MINISTRY OF EDUCATION AND TRAINING

VIETNAM ACADEMY OF SCIENCE AND TECHNOLOGY

GRADUATE UNIVERSITY OF SCIENCE AND TECHNOLOGY

LE THI DIEM

IN VITRO POLYPLOIDIZATION OF NGOC LINH GINSENG (*Panax vietnamensis* Ha *et* Grushv.)

SUMMARY OF DISSERTATION IN PLANT PHYSIOLOGY

Code: 9 42 01 12

HANOI – 2023

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

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The dissertation will be examined by Examination Board of Graduate University of Science and Technology, Vietnam Academy of Science and Technology at ……../2023

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INTRODUCTION

Today, there is an increasing demand for medicinal plants, traditional propagation methods are often dependent on the environment, susceptible to many adverse biotic and abiotic effects, as well as the content of secondary compounds. low grade when harvesting does not meet the demand in terms of both quantity and quality. In this context, the development of artificial polyploid individuals would be a potential approach to increase vigor and secondary compound content.

Ngoc Linh ginseng is a species of ginseng endemic to Vietnam, the scientific name is Panax vietnamensis Ha et Grushv. The group of substances that have a decisive effect on the pharmacological effects of this ginseng species are saponins which are represented as MR2, G-Rb1 and G-Rg1. Moreover, this species of ginseng has the highest content of dammaran saponins and the highest amount of triterpene saponins compared to other species of the genus Panax in the world. However, Ngoc Linh ginseng has a cultivation period lasting from 6 to 7 years. Moreover, diploid Ngoc Linh ginseng grows very slowly and has low weight, so it cannot meet the demand.

Currently, one of the breeding strategies to improve the valuable properties of plants is artificial polyploid mutagenesis. Recently, there are many studies on creating polyploid mutants on medicinal plants, showing that medicinal plants have the entire set of duplicated chromosomes, the content has higher medicinal properties than desired. Therefore, artificial chromosome duplication in medicinal plants can bring about significant economic results. By using the ploidy manipulation by chemical agents such as colchicine, oryzalin. This is a new research direction on Ngoc Linh ginseng in Vietnam, which is meaningful in creating the foundation for mutation research and polyploidy mutations on many precious medicinal plants, which are still quite limited. Besides, this study also lays the foundation for plant physiological research as a basis for further studies on selection, crossbreeding at the cellular level and clonal propagation of polyploid Ngoc Linh ginseng in Vietnam.

Therefore, the topic **"Research on *in vitro* polyploidization of Ngoc Linh ginseng (*Panax vietnamensis* Ha *et* Grushv.)"** was conducted with the aim of creating polyploid bodies as well as obtaining a large number of polyploid seedlings with desirable properties such as: high vitality, good growth and development, thereby contributing to improving and enriching the source of precious medicinal plants in Vietnam.

The objectives of the thesis: Determining the optimal source of materials for somatic embryo induction through plant tissue culture techniques. Determining the polyploid agent and effective polyploidy rate. Generating polyploid Ngoc Linh ginseng by chemical agents.

The main contents of the thesis: Induction of somatic embryos from different sources of Ngoc Linh ginseng polyploid. Determining the polyploid agent, creating a polyploid plant of Ngoc Linh ginseng.

New findings of the thesis: (1) The research evaluated the embryogenesis ability of Ngoc Linh ginseng from different cultures, especially the ability to generate secondary embryos from many primary embryos. (2) Successfully built a process to create tetraploid Ngoc Linh ginseng plants through somatic embryogenesis under the influence of colchicine.

Structure of the thesis: The thesis includes 5 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

Ngoc Linh ginseng is a species of ginseng endemic to Vietnam, scientific name *Panax vietnamensis* Ha *et* Grushv., Ginseng family (Araliaceae). Ngoc Linh ginseng has the highest content of dammaran saponins (about 12-15%) and the highest amount of triterpene saponins compared to other species of the genus Panax in the world. With the above characteristics, Ngoc Linh ginseng is not only a precious ginseng species of Vietnam but also one of the precious ginsengs in the world. However, due to over-exploitation, this species has been included in the Red Book of

Vietnam and is in danger of extinction. Therefore, it is necessary to apply new techniques in production. In particular, tissue culture is an advanced technique that can be applied *in vitro* propagation of Ngoc Linh ginseng, which is considered to be the most effective method to preserve and develop this rare genetic resource.

Over the past 20 years, Ngoc Linh ginseng has been studied in many different fields. Studies on clonal propagation, cell thin layer culture techniques and studies on somatic embryogenesis of Ngoc Linh ginseng have also been carried out. In addition, some other research areas on Ngoc Linh ginseng such as: Research on the germination ability of artificial seeds from Ngoc Linh ginseng somatic embryos. Evaluation of genetic stability of Ngoc Linh ginseng by RAPD indicator; Transgenic hairy roots and growth survey of Ngoc Linh ginseng roots; Research on accumulation and production of secondary compounds.

Induction of polyploidy is a plant breeding method that can generate new genotypes with improved morphological, physiological and biochemical properties by influencing the genome, phenotype, and physiology and plant metabolism. Increased plant cell size, organelle enlargement, increased biomass and secondary metabolite production are typical results of artificial polyploid induction. For medicinal plants, using polyploidization method can change the existing components in their chemical configuration by changing the regulation of biochemical synthesis, adding some new components to their chemical structure.

However, researches on polyploidy on Ngoc Linh ginseng have not been recorded. Therefore, the study of polyploidy on Ngoc Linh ginseng opens up a new research direction on this subject.

Chapter 2. MATERIALS, CONTENTS AND METHODS

2.1. Materials

The source materials used were leaf, petiole and root samples of three-month-old Ngoc Linh ginseng (*Panax vietnamensis* Ha *et* Grushv.) *in vitro* (2n = 24 diploid). Embryo samples in globular, heart-shaped and homogenous cotyledons were isolated from embryo clusters cultured on

MS medium containing 1.0 mg/L 2,4-D; 0.5 mg/L NAA; 0.2 mg/L Kin; 30 g/L sucrose and 8.5 g/L agar at the Central Highlands Scientific Research Institute were used as the primary source of samples for the experiments.

2.2. Contents and methods

2.2.1. Content 1: Optimization of somatic embryogenesis materials from Ngoc Linh ginseng in vitro

Study on the influence of other explant sources: leaf fragments, petioles, roots of *in vitro* plants and embryo cultures in globular, heart-shaped, cotyledonous forms on embryogenesis of Ngoc Linh ginseng.

