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APPLICATION OF BIOTECHNOLOGY TECHNIQUES IN KIWIFRUIT PROPAGATION IN LAMDONG PROVINCE

Major**: Plant Physiology**

Code**: 9 42 01 12**

DOCTORAL THESIS SUMMARY

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INTRODUCTION

1. Rationale of the Study

Mainly found in southern China and widely distributed in Asia, Kiwi (in the genus of Gooseberries (*Actinidia*)) is a monoecious perennial plant producing fleshy berries. The genus *Actinidia* exhibits great biological variation because of its wide distribution in different climatic and geographical environments. This variation is reflected in the common biological characteristics of the species, especially on roots, shoots, stems, leaves, flowers and fruits of the two commercially cultivated species *Actinidia chinensis* var. *chinensis* và *Actinidia chinensis* var. *deliciosa.*

A. chinensis selected species are Red Kiwi, Hort 16, Yellow Kiwi showed diversity and superior traits in comparison with Kiwifruit of other varieties such as fruit size, vitamin C content, soluble solids content thaw and shelf life in cold storage or at room temperature, early flowering, cold and drought tolerant.

Genus *Actinidia* entered Europe, Australia and thrived as an industry at the beginning of the 20th century. *Actinidia chinensis* var. *chinensis*, *A. chinensis* var. *deliciosa*, và *Actinidia eriantha* are cultivars with fruit sizes close to commercial standards. Therein, *Actinidia chinensis* including Red Kiwi, Hort 16, Golden Kiwi were selected to show diversity and superior characteristics compared to kiwifruit of other varieties such as fruit size (average 80-100 g), vitamin C content (250 - 350 mg/100 g fresh weight).), soluble solids content (up to 26%) and shelf life in cold storage (up to 120 days) or at room temperature (40 days), early flowering, cold tolerant, drought tolerant or very fond of adapted to different conditions.

Genus *Actinidia* including 54 species and 75 subspecies. All species in this genus are perennials, vines, and deciduous, and most of these species can crossbreed with each other very easily. Therefore, in the evolutionary process, there have appeared hybrid plants of the same genus of different species. It is one of the main obstacles to study the phylogenetic origin of primitive species within a genus. Furthermore, all known species in the genus are dioecious. Flowering feminie plants are hermaphroditic but produce only empty pollen grains, while masculine plants have unigenderual flowers with many

stamens roughly surrounding a pistil, growth repressed prior to pattern elongation or start ovulating. Therefore, methods of micropropagation, propagation by grafting method to optimize propagation techniques have been studied to meet the demand for high-quality plants, but they can be be determined only when the plants are flowering. On the other hand, there are very few studies applying biotechnological techniques on the golden kiwi (*Actinidia chinensis*), major studies on *in vitro* propagation and there is still no study carried out to investigate and improve the efficiency of TCL priming for micropropagation of this woody vine.

Kiwi has not yet been widely grown in Vietnam, but it is mainly imported from New Zealand, China, Italy, Chile. In the climatic conditions of the Central Highlands, including Lam Dong, where the tropical climate is suitable for the development of kiwifruit plants, there have been some discoveries about forest undistinguished kiwifruit species distributed in Kon Tum and Lam Dong producing small fruit, sour taste. If the research and pilot planting of kiwi is successful, there will be such a contribution to diversify crops, improve farmers' incomes, reduce product costs, and help consumers easily approach this fruit in the context of high cost imported kiwifruit at present. However, studies on genetic diversity of kiwifruit in Vietnam are still limited. There are only few studies towards kiwifruit cloning such as investigating callus formation and the effects of growth regulators, medium pH, sugar concentration and medium volume on single cell culture of green kiwi plant (*Actinidia deliciosa*) of the author group Duong Tan Nhut.

Therefore, the thesis **"Application of biotechnology techniques in kiwifruit propagation in Lam Dong Province**" was conducted with the aim of evaluating genetic relationships and species identification, thereby determining the gender of kiwifruit plants and applying cell thin layer culture technology to effectively evaluate the morphogenesis of pine kiwifruit plants. Callus formation, embryogenesis, shoot formation and adventitious root formation from the petioles and main veins of kiwifruit, generating a large number of gender-determined seedlings to shorten time and be proactive in propagating seedlings outside the nursery, analyzing the viability, growth and development of plants in the nursery environment in Lam Dong Province.

2. Research objectives of the thesis

- Evaluation, identification and analysis of genetic relationships of kiwifruit varieties by molecular biology technique by DNA barcoding method (DNA barcode).

- Application of biotechnological techniques to determine the gender of kiwifruit plant.

- Application of kiwifruit micropropagation to evaluate the efficiency of somatic embryogenesis and embryogenesis of *Actinidia chinensis* from the main leaf veins and petioles through cell thin layer culture (TCL) technology and complete plant regeneration. Kiwi seed production service in Lam Dong Province.

3. Main research contents of the thesis

Identification of Kiwifruit species in Vietnam and determination of kiwifruit gender based on molecular markers.

Application of kiwifruit micropropagation for breeding.

4. New contributions of the thesis

Research results show that the use of 3 gene regions *rbc*L, *mat*K, ITS2 was possible to determine the relative genetic relationship among the collected 20 Kiwi samples, and it was initially possible to determine the origin of these Kiwi cultivars in Vietnam from species of *A. deliciosa* and *A. Latifolia*; The remaining plants were identified from *A. chinensis* and *A. callosa*. This result also shows the ability to determine the genetic relationship among DNA barcode regions of *rbc*L, *mat*K and ITS is different, moreover, the combination of sequence regions together gives higher efficiency in comparison with odd used in assessing genetic relationships as well as species identification. From the above results, it is possible to apply DNA barcode markers in genetic research and evaluation towards plants of the genus Actinidia. However, the accurate identification of each sample at the subspecies level requires further studies and analysis by other techniques and molecular markers to ensure the accuracy and reliability of the method.

The study initially successfully determined the gender of 6 imported golden kiwifruit varieties.

The processes of explant surface sterilization and shoot regeneration obtained from leaf samples at a concentration of 200 ppm AgNPs; On the other hand, the culture medium supplemented with 20% coconut water for the effective growth of shoot clusters is the most optimal one.

This study also demonstrated an efficient procedure for *A. chinenensis* micropropagation through clonal embryogenesis of the TCL specimen (mv-tTCL and mv-lTCL for primary leaf veins; p-tTCL and p-lTCL for petioles). Besides, the growth of shoots of embryogenic origin (with monocotyledon shape) was optimal on MS medium containing 1.0 mg/L BA. In addition, seedlings derived from shoots were cultured on MS medium supplemented with 0.4 mg/L NAA and planted in plastic pots with a combination of humus, coir and biological fertilizers (at a ratio of 2 : 3:1 in weight) showed greater adaptability and growth compared with other treatments in the greenhouse.

CHAPTER 1. OVERVIEW

Kiwi is a fruit of the genus *Actinidia*, consisting of 54 species, commonly distributed in moist and porous soils with altitudes of 800 - 1,400 m, sometimes up to 1,950 m above sea level. Kiwi is a nutrient-rich fruit. Kiwis are exceptionally high in vitamin C and contain a host of other nutrients related to fiber, potassium, vitamin E and folate, as well as various bioactive ingredients.

