MINISTRY OF EDUCATION AND TRAINING

VIETNAM ACADEMY OF SCIENCE AND TECHNOLOGY

GRADUATE UNIVERSITY OF SCIENCE AND TECHNOLOGY

Truong Thi Lan Anh

LANG BIAN GINSENG SOMATIC EMBRYOGENESIS

(*Panax vietnamensis* var. *langbianensis*)

SUMMARY OF DISSERTATION IN PLANT PHYSIOLOGY

Code: 9 42 01 12

HANOI – 2024

The dissertation is completed at: Graduate University of Science and Technology, Vietnam Academy Science and Technology

Supervisors:

1. Supervisor 1: Prof. Duong Tan Nhut, Taynguyen Institute for Scientific Research

2. Supervisor 2: Assoc. Prof. Nguyen Phuong Thao, International University -Vietnam National University Ho Chi Minh City

Referee 1: Assoc. Prof. Nguyen Du Sanh

Referee 2: Assoc. Prof. Nguyen Vu Phong

Referee 3: Assoc. Prof. Hoang Thi Kim Hong

The dissertation will be examined by Examination Board of Graduate University of Science and Technology, Vietnam Academy of Science and Technology at 14h on August 23, 2024

The dissertation can be found at:
1. Graduate University of Science and Technology Library
2. National Library of Vietnam

LIST OF THE PUBLICATIONS RELATED TO THE DISSERTATION

1. **Truong Thi Lan Anh**, Hoang Thanh Tung, Hoang Dac Khai, Nguyen Thi Nhu Mai, Vu Quoc Luan, Do Manh Cuong, Hoang Thi Nhu Phuong, Le Thi Diem, Nguyen Quang Vinh, Doan Manh Dung, Bui Van The Vinh, Nguyen Phuong Thao, Duong Tan Nhut, Micropropagation of Lang Bian ginseng: an endemic medicinal plant. Plant Cell, Tissue and Organ Culture, 2022, 151(3), 565-578. https://doi.org/10.1007/s11240-022-02372-8

2. **Truong Thi Lan Anh**, Nguyen Thi Nhu Mai, Hoang Thanh Tung, Hoang Dac Khai, Do Manh Cuong, Vu Quoc Luan, Hoang Thi Nhu Phuong, Nguyen Van Binh, Bui Van The Vinh, Nguyen Thi Thanh Thuy, Nguyen Phuong Thao, Duong Tan Nhut, Effect of spermidine, glutamine, and proline on somatic embryogenesis and silver nanoparticles supplied culture improved rhizome formation of *Panax vietnamensis* var. *langbianensis*. South African Journal of Botany, 2023, 163, 226-236. https://doi.org/10.1016/j.sajb.2023.10.032.

INTRODUCTION

The genus *Panax*, one of the essential medicinal plant genera, contains ginsenosides with many biological activities and high medicinal values such as anti-stress, anti-fatigue, anti-aging, and enhancement of vitality. Until now, 20 species and subspecies in the genus *Panax* are distributed in East Asia, the Himalayas, Southeast Asia, and North America. However, the precious medicinal plant is rare due to overexploitation, which leads to the threat of extinction. In addition, the propagation of medicinal plants in nature takes much time and depends on natural conditions.

Micropropagation via somatic embryogenesis (SE) is useful for replacing the traditional method with true-to-type plantlets. This method has also been successfully performed on *Panax ginseng* Mayer, *Panax notoginseng*, *Panax quinquefolius*, *Panax japonicus*, *Panax vietnamensis*. The efficiency of SE depends on many factors, such as sterilization, plants, explant types, mineral medium, and plant growth regulators. Besides, primary somatic embryos continue to increase biomass by repeated cycles of secondary SE, thereby improving the propagation process's efficiency. Furthermore, plantlets transferred to the greenhouse had meager survival due to their underdeveloped root systems. There has been some research on *in vitro* rhizome formation to improve plantlet acclimatization in greenhouse conditions such as *P. vietnamensis*, *P. ginseng* and *P. quinquefolius*.

Besides, Lang Bian ginseng (*P. vietnamensis* var. *langbianensis* N.V. Duy, V.T. Tran & L.N. Trieu), an herbaceous medicinal plant that originated from Langbian mountain of Lac Duong District, Lam Dong Province, was discovered by Duy et al. (2016). This plant is famously known as medicine folk, with a small population and number of individuals, scattered distribution, and low genetic diversity. This is due to the exploitation of the indigenous people for medicinal and commercial purposes. Until now, the

micropropagation of Lang Bian ginseng has not been published. Hence, the Lang Bian ginseng somatic embryogenesis (*Panax vietnamensis* var. *langbianensis*) was carried out.

The objectives of the thesis: The thesis objective is to identify factors suitable for primary and secondary somatic embryogenesis and *in vitro* Lang Bian ginseng rhizome formation that accumulate saponin in detail: (1) This study aimed to induce initial materials *in vitro*, (2) Primary somatic embryogenesis using TCL technique, (3) Secondary somatic embryogenesis (4) Rhizome formation with ginsenoside accumulation was also studied.

New findings of the thesis: (1) Silver nanoparticles (AgNPs) have replaced traditional explant surface disinfectants. AgNPs reduced or eliminated the source of Lang Bian ginseng rhizome contamination and positively affected explant somatic embryogenesis. (2) The effectiveness of the TCL technique via SE was evaluated for the first time on Lang Bian ginseng in this study. In particular, primary embryos are an essential material source for secondary SE to improve the efficiency of the propagation process (3) Plantlets derived from secondary embryos grew well with rhizome formation and saponin accumulation *in vitro*.

Structure of the thesis: The thesis includes five main parts: *Introduction*, Chapter 1: *Overview*, Chapter 2: *Materials, content, and research methods*, Chapter 3: *Results and Discussion*, and *Conclusion and Recommendations*.

