CHARACTERIZATION OF SODIUM AZIDE INDUCED BLACK ROT RESISTANT *Dendrobium* 'Earsakul' MUTANTS

Sasitorn Hualsawat

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science Program in Crop Science

Suranaree University of Technology

Academic Year 2019

การศึกษาลักษณะของกล้วยไม้สกุลหวายพันธุ์เอียสกุลสายพันธุ์กลายที่ถูกชักนำโดยโซเดียมเอไซด์ให้ต้านทานโรคเน่าดำ

นางสาวศศิธร หวลสวัสดิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาพืชศาสตร์
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2562

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

Thesis Examining Committee

Thitiporn Machikowa
(Asst. Prof. Dr. Thitiporn Machikowa)
Chairperson

Piyada Alisha Tantasawat
(Prof. Dr. Piyada Alisha Tantasawat)
Member (Thesis Advisor)

Teerayoot Girdthai
(Asst. Prof. Dr. Teerayoot Girdthai)
Member

Sureerat Yenchon
(Dr. Sureerat Yenchon)
Member

Kont Chpt
(Assoc. Prof. Flt. Lt. Dr. Kontorn Chamniprasart)
Vice Rector for Academic Affairs and Internationalization

N. Teaumroong
(Prof. Dr. Neung Teaumroong)
Dean of Institute of Agricultural Technology

ศศิธร หวลสวัสดิ์ : การศึกษาลักษณะของกล้วยไม้สกุลหวายพันธุ์เอียสกุลสายพันธุ์กลายที่ถูกชักนำโดยโซเดียมเอไซด์ให้ต้านทานโรคเน่าดำ (CHARACTERIZATION OFSODIUM AZIDE INDUCED BLACK ROT RESISTANT *Dendrobium* 'Earsakul' MUTANTS) อาจารย์ที่ปรึกษา : ศาสตราจารย์ ดร.ปิยะดา อลิฌาณ์ ตันตสวัสดิ์, 94 หน้า.

กล้วยไม้สกุลหวาย (*Dendrobium*) เป็นไม้ตัดดอกที่มีความสำคัญทางเศรษฐกิจสูงสุดชนิดหนึ่งของประเทศไทย อย่างไรก็ตาม การผลิตยังคงได้รับผลกระทบเชิงลบจากหลายปัจจัยหนึ่งในนั้นคือโรคพืช โดยเฉพาะโรคเน่าดำซึ่งเกิดจากเชื้อ *Phytophthora palmivora* ดังนั้นการปรับปรุง-พันธุ์กล้วยไม้พันธุ์ใหม่ที่มีคุณภาพสูงและต้านทานต่อโรคเน่าดำจึงมีความจำเป็นเร่งด่วน การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาลักษณะของกล้วยไม้สกุลหวายพันธุ์เอียสกุลสายพันธุ์กลายที่ถูกชักนำโดยโซเดียมเอไซด์ให้ต้านทานต่อโรคเน่าดำ ซึ่งการทดลองนี้แบ่งออกเป็น 6 ส่วน คือ 1) การตรวจสอบผลของสูตรอาหารต่าง ๆ เพื่อกระตุ้นการเจริญเติบโตของกล้วยไม้สกุลหวายพันธุ์เอียสกุลในหลอดทดลอง โดยใช้สารควบคุมการเจริญเติบโตของพืชและสารสกัดจากธรรมชาติ 2) การประเมินระดับความต้านทานโรคเน่าดำในกล้วยไม้สกุลหวายพันธุ์เอียสกุลสายพันธุ์กลายและต้นที่ไม่ผ่านการก่อกลายพันธุ์ด้วยวิธีใบตัด 3) การประเมินความแปรปรวนทางพันธุกรรมของสายพันธุ์-กลายที่ต้านทานโรคเน่าดำโดยใช้เครื่องหมายโมเลกุล inter-simple sequence repeat (ISSR) 4) การศึกษารูปแบบของโปรตีนในต้นสายพันธุ์กลายที่ต้านทานโรคเน่าดำและต้นที่ไม่ผ่านการก่อ-กลายพันธุ์หลังจากการปลูกเชื้อ *P. palmivora* 5) การตรวจสอบการเปลี่ยนแปลงโครโมโซมของต้นสายพันธุ์กลายที่ต้านทานโรคเน่าดำและต้นที่ไม่ผ่านการก่อกลายพันธุ์โดยใช้ flow cytometry และ 6) การศึกษาลักษณะทางสัณฐานวิทยาของต้นสายพันธุ์กลายที่ต้านทานโรคเน่าดำและต้นที่ไม่ผ่านการก่อกลายพันธุ์ จากผลการทดลองพบว่า ต้นอ่อนกล้วยไม้สกุลหวายพันธุ์เอียสกุลหลังเลี้ยงบนอาหารเพาะเลี้ยงเนื้อเยื่อที่เติม BAP ความเข้มข้น 1 มก/ล ที่ 90 วัน มีการเจริญเติบโตสูงที่สุด สุขภาพดีกว่า และแข็งแรงมากกว่าต้นอ่อนกล้วยไม้สกุลหวายพันธุ์เอียสกุลที่เลี้ยงบนอาหาร VW0 เมื่อทำการประเมินระดับความต้านทานต่อโรคเน่าดำในกล้วยไม้สกุลหวายสายพันธุ์กลาย จำนวน 35 ต้น และต้นที่ไม่ผ่านการก่อกลายพันธุ์ จำนวน 5 ต้น ด้วยการปลูกเชื้อ *P. palmivora* ไอโซเลต NK-53-9 พบว่า กล้วยไม้สกุลหวายสายพันธุ์กลาย SUT17N05304 และ SUT17N05308 มีความต้านทานต่อโรคเน่าดำสูง และกล้วยไม้สายพันธุ์กลาย SUT17N05305 และ SUT17N05501 มีความต้านทานต่อโรคเน่าดำ จากนั้นนำต้นสายพันธุ์กลายที่ต้านทานโรคเน่าดำทั้ง 4 สายพันธุ์ ไปประเมินความแปรปรวนทางพันธุกรรม รูปแบบของโปรตีน การเปลี่ยนแปลงโครโมโซม และการศึกษาลักษณะทางสัณฐานวิทยาเปรียบเทียบกับต้นที่ไม่ผ่านการก่อกลายพันธุ์ การใช้เครื่องหมาย ISSR จำนวน 16 ไพรเมอร์ เพื่อศึกษาความแปรปรวนทางพันธุกรรมในต้นสายพันธุ์กลายที่ต้านทานโรคเน่าดำ พบว่า