In different climatic and geographical environments, species of the genus *Actinidia* exhibit great biological variability. All species in the genus *Actinidia* appear to have masculine and feminie plants. Propagation of kiwi by traditional methods such as cuttings and grafting is often not very effective due to long-term planting and limited root formation. The current method of kiwifruit micropropagation has achieved some results such as shoot regeneration, organogenesis, somatic embryogenesis and plant regeneration in Vietnam.

Currently, in Vietnam, there is no successful kiwifruit growing area in Vietnam, but mainly experimental planting in some northern mountainous areas and Ky Son in Nghe An province. Up to now, according to the published scientific literature, there are a number of research projects towards some characteristics such as callus formation and the influence of growth regulators, environmental pH, sugar concentration and volume medium for single cell culture of *A. Deliciosa*, cuttings and seeding on *A. latifolia* and *A. Deliciosa*. Lam Dong has a tropical monsoon climate, the average altitude of Lam Dong is from 800 to 1,000 m above sea level. At this temperature, it is relatively suitable for kiwifruit to grow when the Lam Dong region has the same altitude and annual rainfall compared to the kiwifruit distribution area.

However, the kiwi plant is considered a very difficult plant to grow. Micropropagation studies use principal materials such as germinated seeds or whole leaves, endosperm, shoot tips, stem segments, stamens, seedlings, etc. However, those studies mainly focused on centered on *A. deliciosa*, *A. Polygama*, etc... There are very few studies towards the micropropagation of *Actinidia chinensis* Planch, especially the application of TCL technique on *Actinidia chinensis* Planch has not been studied currently.

CHAPTER 2. RESEARCH MATERIALS, CONTENTS AND METHODS

2.1. Material

Leaf samples of 20 varieties of Kiwi (*Actinidia sp.*) were collected at 10 cm from the top from wild plants and commercial sources (1 year old) including: 01 natural sample collected in Dak Long commune, Kon Plong district, Kontum province; 01 natural sample was obtained from Mang ling village, ward 7, Da Lat, Lam Dong province; 18 samples of imported kiwifruit were purchased at the New Seeds Development Center, Hanoi University of Agriculture 1 - Xuan Khuong Seedling Farm, Trau Quy Town, Gia Lam District, Hanoi.

2.2. Research content

Identification of kiwi species in Vietnam and determine gender of kiwifruit plants based on molecular markers

Application of kiwifruit micropropagation for breeding tasks.

2.3. Research Methods

2.3.1. Experimental set-up method

2.3.1.1. Evaluation of genetic diversity and gender determination of kiwifruit plants based on molecular markers

a. Evaluation of genetic diversity

Total DNA extraction was based on the basic CTAB procedure modified under certain conditions.

PCR method was performed to amplify *rbc*L, *mat*K, ITS regions with corresponding primer pairs *rbc*L1-F (5'- ATG TCA CCA CAA ACA GAA AC -3') và *rbc*L 724-R(5'- TCG CAT GTA CCT GCA GTA GC -3'), *mat*K-KIM3-F (5'- CGT ACA GTA CTT TTG TGT TTA CGA G -3') và *mat*K-KIM1-R (5'- ACC CAG TCC ATC TGG AAA TCT TGG TTC -3'), ITS1-5F (5'- GGA AGT AAA AGT CGT AAC AAG G -3') và ITS4-R (5'- TCC TCC GCT TAT TGA TAT GC -3').

Evaluation of similarity and coverage of DNA sequences with available sequences. Sequences were calibrated using ATGC software and checked for biases. Evaluation of similarity and coverage of DNA

sequences with available sequences on NCBI database using BLAST tool.

Phylogenetic plant construction based on DNA sequences of *rbc*L, *mat*K, ITS regions.

b. Kiwi plant gender determination based on molecular markers

Based on the basic CTAB procedure modified under certain conditions, leaf samples of 8 kiwifruit plants were used for total DNA extraction after gender determination.

DNA purity was checked by optical densitometry at 260 nm and 280 nm.

The PCR method was performed with primer pairs SmY1_F (5'-TCG CAA TTC GTT AGG GAT GAT GCG-3') and SmY1_R (5'-CAT AAT CAA CCA TCC ATA AAA ACC AT-3') amplifying the gene region located on the Y chromosome is 770bp in size.

Electrophoresis of PCR product and read the results.

2.3.1.2. Application of kiwifruit micropropagation for breeding work

a. Create *in vitro* sample source

* Sterilize explant surface and regenerate shoots

Leaf samples were obtained from healthy plants in the wild and disinfected with 1 g/L $HgCl_2$, 10 g/L NaOCl and AgNPs at different concentrations (100 ppm, 200 ppm, 300 ppm and 500 ppm). and implanted in callus-inducing medium. After 4 - 8 weeks of culture, data were recorded.

* Proliferation of bud clusters

Bud clusters derived from leaf samples were cut into small shoot clusters containing 3 shoots/explant and were inoculated on shoot growth medium supplemented with organic extracts at concentrations (v/v) of (5%, 10%, 15% and 20%).

b. Study the effect of cutting type and sample size on somatic embryogenesis through TCL of main leaf veins and petioles

The *in vitro* rectangular (1 mm × 10 mm) and rectangular (1 mm × 10 mm) petioles were thinly horizontally and vertically sliced (mv-

tTCL and mv-lTCL for in vitro) with main leaf veins; p-tTCL and p-lTCL for petioles) with different sizes. Samples were cultured on MS medium supplemented with 0.02 mg/L NAA; 0.5 mg/L TDZ; 30 g/L sugar and 8 g/L agar - MSE medium. Whole main leaf veins (1 mm × 10 mm) or entire petioles (1 mm × 10 mm) cultured on MSE medium were used as controls. For the tTCL sectioning method, petiole (p) and main leaf veins (mv) measuring 1 mm × 10 mm (width × length) were cross-sectioned into 2 samples (½ mv-tTCL and ½ p-tTCL), cut into 3 explants (⅓ mv-tTCL and ⅓ p-tTCL and 4 explants (¼ mv-tTCL and ¼ p-tTCL). long) were longitudinally cut into 2 samples (½ mv-lTCL and ½ p-lTCL) and each ½ mv-lTCL and ½ p-lTCL samples were cross-sectioned into 2 samples (¼ mv-lTCL and ¼ p-lTCL) or cut. horizontally into 3 samples (1/6 mv-lTCL and 1/6 p-lTCL) or cross-section into 4 samples (⅛ mv-lTCL and ⅛ p-lTCL).

c. Investigate the effect of a single plant growth regulator towards the shoot regeneration ability of Kiwi somatic embryos

The somatic embryos with cotyledons (2 cm height) derived from p-TCL and mv-TCL samples were cultured in MS medium containing 30 g/L sucrose, 8 g/L agar and different concentrations. difference of BA (0.1; 0.5; 1.0; 1.5 mg/L) or TDZ (0.01; 0.03; 0.05; 0.07 mg/L) to determine suitable concentration during the period of kiwi shoot regeneration. The control was embryos cultured in MS medium without BA or TDZ. The experiment was repeated 3 times with each treatment. Each 350 mL glass flask contains 40 mL of medium culture and each flask holds 3 samples.

d. Investigate the effect of NAA on rooting and adaptability of *in vitro* seedlings

The 6-week-old shoots (4 cm high) were cultured in MS medium containing 30g/L of sugar, 8g/L of agar and different concentrations of NAA (0.1; 0.2; 0.3; 0. 4; 0.5 mg/L) to determine a suitable concentration for *in vitro* rooting. The control was shoots grown on NAA-free medium.

e. Investigate the effect of growing medium on the adaptability of seedlings in the greenhouse

Seedlings after 8 weeks of culture (6 cm high) from shoots grown on MS medium supplemented with optimal NAA concentration were washed agar and planted in plastic pots (15 cm x 12 cm x size).