Chapter 1. OVERVIEW

Ph.D. thesis has been referred to use in this dissertation related to: (1) Overview of the genus *Panax* and *Panax vietnamensis var langbianensis;* (2) Primaly and secondary somatic embryogenesis; (3) The thin cell layer (TCL) technique; (4) Silver nanoparticles enhanced the efficiency of explant surface disinfection and morphogenesis.

Chapter 2. MATERIALS, CONTENTS AND METHODS

2.1. Materials

In content 1, ten-year-old Lang Bian ginseng rhizome explants (1.5 cm in diameter, 9 cm in length) collected from Lang Bian mountain (Lam Dong province, Vietnam) were used as original material for the study. In content 2, the leave, and petiole of Lang Bian ginseng's twelve-week-old shoot were used for somatic embryogenesis. In content 3, the somatic embryos with torpedo shape were used for secondary somatic embryogenesis. In content 4, secondary somatic embryos with cotyledonary shape and adventitious shoot were used for rhizome formation.

2.2. Contents

Content 1: Induction of *in vitro* materials

Content 2: Primary somatic embryogenesis via TCL culture

Content 3: Secondary somatic embryogenesis

Content 4: Rhizome formation and saponin content

2.3. Methods

2.3.1. Experimental design

2.3.1.1. Content 1: Induction of in vitro materials

- **Experiment 1:** Effect of AgNPs on explant surface disinfection and adventitious shoot regeneration

Rhizome explants were pretreated and immersed in AgNPs at different concentrations (0.075; 0.100; 0.125; 0.150 and 0.200%) for 30 min. Then, explants were cut into small pieces (0.5 cm×0.5 cm in size; thickness 0.1 cm) and cultured on MS medium (Murashige and Skoog 1962) supplemented with 1 mg/L 2,4-D, 0.2 mg/L TDZ, 30 g/L sucrose and 8.0 g/L. Eight-week-old calli derived from sterilized-AgNPs rhizomes were cut into small pieces (0.5 cm×0.5 cm in size) and cultured on SH medium added with 2.0 mg/L benzylaminopurine, 50 g/L sucrose and 8.0 g/L agar for adventitious shoot formation.

• **Experiment 2:** Effect of cytokinin on shoot multiplication *in vitro*

Lang Bian ginseng shoots *in vitro* (1 cm) were cultured on SH medium containing different concentrations of BA (0; 0,5; 1,0; 1,5; 2,0 mg/L) or kinetin (0; 0,5; 1,0; 1,5; 2,0 mg/L) for shoot multiplication.

2.3.1.2. Content 2: Somatic embryogenesis via TCL culture

• **Experiment 3:** *In vitro* primary somatic embryogenesis via L-tTCL or P-lTCL explants cultured on auxin-supplemented medium

A Lang Bian ginseng twelve-week-old shoot included five leave (5 mm×10 mm: width×length, each) and a petiole (1 mm×30 mm: width×length). A leaf (L) was cut transverse TCL into 10 explants (L-tTCL: 1 mm×5 mm). Meanwhile, a petiole (P) was cut into 3 explants (1 mm×10 mm in size); then, each P explant was cut longitudinally TCL (P-lTCL) into 2 explants (P-lTCL: 0.5 mm × 10 mm).

The L-tTCL and P-lTCL explants were cultured on MS medium containing different concentrations of 2,4-D (0.5; 1.0; 1.5; 2.0 mg/L), NAA or IBA (1.0; 3.0; 5.0; 7.0; 9.0 mg/L), 30 g/L sucrose and 8.0 g/L agar for somatic embryogenesis.

• **Experiment 4:** Effects of proline, glutamine or spermidine on primary somatic embryogenesis of Lang Bian ginseng L-tTCL or P-lTCL explants

The L-tTCL or P-lTCL explants were cut similarly to experiment 3 and cultured on MS medium containing different concentrations of glutamine (146; 438; 730; 1022 mg/L), proline (100; 200; 300; 400 mg/L) or spermidine (1,5; 7,5; 15 và 30 mg/L) for somatic embryogenesis.

2.3.1.3. Content 3: Secondary somatic embryogenesis

• **Experiment 5:** Effect of medium on secondary somatic embryogenesis

The somatic embryos with torpedo-shape (about 1.5 mm in height) were cultured on different media, including SH; MS; 1/2MS; WPM and B5

supplemented with 1.0 mg/L 2,4-D, 30 g/L sucrose and 8.0 g/L agar for secondary somatic embryogenesis.

• **Experiment 6:** Effect of sucrose concentration on secondary somatic embryogenesis

The somatic embryos with torpedo-shape (about 1.5 mm in height) were cultured on the best medium obtained from experiment 5 supplemented with 1.0 mg/L 2,4-D, 30 g/L sucrose and 8.0 g/L agar and sucrose at different concentrations (0; 10; 20; 30; 40; 50 g/L) for secondary somatic embryogenesis.

• **Experiment 7:** Effect of coconut water concentration on secondary somatic embryogenesis

The somatic embryos with torpedo-shape (about 1.5 mm in height) were cultured on the best medium and sucrose concentration obtained from experiments 5 and 6 supplemented with 1.0 mg/L 2,4-D and 8.0 g/L agar and coconut water at different concentrations (0; 5; 10; 15; 20; 30 %) for secondary somatic embryogenesis.

2.3.1.4. Content 4: Rhizome formation and saponin content

• **Experiment 8:** Lang Bian ginseng rhizome formation and saponin content *in vitro*

Secondary somatic embryos with cotyledonary shape (1.5 mm in height) and adventitious shoot (1.5 cm in height) were cultured on SH medium containing 0.5 mg/L BAP, 0.5 mg/L NAA, 30 g/L sucrose, 1.0 g/ L activated charcoal and 8.0 g/L agar for rhizome formation

• **Experiment 9:** Subsequent growth of Lang Bian ginseng embryos in AgNPs supplement medium

The leaf-derived cotyledonary somatic embryos cultured on SH medium adding 0,5 mg/L BA, 0,5 mg/L NAA, 30 g/L sucrose, 1,0 g/L activated charcoal, 9,0 g/L agar và 1,2 mg/L AgNPs for subsequent growth

and rhizome formation compared to the control (without the addition of AgNPs)

2.4. Morphology observations

Different somatic embryo morphology was cut, stained, and observed under a microscope (Peterson et al. 2008).