Experiment 1: Investigating the effect of 2,4-D and TDZ on embryogenesis from leaf fragments, petioles and roots of Ngoc Linh ginseng

Leaves, petioles, and roots were cultured in MS medium containing fixed 0.5 mg/L NAA + 30 g/L sucrose, supplemented with 2,4-D, control TDZ was MS medium without addition of 2,4-D and TDZ.

Experiment 2: Investigating the effects of 2,4-D and TDZ on secondary embryogenesis from globular, heart-shaped, cotyledon embryos.

Globular, heart-shaped and cotyledonous embryos from the embryo cluster were separated from each embryo and cultured in MS medium + 0.5 mg/L NAA + 30 g/L sucrose, individually supplemented with 2,4- D, TDZ; The control is MS medium without 2,4-D and TDZ.

Experiment 3: Investigating the combined effect of 2,4-D and TDZ on embryogenesis from the sample source with the highest embryo regeneration on the medium supplemented with 2,4-D, TDZ alone of Ngoc Linh ginseng.

Samples with high embryogenesis from experiments 1 and 2 were cultured in MS + 0.5 mg/L NAA + 30 g/L sucrose, supplemented with 2,4-D in combination with TDZ.

2.2.2. Content 2: Optimal material processing of Ngoc Linh ginseng with colchicine and oryzalin

Experiment 4: *Effect of colchicine on secondary embryogenesis*

Ngoc Linh ginseng embryos in globular, uniform shape were treated in liquid MS medium supplemented with colchicine (0; 0.1; 0.2; 0.3; 0.5

and 0.7%), which changed according to time (24, 36, 48 and 72 hours). Then, the treated embryos were transferred to the embryogenesis medium examined in previous experiments to monitor mutations. Embryos not treated with colchicine were used as controls.

Experiment 5: *Investigating the effect of oryzalin on the secondary embryogenesis of Ngoc Linh ginseng*

Ngoc Linh ginseng embryos in globular, uniform shape were treated in liquid MS medium supplemented with oryzalin (0; 0.01; 0.02; 0.03; 0.05 and 0.07%), which changed according to time (24, 36, 48 and 72 hours). Then, the treated embryos were transferred to the embryogenesis medium investigated in previous experiments to monitor mutations.

2.2.3. Content 3: Investigating the growth of mutants and determining the polyploidy level

Experiment 6: Investigating on growth and determing the polyploidy of Ngoc Linh ginseng from secondary embryos arising from embryos that have been treated with colchicine to cause polyploid mutations.

Phenotypically mutant secondary embryos of Ngoc Linh ginseng (*Panax vietnamensis* Ha *et* Grushv.) *in vitro* 6 weeks old after colchicine treatment at the upper stage were separated and cultured on MS medium supplemented with 8 g /L agar, 30g/L sucrose. After 3 months, the plant is monitored for growth and development over time.

Experiment 7: Investigating the growth of tetraploid plants under *in vitro* culture conditions

After determining the polyploidy level of seedlings by counting the number of chromosomes, plants identified as tetraploid were further cultured for 6 months and growth characteristics were recorded. Diploid seedlings were used as controls.

2.2.4. Content 4: Determining the stability of tetraploid plants through regeneration steps

Experiment 8: Research on embryo regeneration from parts of tetraploid Ngoc Linh ginseng on suitable growth medium

The source materials used in this experiment were leaves, stalks and rhizomes of 6-month-old tetraploid Ngoc Linh ginseng that were

cultured to regenerate embryos on the optimal embryogenesis medium of each source. Evaluation of stability of tetraploid plants through methods of chromosomal staining, stomatoscopy, observation of morphology and organs of tetraploid plants, anatomy and observation of anatomical structures.

Experiment 9: Investigating the formation of secondary embryos from embryos derived from tetraploid plants through 3 times of subculture on suitable growth medium.

The regenerated embryos derived from the material sources in experiment 8 were conducted with biomass multiplication. The embryos were subcultured 3 times after 30 days of growth, then the embryos were spread on MS medium to form complete plants. These plants will be evaluated for phenotypic variations, stomatal density, stomatal length, width and chromosome count to determine the ratio of stable tetraploid plants.

2.2.5. Identification of mutations through methods

Anatomy method and observation of plant morphology, method of chromosome staining, method of observing stomata

2.2.6. Statistical processing method

The experiment was arranged in a completely randomized design with 3 replications. Data were processed using Microsoft Excel 2019 software, comparing 1-factor ANOVA with Duncan's test ($p<0.05$) (Duncan 1955) on SPSS 16.0 software.

Chapter 3. RESULTS

3.1. Content 1: Optimization of somatic embryogenesis materials from Ngoc Linh ginseng in vitro

3.1.1. The effect of 2,4-D and TDZ on embryogenesis from leaf fragments, petioles and roots of Ngoc Linh ginseng

The average number of embryos was not proportional to the treatment concentration, the highest number of embryos was supplemented with 0.5 mg/L 2,4-D medium, respectively 51 embryos, but then decreased

to 35.67 embryos when increasing concentration of 0.7 mg/L 2,4-D (Figure 3.1). In medium supplemented with TDZ, the average number of embryos was different between treatments, the highest on medium supplemented with 0.3 mg/L TDZ corresponding to 48.33 embryos (Figure 3.1).

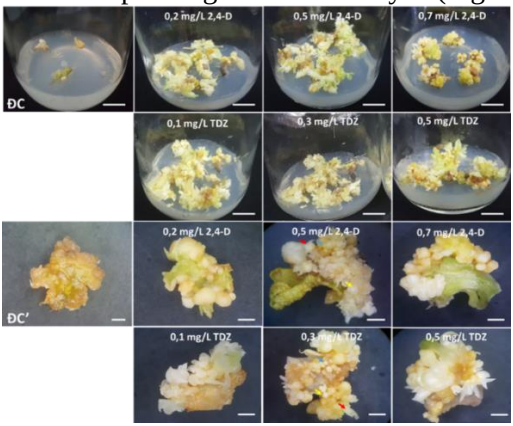

Figure 3.1. Effect of 2,4-D and TDZ on embryogenesis from Ngoc Linh ginseng leaf pieces after 6 weeks of culture (Bar: 1 cm).