มีพันธุกรรมแตกต่างจากต้นที่ไม่ผ่านการก่อกลายพันธุ์ (0.186 GD) นอกจากนี้ SDS-PAGE ระบุแถบโปรตีนขนาด 39 kDa ที่เพิ่มขึ้นในต้นสายพันธุ์กลายที่ต้านทานโรคเน่าดำหลังปลูกเชื้อนาน 3-5 วัน ในขณะที่ต้นที่ไม่ผ่านการก่อกลายพันธุ์พบแถบโปรตีนขนาดเดียวกันหลังปลูกเชื้อนาน 4-5 วัน นอกจากนั้น ยังพบแถบโปรตีนขนาด 13 kDa ที่เพิ่มขึ้นเฉพาะในต้นสายพันธุ์กลายที่ต้านทานโรคเน่าดำ SUT17N05304 และ SUT17N05305 เท่านั้น โปรตีนทั้ง 2 ชนิดนี้อาจเกี่ยวข้องกับ pathogenesis-related (PR) proteins ซึ่งเกี่ยวข้องกับกลไกการต้านทานแบบกระตุ้นขึ้น การวิเคราะห์ flow cytometry แสดงว่าต้นสายพันธุ์กลายที่ต้านทานโรคเน่าดำเหล่านี้มีโครโมโซมแบบ Mixoploid (2n+4n+8n) และมีปริมาณดีเอ็นเอและขนาดจีโนมตั้งแต่ 3.77 ถึง 3.90 pg $2C^{-1}$ และ 3,640 ถึง 3,764 Mbp ตามลำดับ ซึ่งสูงกว่าต้นที่ไม่ผ่านการก่อกลายพันธุ์ นอกจากนั้น การศึกษาลักษณะทางสัณฐานวิทยาของต้นสายพันธุ์กลายที่ต้านทานโรคเน่าดำและต้นที่ไม่ผ่านการก่อกลายพันธุ์ แสดงให้เห็นว่าต้นสายพันธุ์กลายที่ต้านทานโรคมีลักษณะทางสัณฐานวิทยาแตกต่างจากต้นที่ไม่ผ่านการก่อกลายพันธุ์ในด้านจำนวนข้อปล้อง จำนวนใบ และจำนวนราก จึงสรุปได้ว่า กล้วยไม้สกุลหวายพันธุ์เอียสกุลสายพันธุ์กลายที่ชักนำด้วย NaN_3 ทั้ง 4 ต้น ต้านทานต่อโรคเน่าดำที่ระดับห้องปฏิบัติการ และพบว่าต้นสายพันธุ์กลายที่ต้านทานต่อโรคเน่าดำเหล่านี้มีความแปรปรวนทางพันธุกรรมและการเปลี่ยนแปลงทางสัณฐานวิทยารวมถึงรูปแบบของโปรตีนภายหลังจากปลูกเชื้อ แสดงให้เห็นถึงประโยชน์ในการนำไปใช้พัฒนากล้วยไม้สกุลหวายพันธุ์ใหม่ที่ต้านทานต่อโรคเน่าดำในอนาคต

สาขาวิชาเทคโนโลยีการผลิตพืช ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2562 ลายมือชื่ออาจารย์ที่ปรึกษา_____

SASITORN HUALSAWAT : CHARACTERIZATION OF SODIUM AZIDE INDUCED BLACK ROT RESISTANT *Dendrobium* 'Earsakul' MUTANTS. THESIS ADVISOR : PROF. PIYADA ALISHA TANTASAWAT, Ph.D., 94 PP.

CULTURE MEDIA/DENDROBIUM/DETACHED LEAF ASSAY/ISSR/FLOW CYTROMETRY/MORPHOLOGY/MUTATION/SDS-PAGE/SODIUM AZIDE

Dendrobium is one of the most important economic cut-flowers in Thailand. However, its production is negatively impacted by several destructive factors, one of which is disease especially black rot caused by *Phytophthora palmivora*. Therefore, breeding for new orchid varieties with high quality and resistance to black rot is urgently required. This study aimed to characterize sodium azide (NaN_3) induced black rot resistant *D.* 'Earsakul' mutants. The experiment was divided into 6 parts: 1) investigation of the effects of various culture media for stimulation of *D.* 'Earsakul' growth in vitro using plant growth regulators and natural extracts, 2) evaluation of black rot resistance levels in *D.* 'Earsakul' mutants and nonmutagenized controls using detached leaf assay, 3) evaluation of genetic variability of black rot resistant mutants using inter-simple sequence repeats (ISSR) markers, 4) characterization of protein profiles in black rot resistant mutants and nonmutagenized controls following *P. palmivora* inoculation, 5) investigation of chromosomal variation of black rot resistant *D.* 'Earsakul' mutants and nonmutagenized controls using flow cytometry, and 6) morphological characterization of black rot resistant mutants and nonmutagenized controls. The results showed that at 90 days after culture, *D.* 'Earsakul' plantlets cultured on media supplemented with 1 mg/L BAP had the highest growth rate, were healthier and more vigorous than when cultured on VW0 media. When thirty-five *D.* 'Earsakul' mutant lines and five nonmutagenized controls were evaluated for