2.5. Data collection and analysis

2.5.1. Somatic embryogenesis and growth parameters

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

Antioxidant enzyme activity (SOD, CAT and APX) was determined after 12 weeks of culture.

2.5.3. Determination of endogenous hormone content by HPLC-UV

The hormones (2iP, ZEA, KIN, mT, IAA, GA3, SA, ABA, and MEL) of fresh leaf, petiole, and somatic embryo samples (induction phage, embryogenesis callus, globular embryo, and cotyledon embryo) were determined by UHPLC-UV.

2.5.4. Saponin accumulation

The 20-week-old adventitious roots and rhizomes were collected to determine saponin accumulation.

2.6. Cultured condition

The treatments are assigned completely at random with 5 replications and each replication has 30 samples. The explants were incubated in a culture room at a humidity of 55 - 60%, 25±2 °C under darkness or photosynthetic photon flux density (40 – 45 µmol m^{-2} s^{-1}) with 16 h light and 8 h dark photoperiod.

2.7. Statistical analysis

All data were processed by Microsoft Excel 2010 and SPSS 16.0 statistical analysis software based on the Duncan's multiple range test at $p<0.05$ (Duncan 1955). Except experiments 8 and 9 based on the T-test

Chapter 3. RESULTS

3.1. Content 1: Induction of *in vitro* materials

3.1.1. Effect of AgNPs on explant surface disinfection and adventitious shoot regeneration

The results showed that AgNPs were effective in explant surface disinfection and callus induction after 8 weeks of culture. After 2 weeks of culture, the percentage of contaminated explants declined from the highest 76.00% (0.075% AgNPs treatment) to the lowest 25.33% (0.20% AgNPs treatment) but led to necrosis of the rhizome (up to 58.67% in 0.20% AgNPs treatment). After 8 weeks of culture, the disinfected rhizomes in AgNPs treatments formed callus, and the highest callus induction rate was 49.33% for the explants that were surface sterilized with 0.15% AgNPs (Table 3.1).

Table 3.1. Effect of silver nanoparticles on surface disinfection and callus induction of Lang Bian ginseng rhizome after 8 weeks of culture

AgNPs Concentration (%)	Contaminated rate (%) after 2 weeks of culture	Necrosis rate (%) after 2 weeks of culture	Survival rate with callus induction (%) after 8 weeks of culture
0.075	76.00 ± 7.61^{a}	1.34 ± 1.23^{c}	22.66 ± 8.96^{c}
0.100	62.67 ± 8.96^{b}	2.68 ± 1.64^{c}	34.68 ± 8.69^{b}
0.125	54.67 ± 3.00^{b}	6.68 ± 4.70^{c}	38.66 ± 5.60^{b}
0.150	36.00 ± 5.95^{c}	14.68 ± 8.69^{b}	49.34 ± 7.59^{a}
0.200	25.33 ± 5.57^{d}	58.68 ± 7.30^{a}	16.00 ± 3.70^{c}

**Different letters (a, b, ...) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test).*

Calli derived from disinfected rhizomes in AgNPs treatments were transferred to MS medium supplemented with 2 mg/L BAP and formed adventitious shoots after 12 weeks.

Rhizome-derived callus disinfected in 0.15% AgNPs gave a shoot regeneration rate about four times higher than that of 0.2% AgNPs, as shown by the regenerated shoot (45.33% in comparison to 12.00%, respectively), and the number of shoots (5.20 shoots in comparison to 1.20 shoots, respectively) after 12 weeks of culture. Thus, 0.15% AgNPs treatment gave both effective sterilization and adventitious shoot regeneration.

3.1.2. Effects of cytokinin on Lang Bian ginseng shoot multiplication

The results show that BA and kinetin positively affect Lang Bian ginseng shoots multiplication. In the control treatment, no new shoot was recorded. Healthy green shoots were obtained in medium added 1.0 mg/L BA.

3.2. Content 2: Primary somatic embryogenesis via TCL culture

3.2.1. In vitro primary somatic embryogenesis via L-tTCL or P-lTCL explants cultured on auxin-supplemented medium

Morphogenesis from L-tTCL explants of Lang Bian ginseng, cultured on MS medium supplemented with auxin (2,4-D, NAA or IBA), including (1) somatic embryogenesis, (2) callus induction and (3) adventitious root regeneration, were obtained after 12 weeks of culture. (Fig. 3.4). All auxin-treated L-tTCL explants produced somatic embryos, except for 9 mg/L NAA-treated and 1 mg/L IBA-treated explants after 12 weeks of culture. The SE rates (80.00% and 86.66%) were the highest at 5–7 mg/L NAA treatments, while the highest number of SE (40.40) was achieved at 1.5 mg/L 2,4-D treatment. However, it was found significant, the highest GCF of L-tTCL was obtained at 7 mg/L NAA (32.73) (Table 3.3).

The SE rate (100%), the number of SE (51.80 embryos/explant), and GCF of P-lTCL (51.80) in 1 mg/L 2,4-D—treated were significantly higher than those in control and other treatments (Table 3.4). In addition, auxin significantly affects the GCF of SE on both L-tTCL and P-lTCL explants. For L-tTCL explants, control treatment failed to induce SE. In contrast, the

GCF tended to increase with raised auxin concentration. The highest GCF of L-tTCL explants treated with NAA (1636.62) was significantly higher than the highest GCF of explants treated with 2,4-D (1284.89) and IBA (829.98). The total GCF obtained from a single shoot of Lang Bian ginseng, including L-tTCL and P-lTCL, was 1947.42 after 12 weeks of culture.