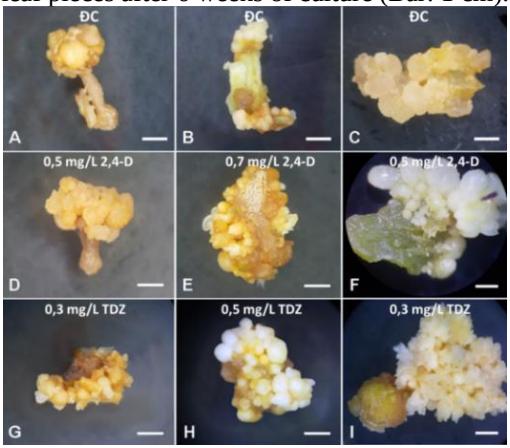

Figure 3.4. Effect of optimal concentrations of 2,4-D and TDZ on embryogenesis from root samples (A, D, G), petioles (B, E, H) and leaf fragments (C, F, I) of Ngoc Linh ginseng after 6 weeks of culture (Bar: 1 mm).

Therefore, the highest average number of embryos per sample was recorded in leaf pieces (51.00 embryos) on medium supplemented with 0.5

mg/L 2,4-D, on petioles (25.33 embryos) and root samples (17.67 embryos) (Figure 3.4).

3.1.2. The effects of 2,4-D and TDZ on secondary embryogenesis from globular, heart-shaped, cotyledon embryos

Secondary embryogenesis is the process by which embryos are formed from embryos, which offers advantages over primary embryogenesis such as a high degree of uniformity and independence from the original sample source. In this study, we investigated the formation of secondary embryos from all 3 embryos, globular, heart-shaped and cotyledon embryos.

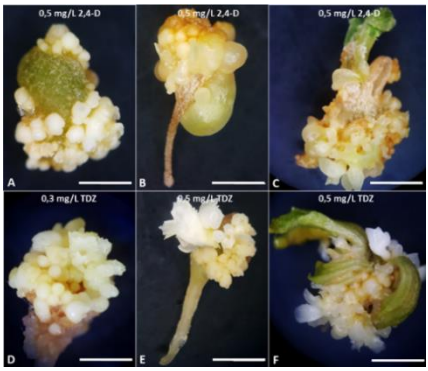

Figure 3.8. Effects of optimal concentrations of 2,4-D and TDZ on secondary embryogenesis from globular (A, D), heart shaped (B, E) and cotyledonous (C, F) embryos) Ngoc Linh ginseng after 6 weeks of culture (Bar: 1 mm).

After 6 weeks of culture, the results showed that the highest average number of embryos per sample was recorded in globular embryos on medium supplemented with 0.3 mg/L TDZ (68.33 embryos). For medium supplemented with 2,4-D, the concentration of 0.5 mg/L for the number of embryos formed on globular, heart-shaped and cotyledonous embryos were all more optimal than other concentrations 65.67 embryo, respectively (Figure 3.8).

3.1.3. The combined effect of 2,4-D and TDZ on embryogenesis from the sample source with the highest embryo regeneration on the medium supplemented with 2,4-D, TDZ alone of Ngoc Linh ginseng

The globular embryo samples were further investigated on embryogenesis under the influence of the 2,4-D and TDZ combination. The results showed that the combination of 2,4-D and TDZ at appropriate concentrations for high secondary embryogenesis. The highest number of embryos formed (73.33 embryos/sample) on medium supplemented with 0.7 mg/L 2,4-D and 0.1 mg/L TDZ (Table 3.3, Figure 3.12) and higher compared with the individual addition of 0.3 mg/L TDZ (67.00 embryos/sample). Fresh weight of embryo clusters was highest at concentrations of 0.7 mg/L 2,4-D and 0.1 mg/L TDZ (2.73 g) (Table 3.3).

Table 3.3. The combined effects of 2,4-D and TDZ on secondary embryogenesis from globular embryos of Ngoc Linh ginseng after 6 weeks of culture.

2,4-D *(mg/L)*	TDZ *(mg/L)*	Callus induction rate*(%)*	Embryo induction rate *(%)*	No. of embryos *(embryo/ sample)*	Fresh weight of embryo cluster *(g)*	Description
0	0	3.50^{b*}	39.67^{b}	4.67^{e}	0.32^{e}	Forming many types of embryos including globular, heart-shaped, cotyledon embryos; embryos are bright white and blue
0	0.3	100.00^{a}	100.00^{a}	67.00^{c}	2.35^{c}	
0.2	0.3	100.00^{a}	100.00^{a}	51.33^{d}	2.05^{d}	
0.5	0.3	100.00^{a}	100.00^{a}	55.67^{d}	2.62^{bc}	
0.7	0.3	100.00^{a}	100.00^{a}	69.00^{b}	2.57^{bc}	
0.7	**0.1**	$\mathbf{100.00^{a}}$	$\mathbf{100.00^{a}}$	$\mathbf{73.33^{a}}$	$\mathbf{2.73^{a}}$	
0.7	0.5	100.00^{a}	100.00^{a}	69.33^{b}	2.62^{bc}	
0.7	0.7	100.00^{a}	100.00^{a}	59.67^{cd}	2.65^{b}	

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

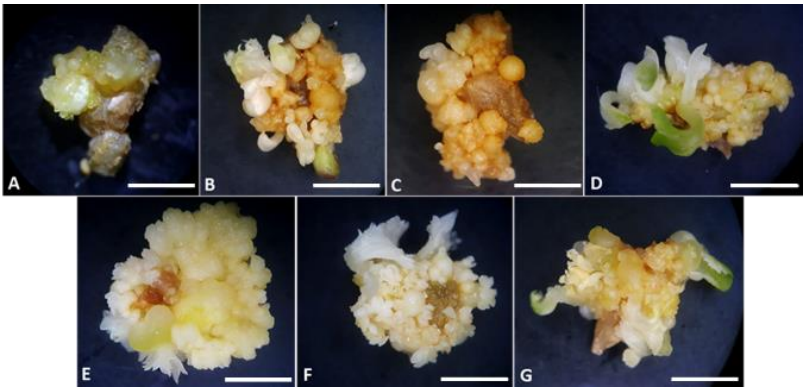

Figure 3.12. The combined effect of 2,4-D and TDZ on secondary embryogenesis from globular embryos of Ngoc Linh ginseng (Bar: 1 mm). A: control. B - D: supplemental medium 0.2; 0.5 and 0.7 mg/L 2,4-D combined with 0.3 mg/L TDZ. E - G: supplemental medium 0.1; 0.5 and 0.7 mg/L TDZ combined with 0.7 mg/L 2,4-D, respectively.

3.2. Content 2: Optimal material processing of Ngoc Linh ginseng with colchicine and oryzalin

3.2.1. Effect of colchicine on secondary embryogenesis

Ngoc Linh ginseng globular primary embryos were treated with colchicine. The results are recorded in Table 3.4.