black rot resistance levels using *P. palmivora* isolate NK-53-9, it was found that two *D.* 'Earsakul' mutants (SUT17N05304 and SUT17N05308) were highly resistant and SUT17N05305 and SUT17N05501 were resistant to black rot disease. These four black rot resistant mutants were further evaluated for their genetic variability, protein profiles, chromosome variation and morphological characteristics compared with nonmutagenized controls. Genetic variability based on 16 ISSR markers demonstrated that black rot resistant mutants were genetically different from nonmutagenized controls (0.186 GD). Furthermore, SDS-PAGE identified up-regulation of a protein with molecular weight of 39 kDa at 3-5 days after inoculation in four black rot resistant mutants while in nonmutagenized controls it appeared at 4-5 days after inoculation. In addition, a 13 kDa protein was uniquely induced only in two of the black rot resistant mutants (SUT17N05304 and SUT17N05305). Both proteins may be related to pathogenesis-related (PR) proteins involved in inducible defense mechanism. Flow cytometry analysis showed that these black rot resistant mutants were mixoploid (2n+4x+8n) and their DNA content and genome size, ranging from 3.77 to 3.90 pg $2C^{-1}$ and 3,640 to 3,764 Mbp, respectively, were higher than all nonmutagenized controls. In addition, morphological characterization of black rot resistant mutants and nonmutagenized control showed that the resistant mutants differed from the non-mutagenized controls on number of nodes, number of leaves and number of roots. It can be concluded that four *D.* 'Earsakul' mutants induced by NaN_3 were resistant to black rot at the laboratory level. Genetic variability and changes in morphology as well as protein profiles after inoculation were observed in these black rot resistant mutants, suggesting their usefulness in future development of new black rot resistant *Dendrobium* varieties.

School of Crop production Technology Student's Signature Sasitorn Hualsawat

Academic Year 2019 Advisor's Signature Pimda Alisha Tantsul

ACKNOWLEDGEMENTS

On the accomplishment of the present study, I would like to take this opportunity to express my gratitude towards those who made this thesis possible.

I would like to express my heartfelt gratitude to my advisor, Prof. Dr. Piyada Alisha Tantasawat who critically reviewed the work and for her tireless guidance, encouragement, and assistance in making this successful thesis. Special thanks to Asst. Prof. Dr. Thitiporn Machikowa, Asst. Prof. Dr. Teerayoot Girdthai and Dr. Sureerat Yenchon for valuable suggestions, advice, and guidance given as committee members.

I also would like to extend my appreciation to all the faculty and staff members of the School of Crop Production Technology, Institute of Agricultural Technology, Suranaree University of Technology and members of the Plant Breeding Laboratory for their kindness and help to facilitate my work.

Finally, I wish to express my deepest gratitude to my parents who always love, encourage, and support me for the successful accomplishment of my study.

Sasitorn Hualsawat

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LIST OF ABBREVIATIONS

BAP	=	Benzylaminopurine
BSA	=	Bovine serum albumin
CRD	=	Completely randomized design
DMRT	=	Duncan's new multiple range test
ISSR	=	Inter-simple sequence repeat
LD	=	Lethal dose
NaN_3	=	Sodium azide
PGRs	=	Plant growth regulators
PLBs	=	Protocorm-like bodies
SDS-PAGE	=	Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis
VW0	=	Vacin and Went 0

CHAPTER I
INTRODUCTION

1.1 Significance of the study

Dendrobium is one of the largest genera in orchid and widely used in the commercial cut- flower production (Rajeevan and Sobhana, 1993). It has become popular worldwide because of its floral characteristics i.e., color, size and shape (Kuehnle et al., 2006), ability to continue blossoming and having life cycle longer than other orchid hybrids (Puchooa, 2004; De et al., 2014). Thailand has become one of the major exporters of fresh cut-flowers, which supplies to Japan, United States, Netherlands, China, India and Italy with an export value of ca. 26,000 million baht per year (Office of Agricultural Economics, 2019). To support the production, micropropagation is very important, particularly the techniques/methods that are capable of producing significant number of quality plants in terms of uniformity in age, size, growth and maturity in order to meet the higher economic values. However, in general, micropropagation of orchids is limited due to slow growth. Thus, the development of culture media to stimulate orchid growth in vitro is very helpful. The medium components including macronutrients, micronutrients, plant growth regulators (PGRs), vitamins, carbon sources and organic compounds as well as state of culture media (Tantasawat and Waranyuwat, 2008) are necessary for growth and development of in vitro cultured plantlets. Previous studies showed that benzylaminopurine (BAP) was efficient for stimulation of growth and development of many orchids i.e.,

Cymbidium finlaysonianum, *Vanda tessellate* and *Violet phalaenopsis* (Rahman et al., 2009; Pradhan et al., 2013; Poonpipat et al., 2018). In addition to PGRs, shrimp paste derived from either shrimp or krill containing kinetin, the essential precursor of chitosan, can increase the cell division of plants (Homjan et al., 2011). Vitamins which are the essential components for plant growth and development when they are directly or indirectly combined with other media components are widely used in plant tissue culture (Abrahamian and Kantharajah, 2011). To reduce the costs of micropropagation, using additives in the form of common products which are available on the market and cheaper than PGRs i.e., the energy drink Ready® containing goji berry and mixed berry juices, V8® and shrimp paste are quite interesting.

Although orchid micropropagation can probably be improved, orchid is still impacted by several destructive factors. One of them is diseases i.e., flower rusty spot (*Curvularia eragrostidis*), fusarium wilt (*Fusarium oxysporum*), anthracnose (*Collectotrichum* sp.), soft rot (*Pseudomonas gladioli*) and black rot (*Phytophthora palmivora*) (Aekaraj, 2008). Black rot is one of the most serious foliar diseases and is caused by polyphagous pathogen. Its severity has been reported worldwide because the pathogen can cause the disease in several host plants i.e., almond, citrus tree, cherry tree as well as ornamental plants including orchid, mainly causing root and crown rot (Uchida et al., 1994; Pane et al., 2006; Cacciola et al., 2008; Türkölmez et al., 2015). The pathogen can infect all plant roots including rhizomes, resulting in rapid plant death (Orlikowski and Szkuta, 2006; Cating et al., 2010). Therefore, breeding for new orchid varieties with high quality and resistance to black rot is urgently required.

Several breeding methods i.e., conventional breeding, molecular breeding, genetic engineering and mutation breeding are very useful for this purpose.