8 cm) with different ratios of humus, coir and micro-fertilizers, each plastic pot has one seedling, the quantity is 300 seedlings. Seedlings are watered twice a day for the first week, then once a day in the early morning.

2.3.2. Statistical analysis

PCR products were sequenced at Macrogen company (10F, 254 Beotkkot-ro geumcheon-gu, Soul 08511, Rep. of Korea).

Sequences were calibrated using ATGC software and checked for biases. Evaluation of similarity and coverage of DNA sequences with available sequences on NCBI database using BLAST tool.

Phylogenetic plant was built using MEGA 7.0 software with bootstrap coefficient 1000.

Data regarding in vitro experiments were processed using Microsoft Excel® 2010 and SPSS 20.0 with Duncan's test at $p < 0.05$.

CHAPTER 3. RESULTS AND DISCUSSION

3.1. Evaluation of genetic diversity and gender determination of kiwifruit plants based on molecular markers

3.1.1. PCR and DNA sequencing

DNA purity test results showed that samples with high purity with OD260/OD280 ranged from 1.8 to 2.0. Total DNA concentration in the samples was in the range of 111.2 - 280 ng/µl to ensure standards and quality.

PCR results of 20 researched Kiwi samples based on *rbc*L, *mat*K, ITS regions with corresponding primers *rbc*L1-F và *rbc*L 724-R; *mat*K-KIM3-F và *mat*K-KIM1-R; ITS1-5F và ITS4-R show clearly visible bands. PCR products of successfully amplified Kiwi samples based on *rbc*L, *mat*K, ITS2 regions. The DNA sequences have sizes *rbc*L (701 -720 bp), *mat*K (773 - 858 bp), ITS (706 - 715 bp).

Results of amplification of *rbc*L, *mat*K, ITS regions with specific primer pairs on 20 Kiwi samples showed that the amplification rates among regions differed by 95%, 80% and 75%, respectively. The sequencing rate of these genomic regions reached 100% on successful amplification samples. This demonstrates genetic variation of some species in the genus *Actinidia*.

3.1.2. Evaluation, identification and analysis of genetic relationships of Kiwi varieties

3.1.2.1. Analysis results of differences among rbcL sequences of 19 collected kiwifruit samples

The amplified *rbc*L, *mat*K, ITS region sequences of the studied samples have a high degree of similarity with those published on NCBI: *rbc*L region (99% - 100%, 97.7 - 100%), *mat*K (99.7% - 100%, 99 - 100%), ITS (87% - 100%, 95.6 - 100%). The conserved region of *rbc*L is 694/701, the variable region is 7/701, the Pi index is 6/701; the conserved region of *mat*K is 766/773, the variable region is 7/773, the Pi index is 3/773 and the conserved region of ITS 675/706, the variable region is 68/706, the Pi index is 22/706 .

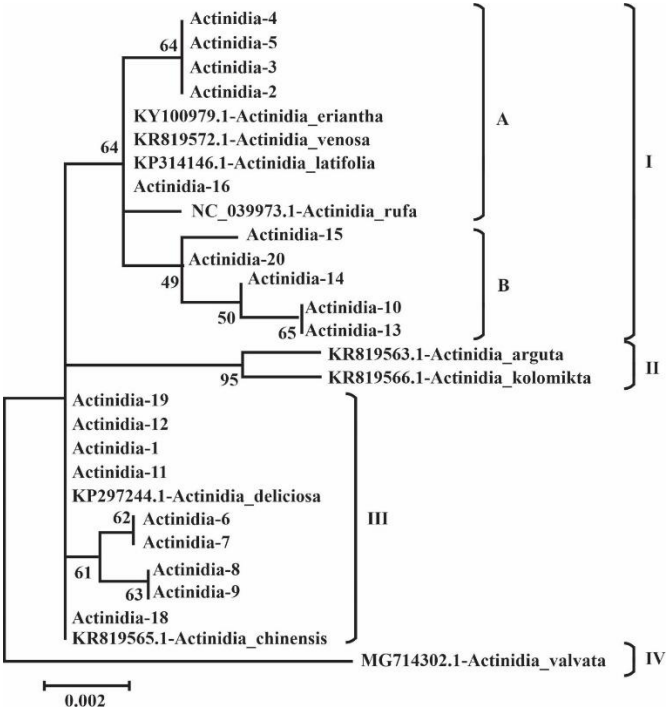

Figure 3.1. Phylogenetic plant of 19 kiwifruit samples based on *rbc*L region DNA sequences and NCBI reference sequences

The results of establishing a phylogenetic plant divided the analyzed Kiwi samples into four main groups. In which, 10 Kiwi samples collected belong to group I, the same group as the reference samples belonging to species such as *A.eriantha, A. venosa, A. latifolia, A. rufa* and the remaining 9 Kiwi samples belong to group III, the same group as the reference samples belonging to species such as *A. deliciosa, A. chinesis*. For group II only reference samples belonging to species *A. arguta, A. kolomika* and group IV consisted of only one reference specimen of species *A. valvata*. The analysis results based on the *rbc*L region sequence of 19 Kiwi samples showed that the genetic origin of the collected Kiwi samples was concentrated in certain species and there was a distinct clustering into 2 sources.

3.1.2.2. Analysis results of differences among rbcL sequences of 16 collected kiwifruit samples

The analysis results based on the *mat*K region sequence of 16 Kiwi samples showed that the genetic origin of 15/16 Kiwi samples collected was closely related to 2 species of *A. deliciosa* and *A. chinesis*, while the remaining Kiwi-2 sample might be closely related to *A. erianth* or *A. latifolia*.

The *mat*K region DNA sequence among 16 Kiwi samples has 7 different positions among the sequences. In which, at the different position 457 there is a change among nucleotides G or A and some sequences lose some nucleotides in this position.

The results of the analysis of the genetic subgroup ratio of the Kiwi samples collected based on the *mat*K region showed that only Kiwi samples collected from Vietnamese natural sources belonged to one group, separate from the rest. In which, Kiwi plant samples collected from Vietnamese natural sources have close genetic relationships with 12 commercial kiwifruit samples.