Table 3.4. Effect of concentration and time of colchicine treatment on the induction of secondary embryos after 1 month of culture.

NT	Colchicine *(%)*	Time of treatment *(hour)*	Survival rate *(%)*	Abnormal embryo ratio *(%)*	Number of secondary embryos *(Embryo/sample)*	
					Normal	Abnormal
1	0.0	0	100.00^{a*}	0.00^{j}	27.22^{a}	0.00^{i}
2	0.1		92.56^{ab}	4.86^{ij}	23.89^{b}	1.22^{h}
3	0.2		89.00^{bcd}	11.16^{i}	21.22^{bc}	2.67^{g}
4	0.3	24	86.11^{bcd}	12.50^{ghi}	21.00^{bc}	3.00^{efg}
5	0.5		88.45^{bcd}	23.50^{fghi}	17.89^{cd}	2.82^{fg}
6	0.7		82.11^{cde}	20.32^{efgh}	14.00^{efgh}	3.56^{cd}
7	0.1	36	90.33^{bc}	11.31^{ghi}	12.22^{fgh}	1.56^{h}

8	0.2		83.44^{bcde}	17.20^{fgh}	13.11^{efgh}	2.67^{g}
9	0.3		80.11^{def}	20.23^{efgh}	12.34^{efgh}	3.11^{defg}
10	0.5		75.22^{efg}	19.36^{efgh}	15.89^{defg}	3.78^{bc}
11	0.7		72.33^{fgh}	23.60^{cdef}	10.45^{ghi}	3.22^{def}
12	0.1		70.33^{ghi}	16.55^{fgh}	16.00^{def}	3.00^{efg}
13	0.2		57.78^{jk}	29.40^{cde}	10.45^{ghi}	4.22^{b}
14	0.3	48	62.45^{ij}	21.65^{defg}	12.44^{efgh}	3.44^{cde}
15	0.5		52.00^{kl}	29.52^{cde}	13.56^{efgh}	5.44^{a}
16	0.7		43.22^{l}	33.88^{bc}	8.11^{i}	5.11^{ab}
17	0.1		65.00^{hij}	18.93^{efgh}	16.89^{de}	3.89^{bc}
18	0.2		50.45^{kl}	21.99^{defg}	9.89^{hi}	2.78^{fg}
19	0.3	72	30.44^{m}	31.27^{bcd}	8.11^{i}	3.56^{cd}
20	0.5		32.33^{m}	42.08^{ab}	$7.44i^{j}$	4.89^{ab}
21	0.7		22.11^{n}	46.49^{a}	5.22^{j}	5.56^{a}

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

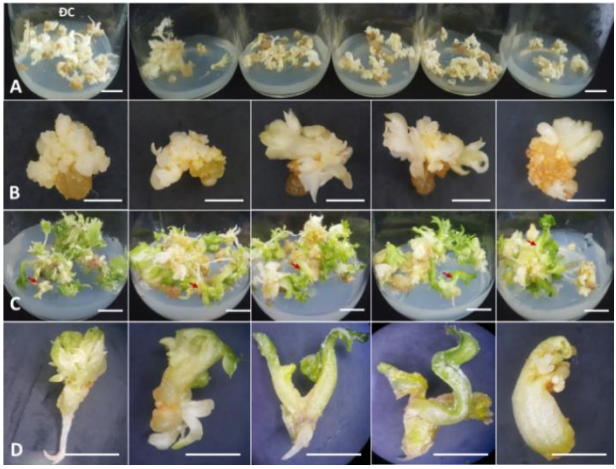

Figure 3.15. Abnormal secondary embryogenesis from globular embryos of Ngoc Linh ginseng under the influence of colchincine concentrations (0.1; 0.2; 0.3; 0.5; 0.7%) during 24 h of treatment (from left to right respectively). A and B: secondary embryos formed after 1 month of culture. C and D: Secondary embryos formed after 2 months of culture.

After 24 hours of treatment, the survival rate of samples decreased from 100% to 82.11%. In contrast, the rate of mutant embryo formation

was 4.86% at 0.1% colchicine concentration and increased to 20.32% at 0.7% colchicine concentration (Table 3.4, Figure 3.15).

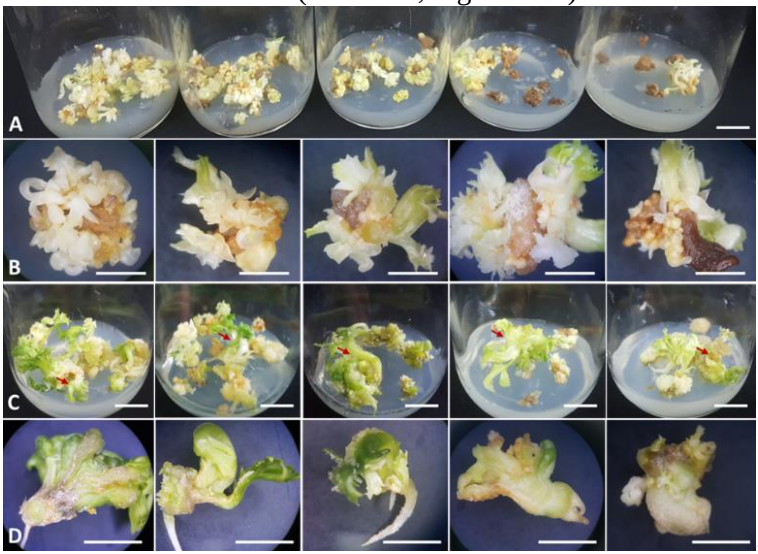

Figure 3.17. Abnormal secondary embryogenesis from globular embryos of Ngoc Linh ginseng under the influence of colchincine concentrations (0.1; 0.2; 0.3; 0.5; 0.7%) during 48 hours of treatment logic (from left to right respectively). B: secondary embryos formed after 1 month of culture. C and D: Secondary embryos formed after 2 months of culture.

After 48 hours of treatment, the survival rate decreased to only 43.22% at 0.7% colchicine concentration (Table 3.4, Figure 3.18A, B). The average number of secondary embryos is also significantly reduced compared to the shorter processing times. At this time point, the number of secondary embryos with abnormal expression increased quite high and reached the highest level in the treatment treatment of 0.5% colchicine (5.44 embryos/implant), corresponding to the rate of 29.52%. (Table 3.4, Figure 3.17).