Conventional breeding is continually employed because of its ease and convenience. However, this method has the major limitation in terms of parents used because there are limited genetic sources of disease resistance in orchids (Wannajindaporn et al., 2014). Genetic engineering is a highly effective method and can modify several desirable characteristics but it is unlikely to be accepted in many countries including Thailand. Interestingly, mutation breeding which can enhance genetic variability and randomly modify gene(s) without altering entire genetic profile is helpful and effective (Wannajindaporn et al., 2014). Therefore, mutation can alternatively be used for the *D.* 'Earsakul' improvement, particularly for disease resistance. Moreover, if this method is used in vitro, it can increase quality and quantity of plants and/or protocorm-like bodies (PLBs) within short period of time (Medina et al., 2004; Wannajindaporn et al., 2014).

Mutation causes a sudden heritable change of chromosomes or genes within a organism. The mutation procured by changes in the base sequences of any genes is called point mutation or gene mutation. This genetic change results in yield and quality improvement in several crops (Pavadai et al., 2009). Generally, there are two major types of mutation, spontaneous and induced mutation. Spontaneous mutation occurs in nature under some environmental conditions by several causes, such as radioactivity and heat etc. or by transposable genetic elements (transposons). This type of mutation was reported widely in barley, lily, sweet corn and several other plants (Jorgensen and Jensen, 1986; Singh et al., 2014) but the frequency of mutation is very low. While induced mutation is artificial through mutagenic agents, which are capable of inducing mutation at higher frequencies (Laosuwan and Thipyapong, 2007). The mutagens include physical mutagen (radiation) e. g., gamma rays, X-rays and neutron and

chemical mutagens e. g., ethyl methanesulfonate (EMS), 5-bromouracil (5-BU), nitrous acid (HNO_2) and sodium azide (NaN_3). NaN_3 is one of the most effective chemicals in mutagenesis because it can induce mutation at high frequency, frequently changing nucleotides from GC to AT (guanine:cytosine to adenine:thymine), resulting in point mutation (transition or transversion), translocation, chromosome lagging and chromosome bridge, which may lead to amino acid changes, thereby altering the function of proteins (Kredich, 1971; Klasterskii et al., 1976; La and Momgold, 1987; Owais and Kleinhofs, 1988; Wannajindaporn et al., 2014). These changes may concurrently change the morphological characters and yield. In addition, they may be related to plant growth and development as well as plant defense mechanisms. It is also inexpensive and has been applied to improve agronomic/horticultural characteristics and resistance/ tolerance to various abiotic/biotic stresses in several plants (Al-Qurainy and Khan, 2009; Wannajindaporn et al., 2014).

Previous reports reviewed that NaN_3 caused chromosomal damage in mitosis and could reduce frequency of chromatid movement on metaphase in barley (Pearson et al., 1975; Veleminsky et al., 1977). In addition, it could also inhibit protein synthesis and DNA replication in bean (Sander et al. 1978). Moreover, Skoric et al. (2008) found that NaN_3 could increase the stearic acid, the main component of fatty acid in sunflower. Rajib and Jagatpati (2011) and Roy and Biswas (2005) also found that it could change morphological characters of ornamental plants and induced flower color modification in carnation, bromeliad and *Spathoglottis plicata*. Our laboratory has recently generated orchid mutants resistant to black rot by NaN_3 and EMS using in vitro selection (Khairum et al., unpublished data). However, these mutants have not been characterized both genotypically and phenotypically. Several methods have been used to characterize

mutant plants i.e., phenotyping, cytology or molecular markers. Wannajindaporn et al. (2016) studied morphological traits i.e., height, internode length, internode number, root length, root number, leaf length and leaf thickness to characterize *Dendrobium* mutants. However, these traits are environment dependent. While cytology based on flow cytometry, which is more rapid, accurate and convenient can also be employed to detect structural chromosome changes and ploidy as well as DNA content. Jones et al. (1998) used flow cytometry to evaluate nuclear DNA content of 26 orchids genera whose DNA content ranged from 1.53 to 4.23 pg $2C^{-1}$. Choopeng et al (2019) also used this method to study the effect of colchicine on survival rate and ploidy level of hydrid between *D. santana* and *D. friedericksianum* orchid. They found that colchicine could increase DNA content from 33 to 50 % of the original DNA content.

To assess genetic variability at DNA and/or RNA levels, molecular markers have been continually used without environmental constraints. Particularly, inter-simple sequence repeat (ISSR) is one of the potential markers for the identification of polymorphisms occurred from mutation because of its multilocus nature and dispersal throughout the genome. ISSR can also be performed without prior sequence information because it uses SSR core sequences as primers. In addition, ISSR is highly polymorphic, simple, rapid, cost-effective and environmentally independent (Reddy et al., 2002; Wannajindaporn et al., 2014). Wannajindaporn et al. (2016) used 10 ISSR marker to evaluate genetic variability in in vitro NaN_3 induced *D.* 'Earsakul' mutants. They reviewed that this marker could characterize genetic profiles of 24 putative mutants compared with controls, and altered DNA profiles were found in 20 from 24 putative mutants (83.33%).

Plants can response to pathogen infection in different ways. One of the most usual responses is the production of pathogenesis related (PR) proteins e.g., chitinase, β-glucanase and phytoalexins during plant defense mechanisms. Previous studies reported that benzothiazole (BTH) considered as abiotic elicitor could increase resistance to *P. palmivora* causing root rot disease in papaya. This response resulted from the accumulation of β-1, 3 glucanase and chitinase which allows plants to resist the pathogen (Qiu et al., 2004). Therefore, the evaluation of protein profiles may help identify the promising protein(s) involving black rot resistance mechanism in *Dendrobium*.

This study aimed to stimulate *D.* 'Earsakul' growth in vitro using PGRs and natural extracts, assess the resistance levels in NaN_3 induced black rot resistant *D.* 'Earsakul' mutants by detached leaf assay and characterize the genetic and chromosomal variation in black rot resistant mutants using ISSR marker and flow cytometry, respectively. In addition, their protein profiles occurring after fungal infection were studied using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The morphological traits of these black rot resistant mutants were also characterized. This knowledge will be beneficial for breeding orchids for black rot resistance in the future.