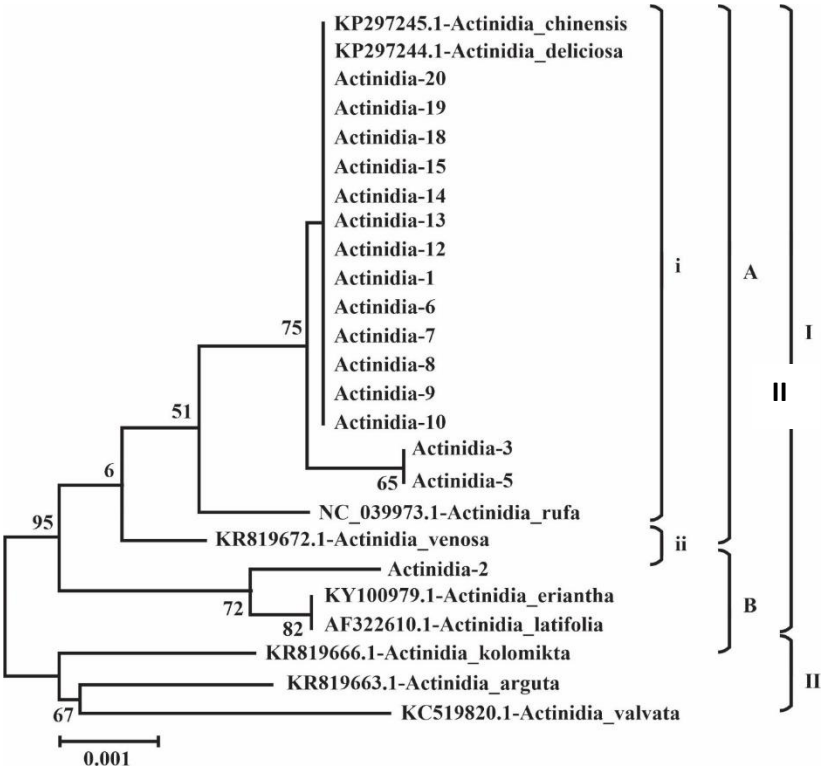

Figure 3.2. Phylogenetic plant of 16 Kiwi samples based on the DNA sequence of the *mat*K region

3.1.2.3. Analysis results of differences among ITS2 sequences of 16 collected kiwi samples

The results of grouping the remaining 11 kiwi samples in the same group with 2 reference sequences. The results of analysis based on the ITS2 region sequence of 15 kiwifruit samples showed that their genetic origin could be from 3 different genetic groups as followed: the first group consisted of 11 closely related Kiwi samples belonging to the same family. 2 species *A. deliciosa* and *A. chinensis*, group 2 includes 3 samples *Actinidia 12*, *Actinidia 19* and *Actinidia 20* belonging to 2 reference sequences of species *A. callosa* and *A. rufa*, the other group only has sample Kiwi-2 and relative separate from the other two groups.

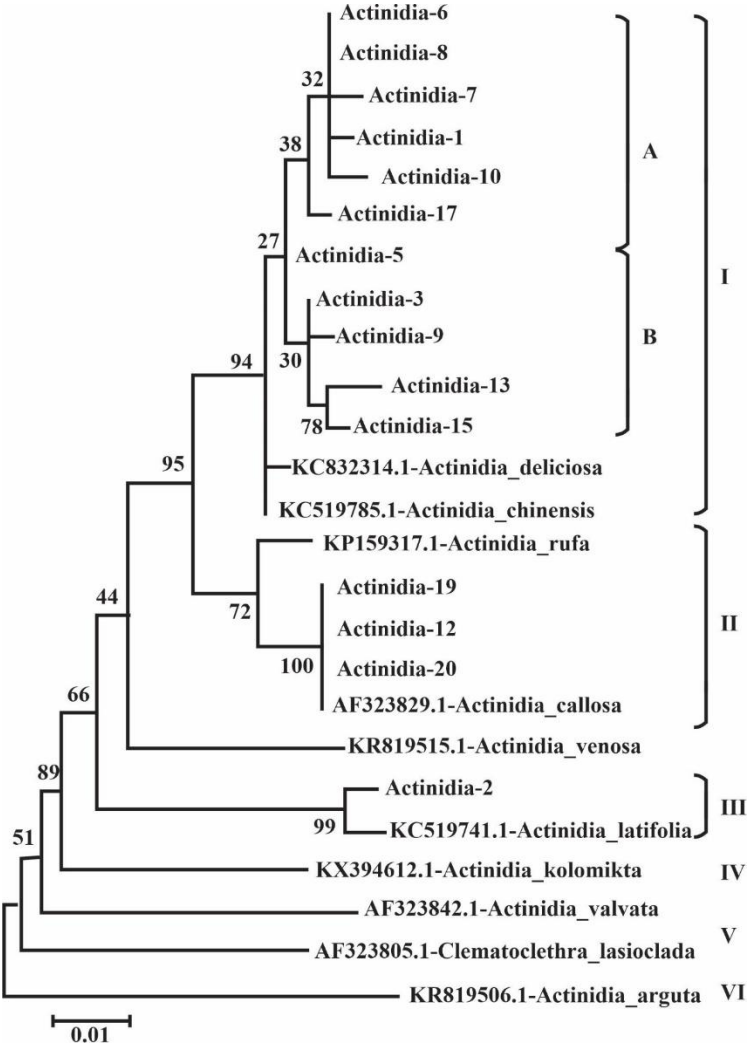

Figure 3.3. Phylogenetic plant of 15 Kiwi samples based on the DNA sequence of the ITS region

The results showed that the DNA sequences of the *rbc*L and *mat*K regions in the chloroplast had 7 different positions while the ITS region in the nucleus had 68 different positions. This shows that the gene region in the chloroplast is more conserved and less variable than that in the nucleus. Because the regions in the nucleus are changed during the hybridization process, there are many differences among the DNA regions in the nucleus.

*3.1.2.4. Region mat*K + ITS

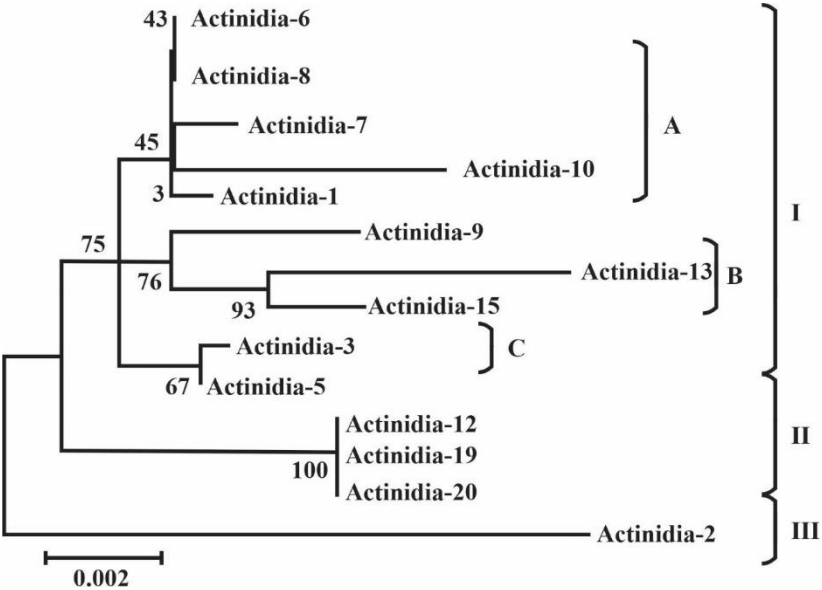

Figure 3.4. Phylogenetic plant of 14 Kiwi samples based on the DNA sequence of the *mat*K + ITS region

Phylogenetic plant of 14 Kiwi samples based on DNA sequences combining *mat*K+ ITS regions divided into 3 main groups (Figure 3.4). Group I was divided into 2 subgroups including group IA including *Actinidia* samples 1, 6, 7, 8,10 and group IB including Kiwi 9, 13, 15 samples, group IC including *Actinidia* samples 3, 5. Group II consisted of 3 *Actinidia* samples 12, 19, 20. Group III includes Actinidia 2 samples.