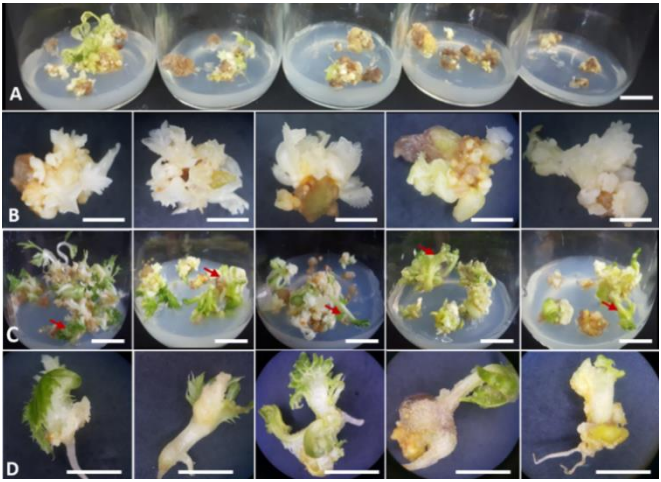

Figure 3.18. Abnormal secondary embryogenesis from globular embryos of Ngoc Linh ginseng under the influence of colchincine concentrations (0.1; 0.2; 0.3; 0.5; 0.7%) in 72 h of treatment logic (from left to right respectively). A and B: secondary embryos formed after 1 month of culture. C and D: Secondary embryos formed after 2 months of culture.

At 72-hour, the survival rate of the samples decreased to only 22.11% in the colchicine treatment of 0.7% (Table 3.4). However, the number of mutant embryos and the percentage of mutant embryos of the sample increased markedly. The highest percentage of mutant embryos was 46.49% at 0.7% colchicine, the corresponding number of mutant embryos was 5.56 embryos/sample (Figure 3.18).

3.2.2. Effect of oryzalin on secondary embryogenesis

The lowest survival rate was recorded at the highest oryzalin concentration of 0.07% at the time points of 24 hours, 36 hours, 48 hours and 72 hours (46.7; 40.0; 33.3 and 13.3%, respectively) (Table 3.5).

At 24-hour, oryzalin concentration as low as 0.01%, secondary embryos were relatively high (17.7 embryos/sample) but the rate of mutant secondary embryos was the lowest (7.9%) and significantly lower at a concentration of 0.07% (31.5%). At the 36-hour point, the percentage of mutant embryos increased with increasing oryzalin concentration (15.7 - 31.6%). Treatment of 0.07% oryzalin for 48 hours gave a high rate of mutant embryos (38.3%). Treatment with a concentration of 0.03 - 0.07%

oryzalin for 72 hours gave the lowest number of secondary embryos (7.7 - 8.0 embryos/sample) and the highest percentage of mutant embryos (42.6 - 45.8%) (Table 3.5, Figure 3.20).

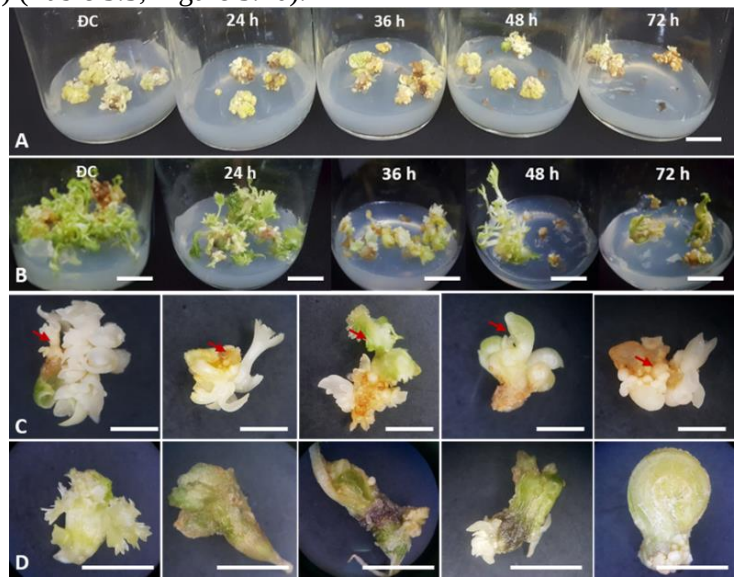

Figure 3.20. Effect of oryzalin on abnormal secondary embryogenesis from globular embryos of Ngoc Linh ginseng at different treatment times (control, 24 h, 36 h, 48 h, 72 h, respectively, from left to right, respectively from left to right) right). A and C: secondary embryos formed after 1 month of culture. B and D: Secondary embryos formed after 2 months of culture.

3.2.3 Plant growth from secondary embryos arising from colchicine-treated embryos

Abnormally expressed secondary embryos (derived from colchicine-treated embryos) were transferred to MS medium containing 8 g/L agar, 30 g/L succrose. After 3 months of culture, the growth and development of the plants were recorded and shown in the table.

3.2.4. Some morphological and anatomical characteristics of seedlings after polyploid treatment with colchicine

Seedlings derived from embryos after polyploidy treatment were observed the change in terms of morphology (Figure 3.22; 3.23), structure (Figure 3.24), anatomical characteristics and counted the number of chromosomes (Figure 3.25).

Figure 3.22. Phenotypic characteristics of some seedlings after polyploid treatment with colchicine after 3 months of culture (Bar: 2 cm).

Figure 3.23. Some mutant leaf forms of seedlings derived from colchicine-treated embryos after 3 months of culture (Bar: 1 cm)

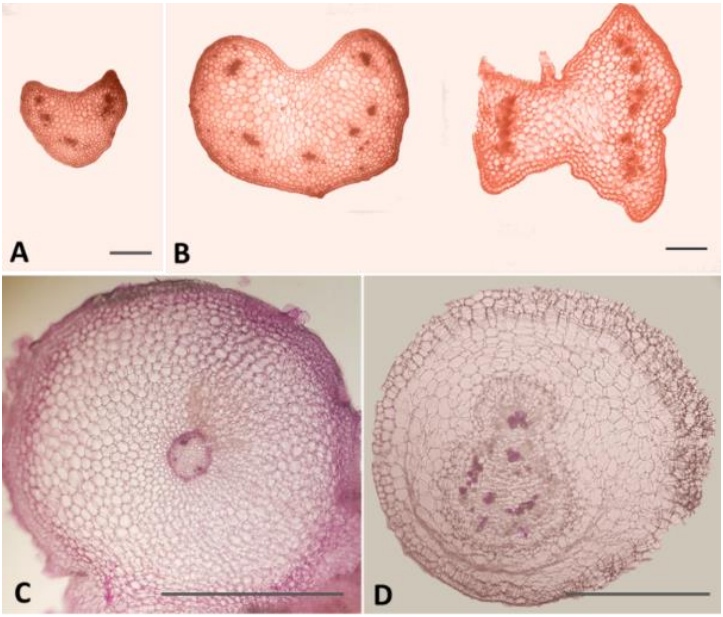

Figure 3.24. Anatomical morphology of leaf petioles and roots of Ngoc Linh ginseng has unusual phenotypes. Petiole samples in control plants (A) and phenotypically abnormal plants (B) (Bar: 1 mm). Root samples in control plants (C) and phenotypically abnormal plants (D) (Bar: 0.2 mm).