1.2 Research objectives

1.2.1 To investigate the effects of various culture media for stimulation of *D.* 'Earsakul' growth in vitro using plant growth regulators and natural extracts.

1.2.2 To evaluate black rot resistance levels in *D.* 'Earsakul' mutants and nonmutagenized control using detached leaf assay.

1.2.3 To evaluate genetic variation in black rot resistant mutants induced by NaN_3 using ISSR markers

1.2.4 To characterize the protein profiles following pathogen infection in black rot resistant mutants and nonmutagenized control.

1.2.5 To investigate the chromosomal variation of black rot resistant mutants using flow cytometry.

1.2.6 To characterize morphological traits of black rot resistant mutants and nonmutagenized control.

1.3 Research hypothesis

1.3.1 Plant growth regulators and natural extracts in culture media may stimulate *D.* 'Earsakul' plantlet growth in vitro.

1.3.2 NaN_3 can induce mutation in *D.* 'Earsakul' that may enable black rot resistance.

1.3.3 ISSR marker can be a powerful tool for assessing genetic changes and genetic variation between resistant mutants and nonmutagenized control.

1.3.4 Resistant mutants may have different protein profiles compared to non-mutagenized control which may be related to plant disease resistance mechanisms.

1.3.5 Resistant mutants may have chromosomal variation compared to nonmutagenized control.

1.3.6 Resistant mutants may have changes in morphological traits compared to nonmutagenized control.

1.4 Scope of the study

This study focused on characterization of putative black rot resistant *D.* 'Earsakul' mutants. The experiment was divided into 6 parts. The first experiment was to stimulate growth of *D.* 'Earsakul' plantlets in vitro using plant growth regulators and natural extracts. The second experiment was to preliminarily evaluate black rot resistance levels at the laboratory level using detached leaf assay. The third experiment was to assess the genetic variability of these mutants using ISSR markers. The fourth experiment was to investigate the chromosomal variation of black rot resistant mutants using flow cytometry. The fifth experiment was to characterize protein profiles of black rot resistant mutants by SDS-PAGE. The sixth experiment was to characterize morphological traits of black rot resistant mutants and nonmutagenized control. The experiments were conducted at the Center for Scientific and Technological Equipment 3, Suranaree University of Technology and Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand.

CHAPTER II
LITERATURE REVIEWS

2.1 General overview of *D.* 'Earsakul'

Dendrobium is the second largest genus in the Orchidaceae family. It was established by Olof Swartz in 1799 and today contains about 1,200 species. The name is from the Greek dendron and bios, which means one who lives on trees, or, essentially epiphyte (Yoocha et al., 2006). Orchid is classified as a monocotyledon with parallel veined leaves. Flowers usually have pink, purple and white color and are ca. 1-2 inches across with 3 colored sepals, 2 petals and the third petal forming a lip. Stems are 12-24 inches long bearing up to 15 flowers (Online 1). They are found in various geographical zones i.e., tropical and subtropical Asia and Northern Australia, and are diverse in growth habits. *D.* 'Earsakul' is one of the most widely distributed genera, being found in Thailand approximately 184 species (Yoocha et al., 2006).

2.2 Economic importance of *D.* 'Earsakul'

D. 'Earsakul' is widely used in the commercially cut flower production due to its floral characteristics i.e., color, size and shape (Kuehnle et al., 2006), and its capability in blossoming and having longer vaselife than other orchid hybrids (Puchooa, 2004; De et al., 2014). Thailand is one of the most important exporter of *D.* 'Earsakul' as fresh cut-flower to Japan, United States, Netherlands, China, India and Italy. The recorded incomes in 2014 to 2019 are shown in Table 2.1 (Office of Agricultural Economics, 2019).

Table 2.1 Export values of *D.* 'Earsakul' as cut-flower and potted orchids from Thailand during 2014-2019.

Year	Export values of orchid *D.* 'Earsakul' (million baht)		
	Cut-flower orchid	Potted orchid	Values
2014	2,047	619	2,666
2015	1,879	790	2,669
2016	1,911	564	2,475
2017	2,041	217	2,258
2018	2,041	519	2,560
2019	2,226	518	2,744

Office of Agricultural Economics (2019)

2.3 Major diseases affecting orchid production

2.3.1 Fusarium wilt

Fusarium wilt is one of the important diseases in orchids that is caused by *F. oxysporum* fungus. It mainly enters the roots and affects the rhizome first before spreading throughout the plants. Early symptoms are observed as yellowing leaves and become quickly blacken. While the outer layers of the rhizomes appear a circle or band of purple or pinkish-purple discoloration. If the disease is extensive, the entire rhizome may turn purple, and the discoloration may extend to the pseudobulbs. Severely infected orchids may die in 3-9 weeks (Online 2).

2.3.2 Flower rusty spot or Botrytis

Botrytis is caused by *Curvularia eragrostidis,* which shows up as small black or light brown spots appearing on sepals or petals of orchid flowers. It thrives in warm humid conditions with restricted air flow. The tiny spots can enlarge and cover the entire flowers (Online 2).

2.3.3 Anthracnose

Anthracnose is caused by *Collectotrichum* sp. Disease symptoms often appear

at leaf tips, which turn brown and sometimes develop as numerous dark bands across the leaves. The affected area is usually sharply defined and somewhat sunken, while the remainder of the leaf appears normal. After that, the fungus develops in the infected area making leaf watery, which usually occurs on the underside of older sepals and petals, and covers the entire flower (Online 1).

2.3.4 Black rot

Black rot is caused by *P. palmivora*, which is considered as a highly polyphagous pathogen usually presented on a wide range of plant species, mainly ornamental plants i.e., lily, tulip and orchids. Under humid wet condition, the pathogen usually causes root and crown rot as well as foliar blighting (Uchida et al., 1994; Pane et al., 2006; Cacciola et al., 2008) and can reduce the qualities and quantities of plants (Uchida, 1994; Cacciola et al., 2008. Early symptoms with small black lesions are often located on the roots or basal portions of the pseudobulbs and the disease continually enlarges throughout the pseudobulbs, rhizomes and leaves, resulting in rapid plant death (Alfieri et al., 1994; Erwin et al., 1996; Orlikowski and Szkuta, 2006 Tsai et al., 2006; Cating et al., 2010).

