaf and petiole of Lang Bian ginseng at the optimal concentration of 0.01 mM.

Plants are sensitive organisms, often facing a variety of abiotic and biotic stresses such as heavy metal toxicity, drought, salinity, and temperature (low and high) throughout their life cycle. The equilibrium between producing and removing reactive oxygen species (ROS) is disturbed under these stresses. Oxidative stress damages biomolecules (DNA, lipids, and proteins) eventually leading to cell death. To survive under oxidative stress, plants have evolved different adaptive strategies, including accumulating metabolites that play a protective role. One such group of metabolites includes polyamines, which have long been thought to play a protective role under various stresses, including oxidative stress, by maintaining the functions and structure of cells in response to oxidative stress. Polyamines regulate ROS homeostasis through their role (directly or indirectly) in modulating the antioxidant system or suppressing ROS production. Plants produce metabolites and molecules such as polyamine and H_2O_2 to combat oxidative stress (Mittler et al., 2011). Besides, polyamines also act as oxidative stress relievers. Plants respond to oxidative stress by implementing enzymatic and non-enzymatic antioxidant pathways (Mandal et al., 2013). Therefore, adding polyamines to the culture medium helps the explant to produce more antioxidant enzymes, thereby helping the explant to limit the adverse effects caused by oxidative stress. Furthermore, a recent study by Zhong et al. (2020) showed that polyamine accumulation plays a vital role in tomato plants under saline stress. They found that polyamine accumulation helped mitigate oxidative stress-induced damage to plant cells through cross-linking between polyamine and antioxidant enzymes; the study also demonstrated that adding spermidine to tomato plants increased the expression of antioxidant enzymes such as SOD, CAT, and APX, improving plant growth and yield under saline stress. In this study, adding 0.01 mM spermidine to the culture medium helped Lang Bian ginseng embryos synthesize more antioxidant enzymes than the control and other treatments. Since then, the formation and the quality of somatic embryos in this treatment were also superior.

SE is both a crucial channel for plant regeneration and a fascinating system for research into the morphology, physiology, and genetic principles of somatic embryo induction and development (Elhiti et al., 2013; Fehér, 2015). To control this process, it is crucial to comprehend the physiological mechanism behind SE induction. By facilitating the signal transduction cascade that reprogrammed gene expression patterns, endogenous hormones are used as signaling molecules for the induction of SE (Elhiti et al., 2013). However, there is no study about the fluctuations of those hormones during the SE of Lang Bian ginseng. Many studies have suggested that exogenous polyamines affect SE by influencing endogenous hormones content. The addition of exogenous polyamines in the embryo culture of *Araucaria angustifolia* increased the levels of endogenous IAA and ABA, thereby showing a direct relationship between polyamines and ABA accumulation, further improving the quality of the somatic embryos, especially during the mature stage (El-Dawayati et al., 2018). Similar results were obtained by Wang et al. (2020), who reported in a study on the effect of exogenous spermidine on SE that the concentrations of four endogenous hormones (IAA, ABA, GA, and ZEA) were all increased after treatment with exogenous spermidine. In particular, the IAA content in the callus was highest and gradually decreased with the process of embryo formation and maturation. Besides, GA content tends to increase rapidly and then gradually decrease during SE, similar to IAA, when explants are treated with exogenous spermidine (Wang et al., 2020).

In contrast, ABA and ZEA tended to increase in the later stages of SE. Thus, the early stages of SE require high levels of IAA and GA, but later stages require lower concentrations of IAA and GA instead of ABA and ZEA (Wang et al., 2020). Furthermore, Khai et al. (2021) also showed that auxin is only present at the stage of SE induction (the 30th day of culture), then gradually decreases, creating favorable conditions for embryo development; after 60–120 days of culture, the auxin content in the Begonia somatic embryo explants was no longer recorded. Our results from this study are similar to those of the above research; after 12 weeks of culture, most of the SE were in the mature stage, and at this stage, the presence of endogenous auxins, specifically IAA, was not detected. The ABA/GAs ratio can indicate a plant's growth and dormancy (Guo et al., 2018), as well as the embryo's pattern of development or late stages (Zheng et al., 2013). At different SE stages of *Ormosia henryi* Prain, the high ratios of IAA/GAs, IAA/ABA, AUX/ABA and AUX/GAs, and the low ratios of GAs/CKs and ABA/CKs were favorable for embryogenesis callus induction; however, the ratios of IAA/CKs and AUX/CKs were lower in cotyledon embryos, which encouraged SE maturation and differentiation. This result was consistent with the actual theory, low AUX and high CKs concentration were required for SE maturation and differentiation (Wu et al., 2021).

The use of nanoparticles in plant tissue culture has gained significant attention in recent years. Nanoparticles, mainly AgNPs, have been reported to enhance the growth and development of plantlets. The presence of AgNPs in the culture medium has been shown to enhance different plant species' morphological, physiological, and biochemical characteristics.

This study showed that the application of AgNPs has a positive effect on the formation and growth of Lang Bian ginseng rhizomes. *Tecomella undulata* micropropagation with 60 μg/L AgNPs has been shown to improve the quality of plantlets (Sarmast et al., 2015). So far, there has been just 1 report on the effects of AgNPs on the rhizome formation of Ngoc Linh ginseng (Cuong et al., 2021), which reported that the growth of Ngoc Linh ginseng rhizomes was significantly improved when cultured on a medium adding with 1.2 mg/L AgNPs. In addition, the presence of AgNPs also helped improve the abnormal phenomena of Ngoc Linh ginseng (Cuong et al., 2021), strawberry (Tung et al., 2021), and gerbera (Tung et al., 2022).

Furthermore, Cuong et al. (2021) also showed that adding AgNPs to the culture medium significantly enhanced the synthesis of secondary compounds in Ngoc Linh ginseng. These secondary metabolites have essential roles in plant defense mechanisms and in producing valuable phytochemicals, such as flavonoids, alkaloids, and phenolic compounds.

Overall, using AgNPs in plant tissue culture has been shown to significantly affect plant growth, development, and secondary metabolite synthesis. However, further research is needed to fully understand the mechanisms involved in enhancing plant growth and secondary metabolite synthesis by AgNPs.

4. Conclusion

The supplement of 0.01 mM spermidine to the culture medium increased SE in both leaf and petiole explants of Lang Bian ginseng through increased synthesis of antioxidant enzymes, thereby reducing adverse effects from ROS on *in vitro* cultures. Besides, the fluctuations of endogenous hormones during SE are also clarified. Moreover, the presence of spermidine and AgNPs in the culture medium increased the rhizome formation rate and improved plantlet quality.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

CRediT authorship contribution statement

Truong Thi Lan Anh: Conceptualization, Methodology, Data curation, Writing – review & editing. **Nguyen Thi Nhu Mai:** Conceptualization, Methodology, Data curation. **Hoang Thanh Tung:** Data curation. **Hoang Dac Khai:** Data curation. **Do Manh Cuong:** Data curation, Writing – review & editing. **Vu Quoc Luan:** Data curation, Writing – review & editing. **Hoang Thi Nhu Phuong:** Data curation, Writing – review & editing. **Nguyen Van Binh:** Data curation, Writing – review & editing. **Bui Van The Vinh:** Data curation, Writing – review & editing. **Nguyen Thi Thanh Thuy:** Data curation, Writing – review & editing. **Nguyen Phuong Thao:** Data curation, Writing – review & editing. **Duong Tan Nhut:** Conceptualization, Methodology, Data curation, Writing – review & editing.

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Supplementary materials

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