Table 3.7. Stomata of mutant forms after colchicine treatment, after 3 months of growth

Ploidy	Stomatal density *(Stomata/mm^2)*	Chloroplast density *(*Chloroplast / *Stomata)*	Stomatal length *(µm)*	stomatal width *(µm)*
Control	125.7^{a*}	27.3^{b}	57.33^{b}	42.33^{b}
Mutant	119.7^{bc}	29.5^{ab}	78.00^{a}	55.33^{a}

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

We evaluated the polyploidy ability of these forms based on the criteria of morphology, size and polyploidization of plants by determining the stomatal parameters such as: stomatal density/cm^2, stomatal chloroplast density/cm^2, stomatal length, stomatal width (Table 3.7, Figure 3.25).

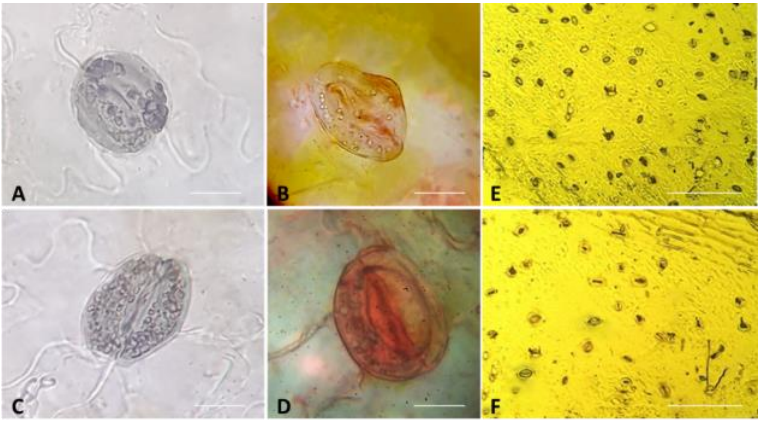

Figure 3.25. Characteristics of stomata in leaves of Ngoc Linh ginseng with phenotypic variation after 3 months of culture from secondary embryos. Stomata shape of control plants (A, B) and phenotypic variation (C, D) (size 0.05 mm); Density of stomata in control (E) and polyploid (F) plants (0.1 mm size).

Morphological observations of this study showed that the stomatal density of the mutants was lower than that of the control plants. In addition, microscopic observations showed a significant difference in the number of chloroplasts in the stomata of the mutant leaves and the control leaves. The number of chloroplasts in the stomata cells of the mutant leaves was higher than that of the control leaves (Figure 3.25). In particular, the number of chloroplasts in the stomata of tetraploid plants is nearly twice that of diploid plants.

3.3. Content 3: Investigating the growth of mutants and determining the polyploidy level

Chromosome counting is the most reliable and obvious method of determining the polyploidy level. In this study, the number of chromosomes in the root tips of plantlets derived from secondary embryos was counted to identify polyploids (Figure 3.27). The results showed that tetraploid and mixoploids were formed under the influence of colchicine; however, the rate of polyploidy induction of seedlings was significantly dependent on concentration and treatment time (Table 3.8).

Table 3.8. Effect of colchicine concentration on polyploid induction rate of Ngoc Linh ginseng at different processing times.

Treatment	Colchicine *(%)*	Treatment time *(hour)*	Polyploid induction rate *(%)*	
			Tetraploid	Mixoploid
1	0.0	0	0.00^{f*}	0.00^{g}
2	0.1	24	0.00^{f}	0.00^{g}
3	0.2		11.11^{de}	0.00^{g}
4	0.3		14.81^{cd}	0.00^{g}
5	0.5		18.52^{bc}	3.70^{fg}
6	0.7		18.52^{bc}	7.41^{ef}
7	0.1	36	14.81^{cd}	7.41^{ef}
8	0.2		18.52^{bc}	11.11^{ef}
9	0.3		18.52^{bc}	14.81^{de}
10	0.5		18.52^{bc}	22.22^{cd}
11	0.7		22.22^{ab}	25.92^{c}
12	0.1	48	18.52^{bc}	11.11^{ef}
13	0.2		22.22^{ab}	14.81^{de}
14	0.3		22.22^{ab}	22.22^{cd}
15	0.5		25.92^{a}	25.92^{c}
16	0.7		18.52^{bc}	29.63^{c}
17	0.1	72	11.11^{de}	22.22^{cd}
18	0.2		7.41^{def}	25.92^{c}
19	0.3		3.70^{ef}	40.74^{b}
20	0.5		3.70^{ef}	48.14^{ab}
21	0.7		3.70^{ef}	55.55^{a}

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

At a treatment time of 24 h, tetraploids began to be detected at treatment concentrations of 0.2% colchicine or higher. At the same time, tetraploid induction gradually increased with increasing treatment concentration. At high concentrations of colchicine 0.5 - 0.7%, haploid bodies were recorded at a low rate (less than 10%). At 36 hours of treatment, 0.1 - 0.7% colchicine treatment gave a tetraploid rate greater than 10%, the highest tetraploid rate was recorded at a concentration of 0.7% (22.22%). Besides, the percentage of mixoploid plants increased

gradually with increasing treatment concentration, the highest percentage of mixoploid plants was recorded.

Treatment of 0.2% - 0.5% colchicine for 48 hours gave the optimal tetraploid induction efficiency (22.22% - 25.93%). At 72 hours, the percentage of tetraploid plants decreased sharply when increasing the concentration of colchicine from 0.1% - 0.7% (from 11.11% to 3.70%), in contrast, the rate of haploid plants formed increased sharply, the highest at concentrations from 0.5% - 0.7% (48.15% and 55.56%).