2.4 Orchid breeding programs

At present, the improvement of orchids has been proceeded through conventional breeding, molecular marker assisted selection, genetic engineering, gene editing, tissue culture and mutation. Conventional breeding is commonly performed because it is easy and convenient. However, conventional breeding for disease resistance is time-consuming and may depend on environmental factors. In addition, resistance gene(s) may not be available. Hence, it may be difficult to breed disease resistant orchids via this method. Genetic engineering/gene editing are highly effective

methods to develop resistant plants without undesirable traits but may not be acceptable in some coutries including Thailand. Mutations can generate genetic/ phenotypic variation including resistance to diseases, and add desirable trait(s) into an otherwise excellent varieties without altering its entire genetic makeup (Wannajindaporn et al., 2014). Mutation breeding has many benefits, for example, cost effective, environmentally friendly and saving more time than other methods for crop improvement. Therefore, mutation is an alternative option to improve disease resistance in *D.* 'Earsakul', especially when used in combination with tissue culture to increase the amount of *Dendrobium* within short period of time (Medina et al., 2004; Wannajindaporn et al., 2014).

2.5 Tissue culture of orchid

Plant tissue culture is useful for propagation because it can enhance the large production of plants throughout the year with high quality. The resulting plants can also be disease-free and genetically true to type (Pillay and Tenkouano, 2011; Pragya et al., 2012). Additionally, micropropagated plants are uniform in age, size, growth, and maturity which are largely considered to increase their economic value. In general, several parts of plants e.g., protocorm-like body (PLB), leaf, shoot tip, node, meristem, or even seed can be used for micropopagation. However, micropropagation of orchids in general is limited due to slow growth and thus necessitates the development of culture media to stimulate orchid growth in vitro. The success of tissue culture depends on the components and state of culture media i.e., macronutrients, micronutrients, plant growth regulators (PGRs), vitamins, carbon sources as well as organic compounds (Tantasawat and Woranyuwat, 2008), which are necessary for the growth and development of in vitro cultured plantlets.

Previous studies showed that BAP was efficient for stimulation of growth and development of orchids i.e., *Doritaenopsis* spp., *Cymbidium finlaysonianum*, *Paphiopedilum callosum* var. sublaeve, *Vanda tessellate* and *Violet phalaenopsis* (Rahman et al., 2009; Pradhan et al., 2013; Poonpipat et al., 2018). Shrimp paste derived from either shrimp or krill contains kinetin, the essential precursor of chitosan, is helpful for increasing the cell division of plants (Homjan et al., 2011). There were a few reports describing the effectiveness of shrimp paste in accelerating the root formation of 'Nam Dok Mai' rose apple *ex vitro* (Chumpookam et al., 2015). However, it has not been used as a supplement in culture media of orchids. In addition, vitamins are also the essential components for plant growth and development when they are directly or indirectly combined with other media components (Abrahamian and Kantharajah, 2011). To reduce the costs of micropropagation, using additives in the form of common products available on the market and cheaper than PGRs i.e., the energy drink Ready® containing goji berry and mixed berry juices, V8® and shrimp paste is interesting. Chaikhiri and Chouychai (2017) reported that 10 mL/L of Ready boot® was the most appropriate supplement for in vitro growth of *C. finlaysonianum*, especially for shoot and root length, number of leaves and roots as well as fresh weight. Vegetable juice 'V8®' is also well considered because it contains water and the concentrated juices of tomatoes, carrots, celery, beets, parsley, lettuce, water cress and spinach. In addition, it also contains vitamin C, beta carotene, as well as citric acid, which are precursors in various biological processes of plants such as photosynthesis, photomorphogenesis, photoprotection, and development (Nisar et al., 2015; Veggies, 2016; Borlongan, 2017).

2.6 Mutation in plants

2.6.1 Chromosomal mutations

Chromosomal mutation is an unpredictable change that occurs in any regions of chromosomal segments. These typically affect more than one gene. There are many types of chromosomal mutations which can be classified into 2 categories as follows:

2.6.1.1 Variation in chromosome structure

There are 4 types of variation in chromosome structure as follows: 1) deletions where some parts of a chromosome or a sequence of DNA is lost during replication. Their effects rely on the size and/or location of deleted sequences, particularly the larger deletions covering many genes that may alter the function of the protein(s) (Lewis, 2004). 2) duplications where extra chromosomal regions are added when a portion of the chromosome is copied and replicated resulting in increasing copy numbers of genes. These extra genes can provide excess proteins (Pongthongkham et al., 2011). 3) inversion is a chromosome rearrangement in which a segment of a chromosome is reversed end to end. Inversion occurs when a single chromosome undergoes breakage and rearrangement within itself. But if the break and reversal occurs at a crucial point, then there can be serious consequences and the inversion can be lethal. 4) translocations when a segment of chromosome breaks off and transfer of one part of a chromosome to another part of the same or a different chromosome, resulting in rearrangement of the genes. In Figure 2.1, the chromosome that originally began with ABC has lost DEF and gained JKL. DEF has been translocated to the second chromosome so that it now reads GHIDEF (Online Etymology Dictionary, 2018).

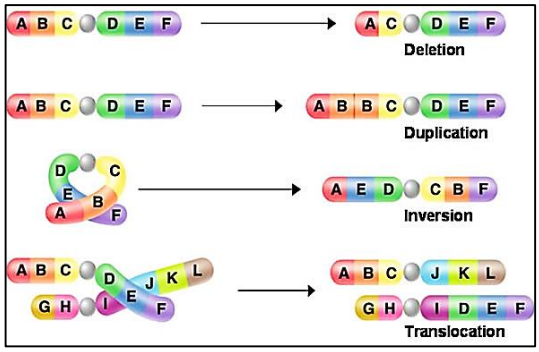

Figure 2.1 Forms of chromosome mutation; deletion, duplication, inversion and translocation (online 1).