*3.1.2.5. rbc*L+*mat*K + ITS *region*

Phylogenetic plant of 14 Kiwi samples based on DNA sequences combining *rbc*L + *mat*K + ITS regions was divided into 3 main groups. (Figure 3.5). Group I is divided into 3 subgroups, including group IA including *Actinidia* samples 6, 7, 8, 1, 10, group IB including *Actinidia* samples 9, 13, 15, group IC including samples *Actinidia* 3, 5. Group II includes 3 *Actinidia* samples 12, 19, 20. Group III includes *Actinidia* 2 samples.

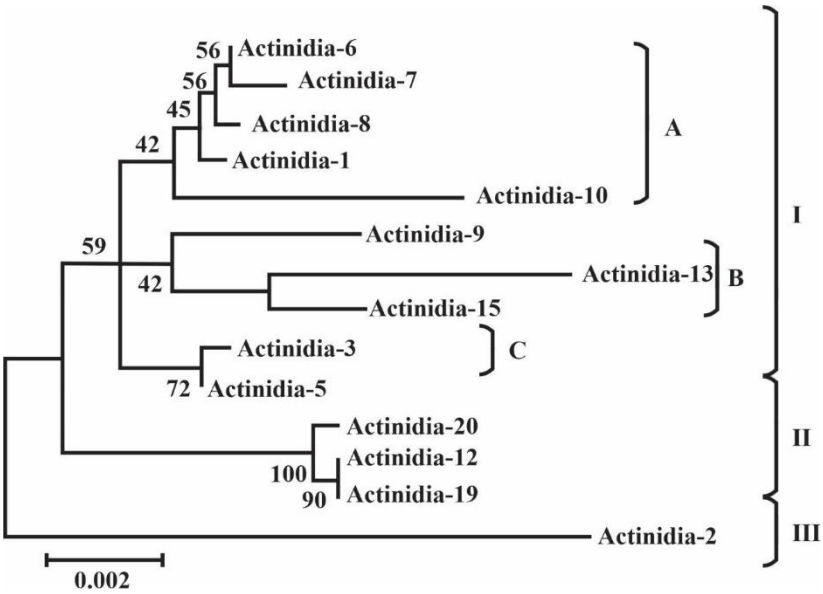

Figure 3.5. Phylogenetic plant of 14 Kiwi samples based *rbc*L + *mat*K + ITS region DNA sequences

3.1.3. Kiwi plant gender determination based on molecular markers

The obtained DNA has high purity (OD260/OD280 range 1.8-2.0). The results of electrophoresis and PCR product testing showed that all feminie samples (AD1, AL2, AC3, AC4, AC5 and AC6) did not donate. Meanwhile, amplification results were successful on a 770 bp gene fragment on the Y chromosome that helped identify two masculine samples AC7 and AC8 (Figure 3.6).

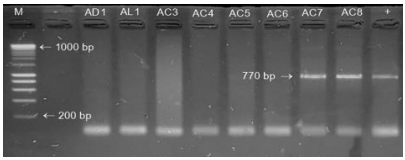

Figure 3.6. Results of electrophoresis and PCR product testing

Marker (-): Negative control of distilled water samples; (+): Masculine plant positive control containing Y chromosome

3.2. Application of kiwifruit micropropagation for breeding work

3.2.1. Create in vitro sample source

3.2.1.1. Sterilize the explant surface and regenerate shoots

The recorded results showed that different explant disinfectants had different effects towards the ability to disinfect the explant surface and induce callus after 4 weeks of culture, the AgNPs concentration increased from 100 ppm to 500 ppm. then the percentage of infected samples decreased (53.33% decreased to 15.56%) and the percentage of necrotic samples increased (6.67% increased to 60.00%) after 4 weeks of culture. In addition, leaf samples that were surface disinfected with AgNPs, HgCl2 and NaOCl all showed callus induction and the highest callus induction rate was 51.11% for surface disinfected leaf samples with 200 ppm. AgNPs.

Table 3.3. Effect of disinfectants on the ability to disinfect the explant surface and induce callus after 4 weeks of culture

Sanitizer	Concentration	Rate of infected samples (%)	Rate of necrotic samples (%)	Sample survival rate and callus induction (%)	Sample morphology
AgNPs	100 ppm	53,33a	6,67d	40,00b	Specimens were almost infected
	200 ppm	35,56b	13,33cd	51,11a	Green specimens, less damaged
	300 ppm	33,33b	31,11b	35,56b	Specimens turned into brown and almost wounded

Sanitizer	Concentration	Rate of infected samples (%)	Rate of necrotic samples (%)	Sample survival rate and callus induction (%)	Sample morphology
	500 ppm	15,56c	60,00a	24,44c	Specimens were necrotic or dead
$HgCl_2$	1 g/L	22,22c	40,00b	37,78b	Specimens turned into white and wounded
NaOCl	10 g/L	48,89a	17,78c	33,33b	Specimens were almost infected

Table 3.4. Regeneration from yellow kiwifruit leaf specimens sterilized with AgNPs, HgCl2 and NaOCl after 8 weeks of culture

Specimen origin	Number of buds/sample	Fresh weight (g)	Dry weight (g)
$HgCl_2$	3,33b	2,33b	0,60b
NaOCl	4,66ab	3,02ab	0,85ab
AgNPs	6,33a	3,82a	1,46a

In this study, 200 ppm AgNPs not only played a role in sterilizing the explants but also had less effect on explant damage than HgCl2 and NaOCl disinfectants. The results of this study once again demonstrate the effectiveness of AgNPs in surface disinfection of kiwifruit leaf explants and can replace traditional disinfectants.

3.2.1.2. Proliferation of bud clusters

Table 3.5. Effect of organic extracts on the proliferation of kiwifruit shoot clusters after 8 weeks of culture

Organic extract	Concentration (%)	Bud height (cm)	Number of leaves/bud	Khối Fresh weight (g)	Bud morphology
Control	0	3,05bc	2,67bcd	1,65c	Small buds
Coconut water	5	2,90bcd	2,33cd	1,83bc	Strong shoots, real, small leaves covered with short, white hairs
	10	3,27b	3,33abc	2,40b	
	15	3,30b	3,33abc	2,46b	Strong shoots, true and large leaves covered with
	20	3,89a	4,33a	3,33a	

Organic extract	Concentration (%)	Bud height (cm)	Number of leaves/bud	Khối Fresh weight (g)	Bud morphology
					short, white hairs
Green bananas	5	2,80cd	2,67bcd	1,59e	Small and irregular buds, small leaves
	10	3,02bc	4,00ab	2,36b	Strong shoots, real, small leaves covered with short, white hairs
	15	2,61de	3,33abc	2,27bc	
	20	2,28ef	2,00cd	1,76cde	Small buds, real and small leaves covered with short, white hairs
Potatoes	5	2,27ef	2,00cd	1,57e	
	10	2,66cde	3,33abc	2,14bcd	Small buds, real and large leaves covered with short, white hairs
	15	1,94g	2,33cd	1,69de	
	20	1,44h	1,67d	1,50e	Small buds, real and small leaves covered with short, white hairs

Culture medium supplemented with 20% *(v/v)* coconut water gave the optimal shoot proliferation efficiency in comparison with other concentrations of coconut water and potato and green banana extracts (Table 3.8 and Fig. 3.7c, d).