Table 3.3. Comparison of primary somatic embryogenesis efficiency from L-tTCL explants in auxin-supplemented media after 12 weeks of culture

Auxin	Concentration (mg/L)	Somatic embryogennesis (%)	No. of somatic embryos	GCF L-tTCL
Control	0	-	-	-
2,4-D	0,5	19.99 ± 7.46^{f}	28.20 ± 1.79^{e}	5.63 ± 2.09^{f}
	1,0	56.66 ± 9.13^{bc}	35.20 ± 2.39^{c}	19.90 ± 3.06^{c}
	1,5	63.33 ± 7.45^{b}	40.40 ± 2.79^{a}	25.70 ± 4.25^{b}
	2,0	26.66 ± 9.13^{ef}	6.40 ± 1.14^{i}	1.70 ± 0.67^{g}
NAA	1,0	16.66 ± 0.00^{f}	13.40 ± 2.88^{h}	2.23 ± 0.48^{g}
	3,0	33.33 ± 0.00^{de}	30.80 ± 2.86^{d}	10.27 ± 0.95^{e}
	5,0	80.00 ± 7.46^{a}	32.80 ± 1.92^{cd}	26.33 ± 3.66^{b}
	7,0	86.66 ± 7.46^{a}	37.80 ± 1.30^{b}	32.73 ± 2.59^{a}
	9,0	-	-	-
IBA	1,0	-	-	-
	3,0	13.33 ± 7.45^{f}	21.80 ± 3.27^{g}	2.80 ± 1.63^{g}
	5,0	30.00 ± 7.46^{de}	25.40 ± 1.82^{f}	7.63 ± 2.03^{ef}
	7,0	53.33 ± 7.45^{c}	31.00 ± 2.00^{d}	16.60 ± 3.13^{d}
	9,0	36.53 ± 7.53^{d}	26.80 ± 1.79^{ef}	9.80 ± 2.15^{e}

**Different letters (a, b, ...) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test).*

Table 3.4. Comparison of primary somatic embryogenesis efficiency from P-lTCL explants in auxin-supplemented media after 12 weeks of culture

Auxin	Concentration (mg/L)	Somatic embryogennesis (%)	No. of somatic embryos	GCF L-tTCL
Control	0	16.70 ± 0.00^{h}	2.00 ± 0.71^{h}	0.33 ± 0.12^{f}
2,4-D	0,5	29.98 ± 7.42^{gh}	2.80 ± 0.84^{h}	0.83 ± 0.33^{f}
	1,0	100.00 ± 0.00^{a}	51.80 ± 6.38^{a}	51.80 ± 6.38^{a}
	1,5	70.02 ± 7.42^{cd}	33.00 ± 2.00^{d}	23.16 ± 3.53^{c}
	2,0	53.34 ± 7.47^{e}	33.40 ± 5.46^{d}	17.87 ± 4.07^{d}
NAA	1,0	20.02 ± 7.42^{gh}	16.00 ± 2.65^{g}	3.30 ± 1.73^{f}
	3,0	63.33 ± 7.45^{e}	21.00 ± 2.35^{f}	13.33 ± 2.40^{e}
	5,0	89.98 ± 9.15^{b}	33.00 ± 1.58^{d}	29.80 ± 4.35^{b}
	7,0	60.02 ± 9.15^{de}	43.00 ± 3.74^{b}	25.57 ± 2.35^{bc}
	9,0	23.34 ± 9.09^{gh}	5.20 ± 1.79^{h}	1.20 ± 0.66^{f}
IBA	1,0	43.32 ± 9.15^{f}	21.40 ± 2.51^{f}	9.40 ± 2.80^{e}
	3,0	79.98 ± 7.42^{c}	28.40 ± 3.85^{e}	22.77 ± 4.12^{c}
	5,0	76.66 ± 9.09^{c}	38.00 ± 3.67^{c}	28.96 ± 2.86^{b}
	7,0	70.20 ± 7.42^{cd}	40.40 ± 3.21^{bc}	28.23 ± 3.16^{b}
	9,0	36.64 ± 7.47^{fg}	29.80 ± 1.52^{de}	10.93 ± 2.29^{e}

**Different letters (a, b, ...) in the same column represent statistically significant differences at p < 0.05 (Duncan's test).*

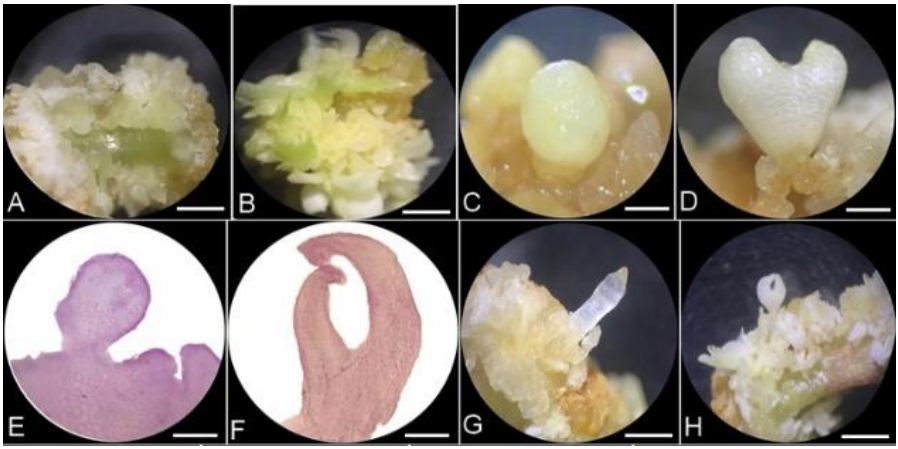


Fig. 3.4. Morphological and histological observation during primary somatic embryogenesis of Lang Bian ginseng. A Embryogenic calli. B Somatic embryogenesis from L-tTCL explants after 12 weeks of culture. C Somatic embryos at the globular and heart embryo stage. D Somatic embryos at the heart embryo stage. E Longitudinal cut of the early globular stage from embryogenic callus. F Longitudinal cut of the cotyledonary stage. G Adventitious root formation. H Somatic embryogenesis from P-lTCL explants after 12 weeks of culture (Bar: 3 mm – A; B; H; Bar: 1mm – C; D; E; F; G).