2.6.1.2 Variation in chromosome number

Variation in chromosome structure may be occurred by spontaneous and induced mutations. Two types of variation in chromosome number are as follows: 1) aneuploidy is the increase or decrease of chromosomes i.e., monosomic (2n-1), double monosomic (2n-1-1), nulliosomic (2n-2) and trisomic (2n+1). In plants, chromosomal abnormal aneuploidies occur more frequently than in animals because aneuploid plants can live. 2) euploidy is the increase or decrease of the entire set of chromosomes i.e. monoploid (2n=x), triploid (2n=3x), tetraploid (2n=4x) and pentaploid (2n=5x). The monoploid is found in some plants i.e., maize, rye, tobacco and pepper etc. Polyploidy is found in about 47% of flowering plants and 65% of grass. It may make plants be highly sterilie (no seeds or seedless). These characteristics sometimes are commercially beneficial (Pongthongkam et al., 2011).

2.6.2 Gene mutation

Gene mutation is the loss or addition of any part of genes, or alteration of gene structure as well as change in nucleotide sequences. This can be classified into 2 categories; point mutation and frameshift mutation.

2.6.2.1 Point mutation or base substitution mutation

Point mutation occurred in DNA or RNA in which one single nucleotide base is added, deleted or changed. DNA and RNA are made up of many nucleotides. There are five different molecules that can make up nitrogenous bases on nucleotides: cytosine, guanine, adenine, thymine (in DNA) and uracil (in RNA), abbreviated C, G, A, T, and U, respectively. The specific sequence of nucleotides encodes all the information for carrying out all cell processes. This mutation includes transition occurring when a pyrimidine or purine base is substituted with another pyrimidine (T to C) or purine (A to G). Transversion occurs when a purine base is substituted with a pyrimidine base, or vice versa (A or G ‹–› T or C). There are 3 levels of point mutation as follows: 1) silent mutation, a single nucleotide is substituted and the same amino acids are produced because of multiple codons coding the same amino acid. For example, AAG and AAA for lysine. When G is changed to A, the same amino acid and protein can be formed. 2) nonsense mutation occurs when a single nucleotide is substituted leading to a translation stop codon. Stop codon is a certain sequence of bases (TAG, TAA, or TGA in DNA, and UAG, UAA, or UGA in RNA) that stops the production of the amino acid chain. It is often found at the end of the mRNA sequence when the protein is produced. A nonfunctional translate protein is consequently produced. And 3) missense mutation alters a codon which encodes a different amino acid resulting in protein change. For example, if a missense substitution changes a codon from AAG to AGG, the amino acid arginine will be produced instead of lysine. However, when there is a change between two amino acids having similar chemical properties, the function of protein may be unchanged (Chulalaksananukul, 2011) (Figure 2.2).

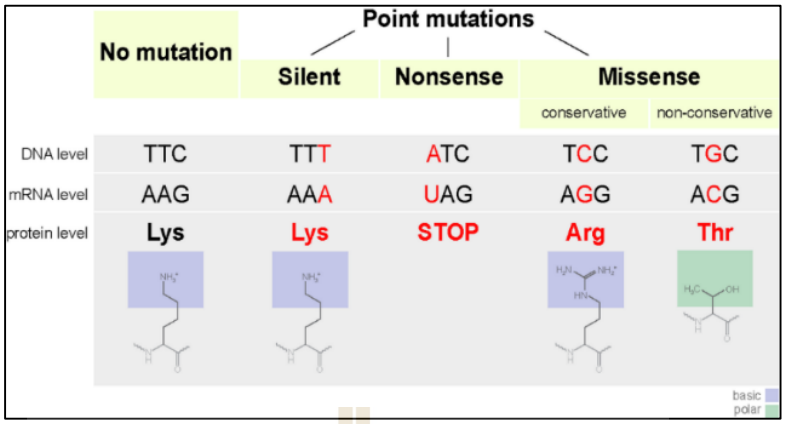

Figure 2.2 The different levels of point mutations: silent, nonsense and missense mutation (online 1).

2.6.2.2 Frameshift mutation

Frameshift mutation results from deletion, duplication and inversion change in the reading frame of nucleotides. Reading frame consists of each 3 base group that encodes for each amino acid. Typically, if it creates stop codons that produce shorter polypeptide chains, protein synthesis is then affected. For example, if a base substitution changes a codon from UUU to UUG, the amino acid phenylalanine will be produced instead of leucine and base GGC to GCA, the amino acid glycine will be produced instead of alanine (Figure 2.3).

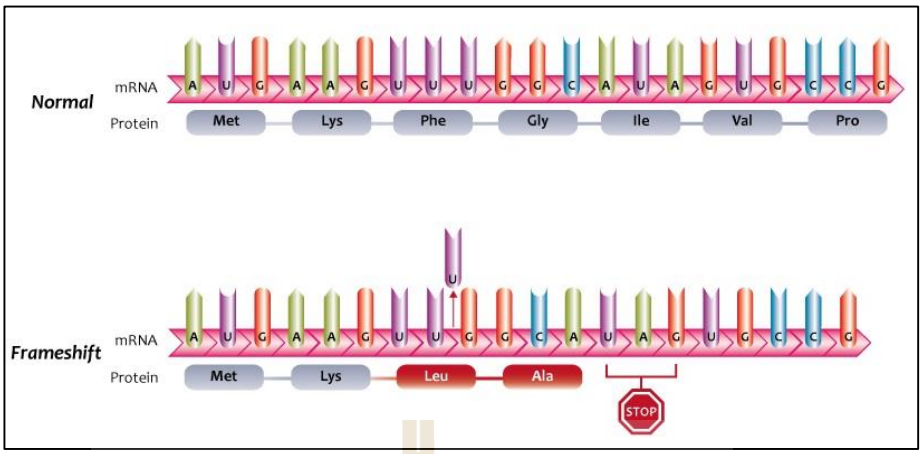

Figure 2.3 Normal DNA molecule and frameshift mutations (online 1).