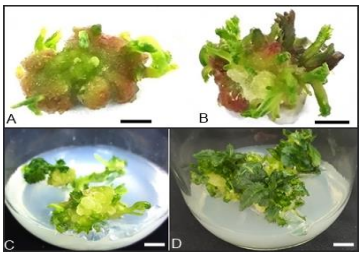

Figure 3.7. *In vitro* prototyping of golden kiwifruit (*Actinidia chinensis*)
A, B: Shoot regeneration from leaf samples disinfected with HgCl2 and AgNPs after 8 weeks of culture *(Bar: 1 cm)*
C, D: Proliferation of shoot clusters on medium without adding organic extracts and supplementing with 20% coconut water after 8 weeks of culture *(Bar: 2 cm).*

3.2.2. Efficacy of somatic embryogenesis and embryogenesis of Actinidia chinensis from main leaf veins and petioles through cell thin layer culture technology (TCL)

3.2.2.1. Clonal embryogenesis through mv-tTCL and mv-lTCL . main vein cultures

Embryogenesis induction showed that the number of clones generated per explant was different and significant among tTCL and lTCL cultures. Embryonic induction (14.00 - 14.66 days) was demonstrated in ¼ mv-lTCL and ½ mv-tTCL samples cultured on

embryo induction medium supplemented with 0.02 mg/L NAA, 0. 5 mg/L TDZ, 30 g/L sugar and 8 g/L agar earlier than others. The highest number of somatic embryos per sample (10.66 embryos) and GCFTCL (21.04) were recorded on the ½ mv-tTCL sample. Meanwhile, in culture 1/8 mv-lTCL on embryo induction medium showed the latest induction of clonal embryogenesis (20.66 days). For the ½mv-tTCL sample cultured on embryo induction medium, embryo clonal induction was 98.67% (Figure 3.8f). The results also showed that with smaller explants, the number of somatic embryos per sample formed was lower in both mv-tTCL and mv-lTCL samples. For the entire main leaf veins (control), the fresh weight of the clones (1568.00 mg) was the largest. In summary, clonal embryogenesis from mv-tTCL sample cultures is optimal when the leaf major veins are used as explants during somatic embryo induction.

3.2.2.2. Clonal embryogenesis by petiole culture of p-tTCL and p-lTCL

Embryogenesis was also observed in all TCL samples for petiole cultures,. Induction of clonal embryogenesis (15.33 - 16.33 days) was recorded in the sample ½ p-tTCL, p, ¼ p-lTCL, 1/6 p-lTCL and 1/8 p-lTCL earlier in ½ p-lTCL samples, 1/3 p-tTCL samples and ¼ p-tTCL samples were cultured on embryogenesis medium (Table 3.10). The p-tTCL sample showed the highest number of clones per sample (8.66 embryos) and the obtained GCFTCL was 15.2. For the smaller sized p-lTCL sample, embryo induction was earlier, while for the larger p-tTCL sample, SE induction was also earlier (Table 3.10). Asexual embryogenesis (92.33%) was highest in the 1/6 p-lTCL sample (Table 3.10). The results also show that the fresh weight of the explant is directly proportional to the explant size. Furthermore, fresh weight of SE was highest (1658.66 mg) in the control sample.

Table 3.6. Somatic embryogenesis of *Kiwi* through cell-thin layer culture of leaf main vein (mv) samples after 8 weeks of culture

TCL sample	Induction of somatic embryogenesis (day)	Embryogenesis rate (%)	Number of somatic embryos per culture **	GCF_{TCL}	Fresh weight of somatic embryo cluster (mg)	Morphology of somatic embryo
mv (ĐC)	19,66b*	92,00b	10,00a	9,20cd	1568,00a	Somatic embryo (sphere,
½ mv-lTCL	27,00a	72,33c	6,00b	8,68d	937,33b	heart and torpedo) and
¼ mv-lTCL	14,00c	72,66c	6,66b	19,36ab	531,33d	color (white or light

TCL sample	Induction of somatic embryogenesis (day)	Embryogenesis rate (%)	Number of somatic embryos per culture **	GCF_{TCL}	Fresh weight of somatic embryo cluster (mg)	Morphology of somatic embryo
1/6 mv-lTCL	19,66b	54,00e	5,33b	17,27ab	419,66f	green)
1/8 mv-lTCL	20,66b	61,00d	2,66c	12,98bcd	412,33f	
½ mv-tTCL	14,66c	98,67a	10,66a	21,04a	945,00b	Somatic embryo (spherical,
1/3 mv-tTCL	19,66b	59,33d	6,00b	10,68cd	595,33c	heart-shaped, torpedo and
¼ mv-tTCL	20,00b	72,66c	5,33b	15,49abc	459,33e	cotyledon) and color (white or dark green)

The study showed that 1/8 p-lTCL cultures cultured on embryogenesis medium resulted in clonal embryos with the highest total number of embryos (32.0 embryos). However, clones formed by the 1/8 p-lTCL implant were spherical, heart, and torpedo-shaped while the ½ p-tTCL culture fully captured the developmental shapes of the asexual embryos. (Spherical, heart, torpedo, and cotyledon), these clones had higher shoot regeneration compared with other TCL samples. Table 3.10 also shows that the size of the explant also affects the development of the cloned embryos. For the whole petiole (1 × 10 mm) and ½ p-lTCL samples (Figure 3.8o), the clonal embryos reached the torpedo-shaped stage; while p-lTCL with smaller size TCL did not show torpedo and cotyledon appearance after 8 weeks of culture.