3.2.2. Effects of proline, glutamine or spermidine on primary somatic embryogenesis of Lang Bian ginseng L-tTCL or P-lTCL explants

3.2.2.1. Effects of glutamine, proline or spermidine on primary somatic embryogenesis of Lang Bian ginseng L-tTCL explants in vitro

The effects of glutamine, proline, and spermidine on primary SE from leaf explants after 12 weeks were recorded in Table 3.6 and Fig. 3.5.

Table 3.6. Comparison of primary somatic embryogenesis efficiency from L-tTCL explants on MS medium supplemented with glutamine, proline or spermidine after 12 weeks of culture

Treatment	**Concentration (mg/L)**	**Somatic embryogenesis rate (%)**	**No. of embryos/ explant**	**Somatic embryo morphology**
Control	0	76.68 ± 14.88^{b}	33.40 ± 4.67^{c}	Globular
Glutamine	146	83.34 ± 16.65^{ab}	52.20 ± 5.26^{a}	Globular, heart, torpedo
	438	83.32 ± 11.77^{ab}	22.60 ± 4.67^{d}	
	730	60.02 ± 9.15^{c}	12.60 ± 2.07^{ef}	
	1022	56.68 ± 9.15^{cd}	10.40 ± 2.07^{ef}	
Proline	100	53.34 ± 7.47^{cd}	21.00 ± 4.06^{d}	
	200	29.98 ± 7.42^{e}	14.60 ± 3.65^{e}	
	300	0.00 ± 0.00^{f}	0.00 ± 0.00^{g}	-
	400	0.00 ± 0.00^{f}	0.00 ± 0.00^{g}	-
Spermidine	1,5	93.32 ± 9.15^{a}	54.20 ± 4.44^{a}	Globular, heart, torpedo, and cotyledon
	7,5	80.00 ± 13.92^{ab}	38.60 ± 3.43^{b}	
	15	60.02 ± 9.15^{c}	8.60 ± 1.14^{f}	
	30	43.32 ± 14.94^{de}	3.40 ± 0.89^{g}	

**Different letters (a, b, ...) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test).*

Besides, spermidine at low concentrations (1,5 - 7,5 mg/L) posed positive effects on SE, whereas this polyamine ceased somatic embryo formation when applied at higher concentrations (Table 3.6). Moreover, somatic embryos were observed in globular, heart, torpedo, and cotyledon shapes in all spermidine treatments (Fig. 3.5D), with the highest outcome of SE rate and somatic embryos/explant were 93.32 % and 54.20, respectively, in the 1,5 mg/L treatment. The somatic embryo regeneration coefficient gave the same result; the highest GCF was obtained at 1,5 mg/L spermidine.

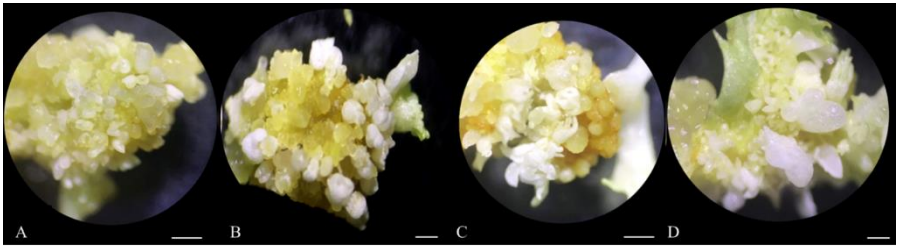


Fig. 3.5. Primary somatic embryogenesis from L-tTCL explant of Lang Bian ginseng after 12 weeks of culture (Bar: 1 mm) A. Control; B. 146 mg/L glutamine; C. 100 mg/L proline; D. 1,5 mg/L spermidine

3.2.2.2. Effects of glutamine, proline or spermidine on primary somatic embryogenesis of Lang Bian ginseng P-lTCL explants in vitro

For petiole explants, adding glutamine and proline to the MS medium did not stimulate the explants' SE rate. 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 1,5 mg/L spermidine, which is a statistically significant difference from the other treatments (Table 3.7, Fig. 3.6). Like the leaf explant, the petiole showed the lowest embryogenesis when increasing the concentration of spermidine supplement up to 30 mg/L. Besides, the SE coefficient also gave similar results.

Table 3.7. Comparison of primary somatic embryogenesis efficiency from P-lTCL explants on MS medium supplemented with glutamine, proline or spermidine after 12 weeks of culture

Treatment	**Concentration (mg/L)**	**Somatic embryogenesis rate (%)**	**No. of embryos/ explant**	**Somatic embryo morphology**
Control	0	93.32 ± 9.15[a]	54.20 ± 4.27[b]	Globular
Glutamine	146	73.34 ± 9.09[c]	35.00 ± 5.48[d]	Globular, heart, torpedo
	438	63.36 ± 7.47[cd]	34.60 ± 4.22[d]	
	730	53.34 ± 7.47[de]	23.00 ± 8.37[e]	Globular
	1022	43.32 ± 9.15[e]	18.20 ± 5.07[ef]	
Proline	100	79.98 ± 7.42[b]	42.00 ± 8.60[c]	Globular, and heart
	200	73.34 ± 9.09[c]	29.80 ± 6.14[d]	Globular
	300	60.02 ± 9.15[d]	19.60 ± 3.78[ef]	
	400	56.68 ± 9.15[d]	13.40 ± 4.22[f]	
Spermidine	1,5	96.66 ± 7.47[a]	68.80 ± 2.95[a]	Globular, heart, torpedo, and cotyledon
	7,5	93.32 ± 9.15[a]	60.40 ± 5.13[b]	
	15	79.98 ± 7.42[b]	46.40 ± 2.97[c]	
	30	76.66 ± 9.09[b]	15.20 ± 3.11[f]	