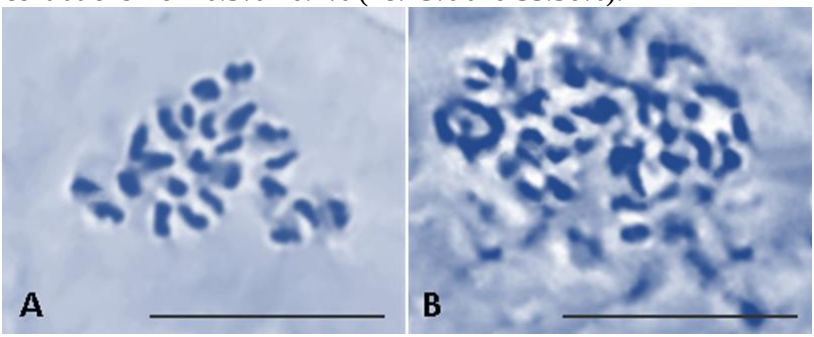

Figure 3.27. Number of chromosomes at the root tips of diploid (2n = 24) (A) and tetraploid (2n = 48) (B) roots in Ngoc Linh ginseng after 3 months of culture (Bar: 5 µm).

In this study, tetraploid Ngoc Linh ginseng (2n = 48) was successfully created from secondary embryos arising from embryos treated with colchicine with a high induction rate (25.92%). At the same time, polyploid bodies were also recorded during the polyploid treatment with Ngoc Linh ginseng embryo samples at a high rate (Table 3.8).

On the other hand, a change in the characteristics of the stomata is considered as one of the common signs in polyploids. According to the results of determination of the above polyploidization level, the leaves of tetraploid and diploid plants were obtained and the characteristics of the stomata were observed by optical microscope. The results showed that the leaf stomata of the tetraploids were ovoid, scattered and larger in size than the control. The length and width of the stomata in the tetraploid leaves (75.46 µm and 52.63 µm) were significantly larger than those in the control (57.10 µm and 42.03 µm) (Table 3.9, Figure 3.28A, B). However, the

stomatal density of the tetraploid (110.20 stomata/mm2) was lower than that of the control (122.67 stomata/mm2) (Table 3.9). In addition, microscopic observation revealed a significant difference in the number of chloroplasts in the stomata of tetraploid and diploid leaves. The number of chloroplasts in the stomata of tetraploid leaves was higher than that of the control (Fig. 3.28C, D).

Table 3.9. Characteristics of stomata on leaf epidermis of tetraploid Ngoc Linh ginseng after 3 months of culture

Ploidy	Stomatal density (mm^2)	Stomatal length (µm)	Stomatal width (µm)	Stomatal ratio[z]	
				Length	Width
Diploid	122.67[a*]	57.10[b]	42.03[b]	1	1
Tetraploid	110.20[b]	75.46[a]	52.63[a]	1.32	1.24

**Different letters (a, b,…) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test). ZRatio of length (or width) of stomata of tetraploid and diploid plants.*

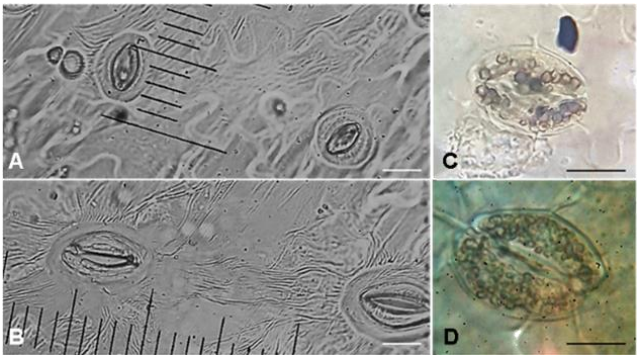

Figure 3.28. Comparison of stomatal characteristics of diploid and tetraploid bodies in Ngoc Linh ginseng after 3 months of culture. Shape and size of stomata in diploid (A) and tetraploid (B). Density of chloroplasts on stomatal guard cells of diploid (C) and tetraploid (D) (Bar: 20 µm).

3.4. Content 4: Investigating the growth of mutants and determining the polyploidy level

The tetraploid plants had significant changes in some morphological characteristics compared to the diploid seedlings after 6 months of culture, the highest seedling height (12.10 cm), significantly higher than that of the diploid seedlings (7.59 cm). The leaves of the tetraploid seedlings were well developed in terms of petiole diameter, petiole length, leaf width, and leaf length. In addition, rhizome fresh weight and seedling fresh weight were significantly greater (40.00% and 54.01%, respectively) than diploids (Figures 3.29 and 3.30).

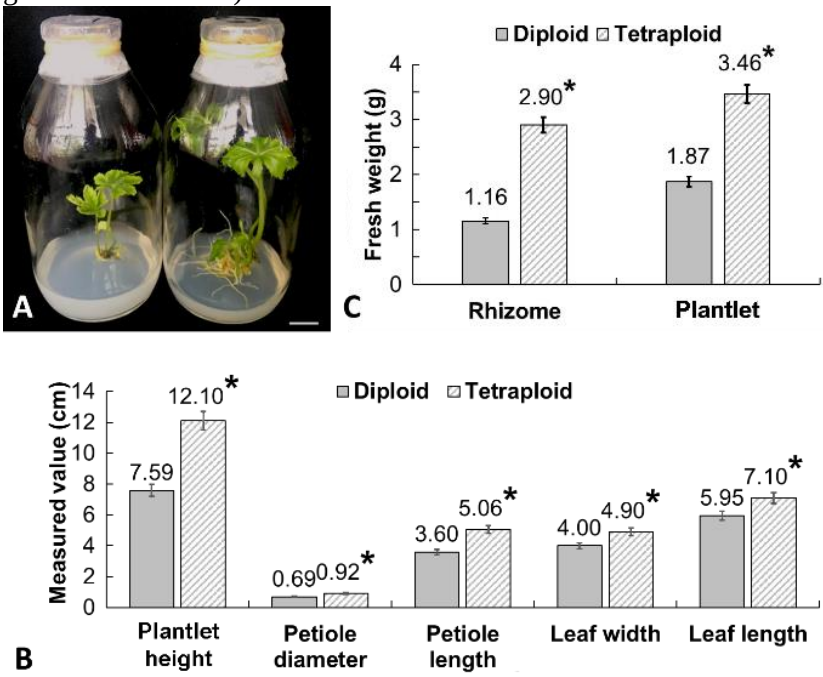

Figure 3.29. Comparison of some morphological characteristics between diploid and tetraploid of Ngoc Linh ginseng after 6 months of culture. A: diploid (left) and tetraploid (right) in vitro seedlings (1 cm measure). B and C: statistics on some growth characteristics of diploid and tetraploid seedlings (*$p < 0.05$).

Figure 3.30. Ngoc Linh ginseng plants *in vitro* diploid (A) and tetraploid (B - D) after 6 months of culture (Bar: 1cm).