Figure 3.8. Somatic embryogenesis from the main veins of the leaves and petioles of *Actinidia chinensis* Planch. through thin layer culture of cells after 8 weeks of culture

a-h: Clusters of somatic embryos derived from thin-layer cultures of primary tendinous cells (mv); (mv; ½ mv-lTCL; ¼ mv-lTCL; 1/6 mv-lTCL; 1/8 mv-lTCL; ½ mv-tTCL; 1/3 mv-tTCL; ¼ mv-tTCL; left to right) *(Bar: 1cm)*

i-q: Clusters of somatic embryos derived from petiole cell thin layer cultures (p); (p; ½ p-lTCL; ¼ p-lTCL; 1/6 p-lTCL; 1/8 p-lTCL; ½ p -tTCL; 1/3 p-tTCL; ¼ p-tTCL; left to right) *(Bar: 1cm).*

r, s, t: Somatic embryos with spherical, torpedo-shaped and cotyledons were observed under a stereo microscope (Bars: 1 mm); u, v x: Anatomy of spherical, heart-shaped and cotyledonous somatic embryos observed under an optical microscope (*Bar:: 40 µm).*

Table 3.7. Somatic embryogenesis of *Actinidia chinensis* through cell-thin layer culture of petiole specimen (p) after 8 weeks of culture

TCL sample	Induction of somatic embryogenesis (day)	Embryogenesis rate (%)	Number of somatic embryos per culture **	GCF_{TCL}	Fresh weight of somatic embryo cluster (mg)	Morphology of somatic embryo
p (ĐC)	15,66c*	88,66ab	6,66b	5,90c	1658,66a	Somatic embryo (sphere, heart and torpedo) and color (white or light green)
½ p-lTCL	19,33ab	87,33ab	4,33c	7,56c	977,66b	
¼ p-lTCL	16,00c	74,33c	5,00bc	14,87b	545,00e	
1/6 p-lTCL	16,33bc	92,33a	5,66bc	31,36a	464,66f	
1/8 p-lTCL	16,00c	66,66d	6,00bc	32,00a	459,66f	
½ p -tTCL	15,33c	87,66ab	8,66a	15,18b	954,33c	Somatic embryo (spherical, heart-shaped, torpedo and cotyledon) and color (white or dark green)
1/3 p-tTCL	20,00a	75,66c	6,33b	14,37b	596,33d	
¼ p-tTCL	20,33a	78,00c	5,66bc	17,66b	493,66f	

3.2.2.3 Growth adjustment coefficient of asexual embryogenesis

The growth adjustment coefficient of embryogenesis among mv-tTCL and p-tTCL compared with the control showed that the highest number of somatic embryos per sample was observed with the ½ mv-tTCL sample (10, 66 embryos) and ½p-tTCL (8.66 embryos) and GCFTCL were obtained with ½mv-tTCL and ½p-tTCL of 21.0 and 15.2 respectively.; The present study has shown that the procedure for obtaining TCL - SEMs of the leaf veins and petioles of kiwifruit is the optimal procedure. A large amount of embryogenic induction was observed from mv-TCL and p-TCL, the embryos were of good quality, strong and showed high shoot regeneration compared with other methods.

3.2.3. Effect of BA and TDZ on shoot regeneration

Table 3.9. Effect of BA and TDZ on shoot regeneration of *Kiwi* after 6 weeks of culture

Cytokinin (mg/L)		Number of buds		Bud height (cm)	Number of leaves	Fresh weight of buds (g)	Morphology of buds
		Tổng	≥ 4 cm				
ĐC	0	2,33d*	1,00d	3,30cd	2,67e	1,68g*	Thin, small shoots, light green leaves
BA	0,1	3,33cd	2,67bc	4,16abc	3,33de	1,96f	
	0,5	3,67bc	3,33b	4,18abc	4,67abc	2,21e	Buds are large, strong, leaves are light green and covered with shiny white hairs
	1,0	5,67ab	5,00a	4,86a	5,67a	3,75b	Buds are large and strong; leaves are light green and covered with shiny white hairs
	1,5	6,67a	3,33b	4,36ab	5,33ab	3,10d	Buds are large and strong; leaves are yellow and covered with white hairs
TDZ	0,01	2,33d	1,33cd	3,35bcd	3,33de	3,13d	Thin and small buds with light green leaves
	0,03	2,67cd	1,33cd	2,41d	3,67cde	3,16d	
	0,05	3,67bc	2,00c	3,90bc	4,33bcd	3,94a	Buds are large and strong; leaves are yellow and covered with white hairs
	0,07	3,67bc	2,00c	2,87d	4,67abc	3,53c	

In this study, the addition of BA or TDZ to the culture medium significantly affected shoot regeneration in A. chinensis; in which, shoot regeneration medium supplemented with 1.0 mg/L BA resulted in a higher number of shoots per explant (total number of shoots and shoots with a height greater than 4 cm); Moreover, this concentration of BA also showed a higher efficiency of shoot elongation than that of TDZ treatment.

3.2.4. Effect of NAA towards in vitro rooting

All shoots grown in MS medium supplemented with or without NAA showed in vitro rooting after 8 weeks. The number of roots per seedling and root length increased with the increase of NAA concentration from 0.1 to 0.4 and reached the optimal value at 0.4 mg/L (10.67 cm, respectively). and 5.85 cm) (Figure 3.9c and Figure 3.10a). Meanwhile, the number of roots per seedling and root length decreased at NAA concentrations higher than 0.4 mg/L. In addition, shoots grown on medium supplemented with 0.4 mg/L NAA gave better seedling quality than other shoots through growth parameters

such as plant height (7.08 cm), number leaves (7.67 leaves), leaf length (3.64 cm) and SPAD index (52.87). In addition, fresh weight and leaf width did not show statistically significant differences among NAA concentrations from 0.3 to 0.5 mg/L. Therefore, the addition of 0.4 mg/L NAA to the culture medium to produce the efficiency and quality of seedlings was optimal in this study.

Table 3.10. Effect of NAA on *in vitro* rooting of *Actinidia chinensis* shoots after 8 weeks of culture

NAA (mg/L)	Seedling height (cm)	Number of roots per seedling	Root length (cm)	Number of leaves per seedling	Leaf length (cm)	Leaf width (cm)	SPAD Index	Fresh weight of seedlings (g)
0	5,03c*	2,33f	0,90e	5,67bc	2,16d	1,73d	39,42e	0,47c
0,1	5,17c	4,67e	1,77d	6,67ab	2,48c	2,36c	42,70d	0,88b
0,2	5,61b	6,33d	2,06d	6,67ab	2,78b	2,39c	48,47bc	0,92b
0,3	5,30bc	8,33c	3,77c	6,33bc	2,76b	2,50ab	45,50c	1,29a
0,4	7,08a	10,67a	5,85a	7,67a	3,64a	2,67a	52,87a	1,22a
0,5	5,59b	9,33b	4,31b	5,33c	2,47c	2,52ab	47,00bc	1,31a

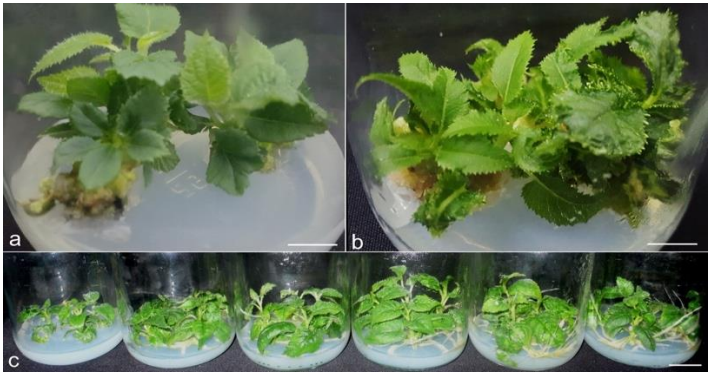

Figure 3.9. *In vitro* regeneration and rooting of *Kiwi*

a: Shoot regeneration on MS medium containing 30 g/L sugar, 8 g/L agar;

b: Shoot regeneration on MS medium containing 30 g/L sugar,

8 g/L agar and 1 mg/L BA;

c: *In vitro* rooting on MS medium containing 30 g/L sugar, 8 g/L agar and different concentrations of NAA (0.1; 0.2; 0.3; 0.4; 0.5 mg) /L or without NAA (left to right).