**Different letters (a, b, ...) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test).*

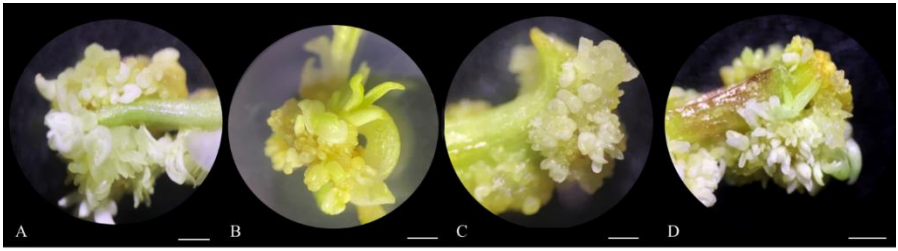


Fig. 3.6. Primary somatic embryogenesis from petiole explant of Lang Bian ginseng after 12 weeks of culture (Bar: 1 mm) A. Control; B. 146 mg/L glutamine; C. 100 mg/L proline; D. 1,5 mg/L spermidine

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

For L-tTCL leaf-explants, the levels of antioxidant enzymes, including CAT, SOD, and APX, in somatic embryos cultured on 1,5 mg/L spermidine medium were significantly higher than those on 146 mg/L glutamine and 100 mg/L proline. With 146 mg/L glutamine (186.89 U/g, 1145.52 U/g) treatment, and 100 mg/L proline (136.46 U/g; 930.22 U/g), 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 3.8).

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 explant, the addition of proline was able to enhance the activity of SOD and APX compared with the control (Table 3.9).

Table 3.8. Effects of proline, glutamine and spermidine on the activities of SOD, CAT and APX in L-tTCL 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}
146 mg/L Glutamine	186.89 ± 1.47^{c}	1145.52 ± 3.41^{c}	2.65 ± 0.04^{b}
100 mg/L Proline	136.46 ± 1.27^{d}	930.22 ± 1.28^{d}	1.78 ± 0.04^{c}
1,5 mg/L Spermidine	355.10 ± 10.87^{a}	2047.87 ± 13.76^{a}	3.18 ± 0.15^{a}

**Different letters (a, b, ...) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test).*

Table 3.9. Effects of proline, glutamine and spermidine on the activities of SOD, CAT and APX in P-lTCL explants of Lang Bian ginseng after 12 weeks of culture.

Nghiệm thức	**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}
146 mg/L Glutamine	181.60 ± 2.73^{b}	1183.11 ± 7.39^{b}	2.50 ± 0.05^{b}
100 mg/L Proline	151.81 ± 1.09^{c}	689.20 ± 6.68^{d}	1.64 ± 0.06^{c}
1,5 mg/L Spermidine	208.19 ± 2.16^{a}	1794.30 ± 16.20^{a}	2.87 ± 0.03^{a}

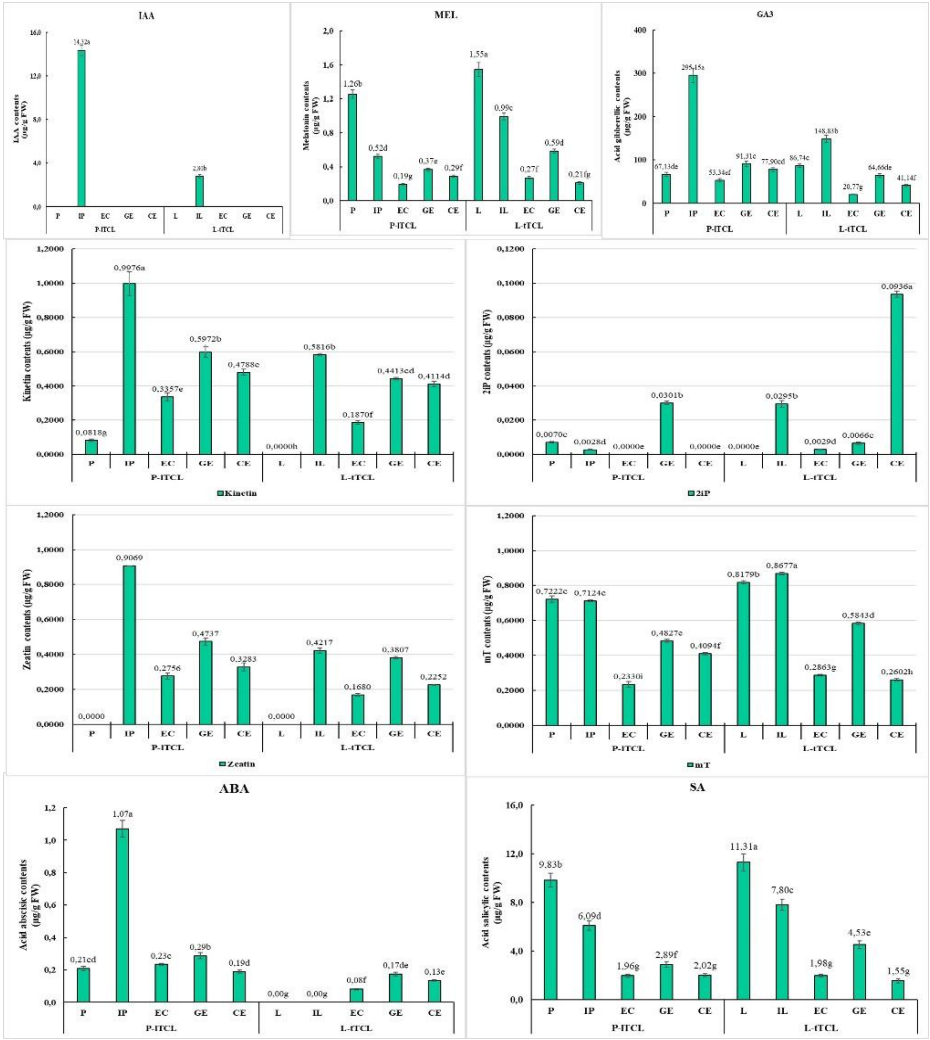
**Different letters (a, b, ...) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test).*