The tetraploid explants gave good regeneration results on suitable media. In leaf fragment samples, the average number of embryos (42 embryos/sample) was not different from that of diploid control leaves (41.7 embryos/sample). Similarly, the petiole and root explants also had a high number of embryos formed, respectively 35 embryos/ sample for petiole and 25.3 embryos/ sample for root samples (Table 3.10, Figure 3.31).

Table 3.10. Embryo regeneration from tetraploid parts of Ngoc Linh Ginseng on suitable growth medium

Sample	Embryo induction rate *(%)*	The number of embryos *(embryos/ sample)*	Fresh weight *(g)*		
			1st time	2nd time	3rd time
Control	100	41.7^{a*}	2.02^{b}	2.09^{b}	2.14^{b}
Leaf	100	42.0^{a}	2.99^{a}	2.82^{a}	2.72^{a}
Petiole	100	35.0^{ab}	2.04^{b}	2.21^{b}	2.14^{b}
Root	100	25.3^{b}	1.25^{c}	1.25^{c}	1.37^{c}

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

The embryos were carried out with biomass multiplication on the optimal medium for creating secondary embryos, after 3 times of transplanting, the embryos still had good formation. Therefore, secondary embryogenesis of embryos derived from embryos of tetraploid plants has relatively strong growth and stability. Simultaneously, tetraploid plants grown from secondary embryos (embryos regenerated at 3rd transfer) on MS medium showed phenotypic stability and polyploidy after 6 months of culture (Figure 3.32).

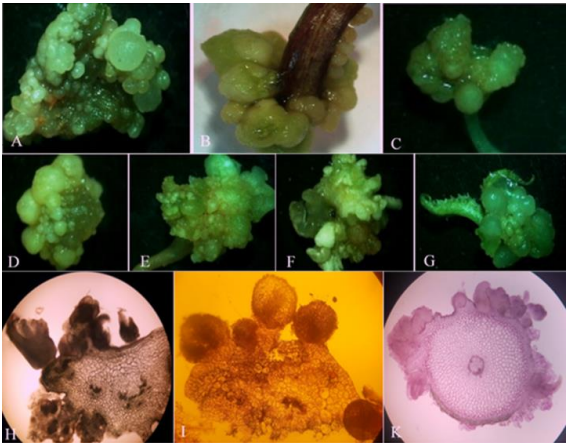

Figure 3.31. Embryo regeneration of tetraploid Ngoc Linh ginseng from different sources. A, B, C: Embryos arise from leaves, stalks, and roots. D, E, F, G: Secondary embryo regeneration from embryos of Ngoc Linh's tetraploid plants.

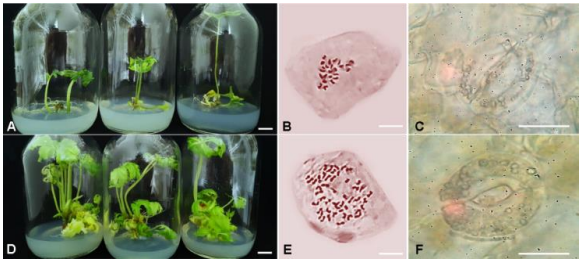

Figure 3.32. *In vitro* plants, number of chromosomes and stomata of diploid (A, B, C) and tetraploid (D, E, F) Ngoc Linh ginseng plant after 6 months of culture.

CONCLUSION

Content 1: Optimization of somatic embryogenesis materials from Ngoc Linh ginseng *in vitro*

Optimization of material resources for successful somatic embryogenesis and secondary embryogenesis was studied. Of the three sources of materials: leaf fragments, petioles and roots, the best somatic embryogenesis was leaf fragments on MS medium containing 0.5 mg/L NAA and supplemented with 0.5 mg/L 2.4- D. For materials from somatic embryos, the embryogenesis efficiency was higher than that of leaves, petioles and roots of 3-month-old *in vitro* plants. In which, the highest

number of embryos generated was recorded in globular embryos cultured on MS medium with 0.5 mg/L NAA and supplemented with a combination of 0.7 mg/L 2,4-D with 0 ,1 mg/L TDZ.

Content 2: Treatment of polyploid formation on Ngoc Linh ginseng with colchicine and oryzalin

Globular embryos for mutant embryogenesis at all concentrations and experimental times. The longer sample-processing time combined with the high concentration, the number of mutant embryos was increased. The highest number of mutant embryos was formed when colchicine was treated with a concentration of 0.5% - 0.7% in 48 hours. Furthermore, treatment of 0.7% colchicine for 72 hours had the most profound effect on secondary embryos, and those embryos were mostly abnormal.

Content 3: Determination of polyploidy of Ngoc Linh ginseng seedlings derived from primary embryos treated with colchicine.

Colchicine treatment, increasing colchicine concentration and time reduced sample survival and number of secondary embryos but increased the rate of mutant secondary embryo formation, colchicine concentration at 0.3% to 0.5% in 48 hours for a high rate of tetraploid (22.22 - 25.92%). The results also show that tetraploid (2n = 48) has larger stomatal size, lower stomatal density, thicker chloroplast density in stomata, and better growth than diploid (2n = 24).

Content 4: Investigating the growth, regeneration and stability of tetraploid Ngoc Linh ginseng.

The tetraploid Ngoc Linh ginseng showed significantly higher growth indicators than the tetraploid plant. Parts of tetraploid plants were further cultured to investigate embryo regeneration on suitable embryogenesis medium. The results show that the explants derived from tetraploid plants reproduce stably and the plants grown from embryos still have the characteristic properties of tetraploid plants through testing methods.

LIST OF THE PUBLICATIONS RELATED TO THE DISSERTATION

1. **Le Thi Diem**, Truong Hoai Phong, Hoang Thanh Tung, Hoang Dac Khai, Vu Quoc Luan, Do Manh Cuong, Nguyen Thi Nhu Mai, Trinh Thi Huong, Bui Van The Vinh, Duong Tan Nhut, Comparison of somatic embryogenesis efficiency from *in vitro* explant sources of Ngoc Linh ginseng (*Panax vietnamensis* Ha *et* Grushv.), *Vietnam Journal of Science and Technology - Series B, **2022*** (Accepted article).

2. **L.T. Diem**, T.H. Phong, H.T. Tung, H.D. Khai, T.T.L. Anh, N.T.N. Mai, D.M. Cuong, V.Q. Luan, T. Que, H.T.N. Phuong, B.V.T. Vinh, D.T. Nhut, Tetraploid induction through somatic embryogenesis in *Panax vietnamensis* Ha *et* Grushv. by colchicine treatment, *Scientia Horticulturae, **2022**, 303,* 111254.