3.2.5. Greenhouse adaptation and growth

The results of the best in vitro rooting shoots after NAA supplementation were introduced into the greenhouse nursery after 6 weeks as shown in Table 3.13 seedlings grown on a combination of HM, CF and BF medias (with a 2:3:1 weight ratio) for survival (93.67%), seedling height (10.36 cm), leaf length (3.83 cm) and leaf width (2. 92 cm) is the highest among the other substrate ratios. Meanwhile, the number of leaves of the seedlings grown on the substrate was not significantly different. Seedling adaptation and

Figure 3.10. *In vitro* and *ex vitro* seedlings of *Kiwi* derived from somatic embryos

a: *In vitro* seedlings regenerated from shoots derived from somatic embryos were cultured on MS medium containing 0.4 mg/L NAA after 8 weeks of culture *(Bar: 5 cm)*

b: Seedlings planted in plastic pots with a combination of humus, coir and biological fertilizers (2: 3: 1) after 2 weeks in the greenhouse *(Bar: 2 cm)*

c: Seedlings planted in plastic pots with a combination of humus, coir and biological fertilizers (2: 3: 1) after 6 weeks in the greenhouse *(Bar: 3 cm)*

seedling growth on HM, CF, substrates and BF (with a 2:3:1 weight ratio) was optimal after 2 and 6 weeks in the greenhouse (Fig. 3.10b-c)

The results of this study indicate that the combination of substrates helps plants to adapt and grow well in the greenhouse.

Table 3.11. Adaptation and growth of *Kiwi*seedlings after 6 weeks in a greenhouse

Substrate Ratio (HM:CF:BF)	Survival rate (%)	Seedling height (cm)	Number of new leaves	Leaf length (cm)	Leaf width (cm)
1:0:0	76,00bc*	7,71c	0,67b	2,30bc	1,88c
1:3:1	74,67c	8,00c	1,33ab	1,95c	1,88c
2:4:1	88,67a	9,61b	1,67ab	2,80b	2,47b
2:3:1	93,67a	10,36a	2,33a	3,83a	2,92a
2:2:1	85,33ab	9,20b	1,67ab	2,57bc	2,15bc

CHAPTER 4. CONCLUSIONS AND RECOMMENDATIONS

4.1. Conclusion

Content 1: Identification of Kiwi species in Vietnam and gender determination of Kiwi plants based on molecular markers

The study shows the determination ability of genetic relatively relationship among 20 collected Kiwi samples based on 3 regions *rbc*L, *mat*K, ITS, and the 2 Kiwi varieties in Vietnam can be initially identified from of species *A. deliciosa* and A. *latifolia*; The remaining plants were identified from A. *chinensis* and A. *callosa*. This result also shows the ability to determine the genetic relationship among the DNA barcode regions *rbc*L, *mat*K and ITS is different, moreover, the combination of sequence regions together gives higher efficiency in comparison with discretely used in assessing genetic relationships as well as species identification. From the above results, it is possible to apply DNA barcode markers in genetic research and evaluation towards plants of the genus *Actinidia*. However, the accurate identification of each sample at the subspecies level requires further studies and analysis by other techniques and molecular markers to ensure the accuracy and reliability of the method.

The study initially successfully determined the gender of 6 imported golden kiwifruit varieties.

Content 2: Application of kiwifruit micropropagation for breeding tasks

The explant surface sterilization and shoot regeneration were obtained from leaf samples disinfected with 200 ppm AgNPs; In addition, the culture medium supplemented with 20% coconut water for the effective growth of shoot clusters was the most optimal.

This study builts an efficient procedure for micropropagation of *A. chinenensis* through clonal embryogenesis of the TCL specimen (mv-tTCL and mv-lTCL for the main vein explant; p-tTCL and p. -lTCL for petioles). Besides, the growth of shoots of embryogenic origin (with monocotyledon shape) was optimal on MS medium containing 1.0 mg/L BA. In addition, seedlings derived from shoots were cultured on MS medium supplemented with 0.4 mg/L NAA and planted in plastic pots with a combination of humus, coir and biological fertilizers (at a ratio of 2 : 3:1 in weight) showed greater adaptability and growth compared with other treatments in the greenhouse.

4.2. Recommendations

The results of the thesis have evaluated genetic diversity, determined gender and built an *in vitro* propagation process through cell thin layer culture technology. My recommendation to conduct some more research are as followed:

- Studying the influence of other mineral media and other plant growth regulators on the morphogenesis of the explants.

- Continue to investigate more about gender determination of kiwifruit varieties to find feminie plants to improve kiwifruit micropropagation performance.

- Further investigation on the growth and development conditions of Kiwi seedlings with suitable substrates in nursery conditions.

- Based on the knowledge about the genome as well as the sequencing of the kiwi's genome, research on crossbreeding new lines or varieties to create hybrid plants suitable for the climatic conditions

in Vietnam.

- Further, larger-scale research is needed to provide gender-disaggregated seedlings in a large number of quality seed sources that contribute to the supply of quality commercial seed in the market.

NEW CONTRIBUTIONS OF THE THESIS

The topic focuses on research on genetics, gender determination of golden kiwifruit plants (*Actinidia chinensis*), determination of culture material, explant type and size, and growth regulators capable of stimulating morphogenesis of kiwifruit through cell thin layer culture (TCL) of tendons main leaves and petioles of kiwifruit. This is the first study in Vietnam towards gender determination and kiwifruit micropropagation through TCL technique to shorten the propagation time and be proactive in the propagation of seedlings outside the nursery of golden kiwifruit (*Actinidia chinensis).*

LIST OF PUBLICATIONS

1. Nguyen Thi My Hanh, Hoang Thanh Tung, Hoang Dac Khai, Do Manh Cuong, Vu Quoc Luan, Nguyen Thi Nhu Mai, Truong Thi Lan Anh, Bui Van Le, Duong Tan Nhut, (2022), Efficient somatic embryogenesis and regeneration from leaf main vein and petiole of *Actinidia chinensis* planch. via thin cell layer culture technology, *Scientia Horticulturae*, 298, 110986.

2. Nguyen Thi My Hanh, Hoang Thanh Tung, Hoang Dac Khai, Do Manh Cuong, Vu Quoc Luan, Nguyen Thi Nhu Mai, Huynh Van Biet, Huynh Huu Duc, Hoang Thi Nhu Phuong, Bui Van Le, Duong Tan Nhut (2022), Gender determination of golden kiwi plants using molecular markers and micropropagation (*Actinidia chinensis*), *Vietnam Journal of Science and Technology - VJS (Version B)* 64(7) 54-59.

3. Nguyen Thi My Hanh, Hoang Thanh Tung, Hoang Dac Khai, Nguyen Thi Nhu Mai, Vu Quoc Luan, Do Manh Cuong, Huynh Huu Duc, Nguyen Truong Giang, Hoang Thi Nhu Phuong, Bui Van Le, Duong Tan Nhut (2022), The use of DNA barcode in genetic diversity analysis and identification of some Kiwi species (*Actinidia spp*.), *Vietnam Journal of Science and Technology - VJS (Version B)* (Accepted for publication).