3.2.2.4. 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. 3.9). Endogenous CKs (ZEA, 2iP, KIN, mT), IAA, and GA_3 concentrations were the highest during the induction stage in both types of 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 and not at any other stages. 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).

Depending on the explant type, endogenous hormone ratios varied widely during SE (Fig. 3.13). In both types of explants, the ratio of IAA and MEL/CKs 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. 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 the 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 ratios decreased in the remaining stages



of SE for leaf explants.

Fig. 3.9. 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.

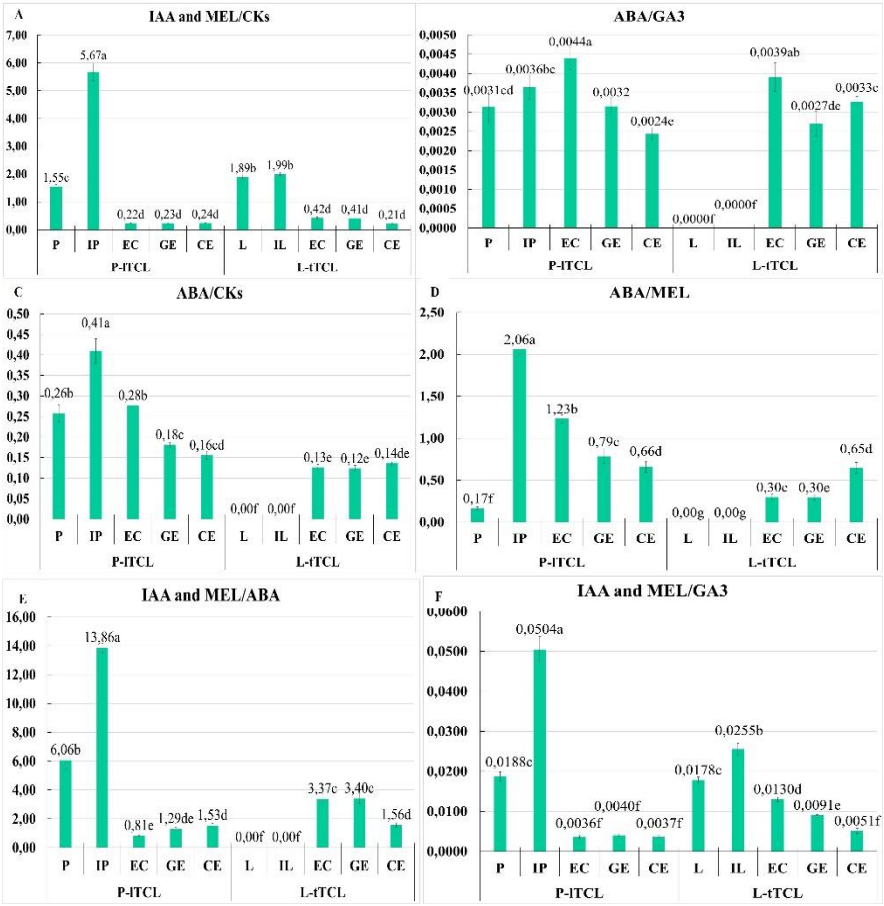


Fig. 3.13. The ratio of endogenous hormones in somatic embryogenesis of Lang Bian ginseng.

3.3. Content 3: Secondary somatic embryogenesis

3.3.1. Effect of medium on secondary somatic embryogenesis

The highest number of secondary somatic embryos (39.20 embryos/explant), fresh weight (1005.34 mg) and dry weight (77.90 mg) were observed on 1/2 MS medium after 12 weeks of culture. Besides, the secondary somatic embryos formed in 1/2 MS medium were mainly in the cotyledonary stage. This embryo type was suitable for regenerating plantlets**.**

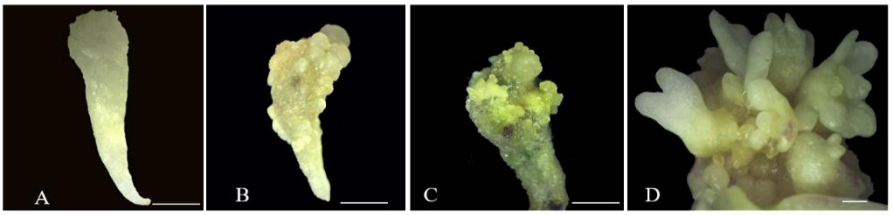


Fig. 3.14. Secondary somatic embryogenesis Lang Bian ginseng. A Primary embryo. B Primary-derived secondary embryo formation with globular stage. C Secondary embryo multiplication. D The developmental stages of secondary embryos (Bar: 1 mm – A, B, C; Bar: 2 mm – D)

3.3.2. Effect of sucrose concentration on secondary somatic embryogenesis

The highest number of secondary somatic embryos/explant (47.40 embryos), fresh weight (1062.29 mg) and dry weight (82.77 mg) were recorded in 40 g/L sucrose treatment after 12 weeks of culture. In addition, sucrose was influential in the subsequent secondary somatic embryo development. In the control treatment, secondary somatic embryos were formed mainly in globular shape and needed to be developed through subsequent stages to create complete plantlets. Meanwhile, secondary somatic embryos were formed in a medium supplemented with 40 g/L sugar, mainly at the torpedo and cotyledon stages (79.48%).

3.3.3. Effect of coconut water concentration on secondary somatic embryogenesis

Secondary somatic embryogenesis, mainly in the torpedo and cotyledon stages, was the highest in 15% of coconut water-supplemented medium.

3.4. Content 4: Rhizome formation and saponin content

3.4.1. Rhizome formation

Healthy morphological normalities of the secondary somatic embryos and adventitious shoot-derived plantlets with large and dark green leaves were obtained after 20 weeks of culture. Adventitious shoot-derived plantlets induced adventitious roots, whereas rhizome and adventitious roots were obtained from secondary embryo-derived plantlets (Fig. 3.18).

Fig. 3.18. Rhizome formation from secondary embryo after 12 weeks of culture (Bar-1 cm)

3.4.2. Saponin content

The analysis of saponins using the HPLC method showed that both twenty-week-old *in vitro* Lang Bian ginseng rhizomes and adventitious roots had Rg1, Rd and Rb1. The concentrations of Rg1, Rd, Rb1, and total saponins in rhizome explants (703.38, 770.67, 174.81, and 1648.86 µg/g, respectively) were higher than those in adventitious roots (325.79, 171.08, 4.78, and 501.65 µg/g, respectively).

3.4.3. Subsequent growth of Lang Bian ginseng embryos in AgNPs supplement medium

The leaf-derived cotyledonary somatic embryos cultured on MS medium adding 1,5 mg/L spermidine after being transferred to SH medium with the additions of 0.5 mg/L BA, 0.5 mg/L NAA, 30 g/L sucrose, 1.0 g/L activated charcoal, 9 g/L agar and 1.2 mg/L AgNPs showed an improved rate of rhizome formation compared to the control (without the addition of AgNPs). In addition, adding AgNPs to the MS medium also helped increase the rhizome formation rate and improve crop quality.

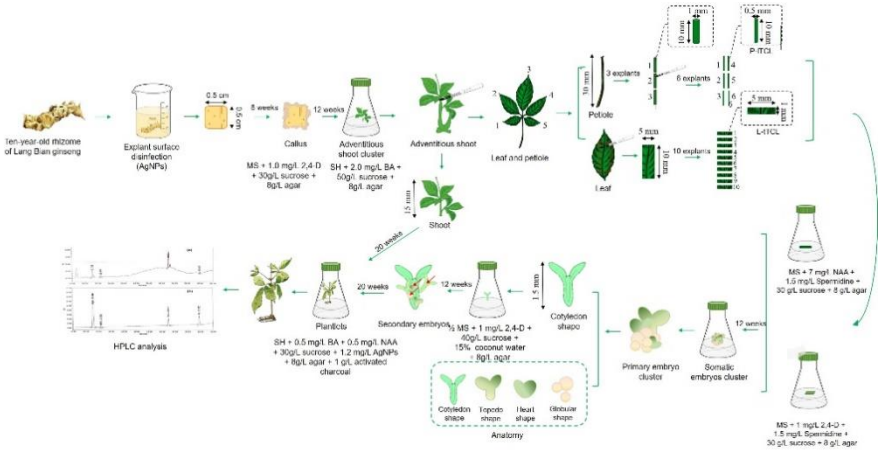


Fig. 3.20. Schematic of Lang Bian ginseng micropropagation via somatic embryogenesis

CONCLUSION AND RECOMMENDATIONS

1. Conclusion

Induction of in vitro materials

Rhizome explants disinfected with 0.15% AgNPs for 30 min resulted in the most effective surface disinfection. Moreover, adventitious shoot regeneration from 0.15% AgNPs disinfected-rhizome shoots multiply

rapidly in SH medium supplemented with 1 mg/L BA, 30 g/L sucrose, 8,0 g/L agar.

In vitro primary somatic embryogenesis via TCL explants cultured

A positive role for NAA or 2,4-D in Lang Bian ginseng somatic embryogenesis was demonstrated in this study. Besides, glutamine, proline and spermidine affect somatic embryogenesis. In particular, the optimal somatic embryogenesis was achieved on MS medium supplemented with 1,5 mg/L spermidine in both L-tTCL and P-lTCL through an enhanced synthesis of antioxidant enzymes (APX, CAT and SOD). In addition, at different SE stages of Lang Bian ginseng, there were significant differences in endogenous hormone contents. Endogenous CKs (ZEA, 2iP, KIN, mT), IAA, and GA3 concentrations were the highest during the induction stage in both types of explants. IAA was only detected at the induction stage of SE in both explants and not at any other stages. At the same time, the presence and concentration of these endogenous hormones also differed between leaf and petiole explants.

Secondary somatic embryogenesis

Secondary somatic embryos, mainly in the cotyledonary stage, were obtained from the primary torpedo somatic embryos culture on 1/2 MS medium supplemented with 1.0 mg/L 2,4-D, 40 g/L sucrose and 15% coconut water.

Rhizome formation and saponin content

Healthy, morphological normalities of secondary somatic embryo-derived plantlets with large and dark green leaves; rhizomes were obtained after 20 weeks of culture in a SH medium supplemented with 0.5 mg/L BA, 0.5 mg/L NAA, 30 g/L sucrose, 1.0 g/L activated charcoal, 8,0 g/L agar and 1.2 mg/L AgNPs. Moreover, the analysis of saponins using the HPLC method showed that twenty-week-old *in vitro* Lang Bian ginseng rhizomes had Rg1, Rd and Rb1.

2. Recommendations

The study has shown factors affecting the primary and secondary embryogenesis of Lang Bian ginseng. However, it is necessary to continue in-depth research on physiological changes and gene expression to clarify this impact.

Further, study the plantlet acclimatization and growth in the greenhouse.

Further study and application of Lang Bian ginseng micropropagation via somatic embryogenesis on a larger scale are needed.