Spontaneous mutation occurs from environmental agents (i.e. chemicals and radiation), transposable elements that can move to the other sites of the genome causing mutation or chromosomal changes. As a result, there is a tautomeric shift or ionization that causes variation of genetic code (Pongthongkam et al., 2011). Spontaneous mutation occurs at low frequency (ca. 10^{-6}) in the nature while induced mutation caused by treatment with agents called mutagens occurs at higher frequencies. Mutagens are classified into two broad categories, chemical mutagens and radiation. NaN_3 is one of the most effective chemicals in mutagenesis because it can induce mutation at high frequencies and contribute to variation of genes and chromosomes (Laosuwan and Thipyapong, 2007). Variation of genes and chromosomes may result in different gene expression. Macro mutation is visible, or can be detected with simple methods e.g., chlorophyll changes, disease resistance and protein percentage. While micro mutation often focuses on quantitative traits, resulting from alteration of groups of genes. Detection is possible only by statistical analysis i.e., height, seed size and yield.

2.7 Sodium azide as potential mutagen for mutagenesis

Sodium azide is an ionic compound consisting of N_3^-group which is centrosymmetric with N-N distances of 1.18 Å. It is frequently used in the field of food and pharmacy industry including agricultural applications, and can be applied to induce mutation in plants and animals (Khan and Al-Quariny, 2009). It can induce mutations at high frequencies i.e., point mutation (transition and tranversion), translocation, chromosome lagging and chromosome bridge (Kredich, 1971; Klasterskii et al., 1976; La and Momgold, 1987; Owais and Kleinhofs, 1988). Its mutagenic activity is associated with the synthesis of an organic metabolite such as an amino acid analogue L-azidoalanine [N_3-CH_2-CH (-NH_2)-COOH] or L-cysteine identified in bacteria and barley, and O-acetylserine sulfhydrylase is simultaneously found in this process. This enzyme can catalyze the binding of azide (N^-_3) or sulfide (S^-_2) with O-acetylserine to produce L-azidoalanine or L-cysteine (Klasterskii et al., 1976). This process can induce point mutation during DNA replication (Kredich, 1971; La and Momgold, 1987; Owais and Kleinhofs, 1988) causing the transversion such as purine replacing pyrimidine base or vice versa (A or G to T or C). These variations can affect the metabolisms related to plant growth and development (Khan and Al-Quariny, 2009). Previous studies have reported that NaN_3 caused chromosomal damage during mitosis stage and could reduce the frequency of chromatid movement during metaphase in barley. It could also inhibit protein synthesis and DNA replication in bean (Pearson et al., 1975; Veleminsky et al., 1977 and Sander et al., 1978). Skoric et al. (2008) found that it could increase the stearic acid which is significant content of fatty acid composition in sunflower. In addition, Rajib and Jagatpati (2011) and Roy and Biswas (2005) found that NaN_3 could change morphological characteristics of ornamental plants and induce flower color

modification in carnation, bromeliad and *Spathoglottis plicata*. Recently, *Dendrobium* mutants have been generated using NaN_3, EMS and in vitro selection (Khairum et al., unpublished data). To induce mutation, significant factors affecting mutagenesis should be considered; concentration of mutagens, time and lethal dose (LD). Particularly, LD_{50} which is a significant index to determine effective dose for the mutagenic effect resulting in a 50% reduction of viable plants and the highest frequency of mutation. It should be noted that the survival rate is highly reduced when the concentration of mutagens is increasing. Therefore, it is necessary to use the appropriate conditions to increase the chances of success and high rates of mutation. Plant tissue culture is a technique used to maintain or grow plant cells, tissues or organs under aseptic conditions on a culture medium and widely used for micropropagation. Owing to its high efficiency for rapid multiplication with large number of plants, this technique is commonly used to propagate orchids because germination by seed is low rates in nature (Aktar et al., 2008). In addition, plant tissue culture may generate genetic variability i.e., somaclonal variations as a result of gene or chromosomal mutation. These variations may be caused by nutrients in culture medium or environmental conditions (Laosuwan and Thipyapong, 2007). Therefore, tissue culture is helpful for mutiplying and increasing survival rates of *D.* 'Earsakul' mutants.

2.8 The identification of mutants

The identification of mutants can be accomplished by several methods i.e., phenotyping, cytogenetic or molecular markers. *D.* 'Earsakul' has specific morphology and patterns of growth, the deviation from which may indicate mutation. Wannajindaporn et al. (2014) found that morphological differentiation were observed

in some *D.* 'Earsakul' putative mutants i.e., reduced height, higher numbers of nodes, reduced node length, shorter and thicker leaves, and shorter and fewer roots, compared with nonmutagenized control after cultured for 6 months. However, the evaluation of morphological characteristics has limitation in terms of time-consuming and unreliable due to the influence of environmental conditions.

Cytogenetic analysis can be used to detect DNA content, genomic size, and variation of chromosome number and structure. Jones et al. (1998) used flow cytometry to evaluate nuclear DNA content of 26 orchids genera whose DNA content ranged from 1.53 to 4.23 pg $2C^{-1}$. Yenchon and Te-chato (2014) reported that this method could characterize the polyploidy induction of *D. formosum*. Chromosome observation of tetraploid plants revealed that they had chromosome number 2n=4x=76 whereas diploid plants had 2n=2x=38. Choopeng et al (2019) also used this method to study the effect of colchicine on survival rate and ploidy level of hydrid between *D. santana* and *D. friedericksianum* orchid.

Molecular markers have been offered as significant tools for evaluation of genetic variability and diversity in plants due to the highly polymorphic nature, simplicity, rapidity, and cost-effectiveness (Wannajindaporn et al., 2014). Several molecular markers have been used to identify mutants and somaclonal variants including restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR) and inter-simple sequence repeat (ISSR) (Khawale and Singh, 2006; Khosravi et al., 2009; Kuchma et al., 2011; Kumar et al., 2011; Mostafa and Alfrmawy, 2011; Bidabadi et al., 2012). These molecular markers could detect genetic differences between mutants and nonmutagenized plants, perform DNA fingerprinting

and chromosome mapping to breed new orchid cultivars in the future (Yoocha et al., 2006). ISSRs for assessing genetic variability of mutants are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors on the non-repeat adjacent regions. The main advantages of ISSRs are their random distribution throughout the genome, no requirement of genome sequences and high polymorphism. ISSR markers have been used successfully in numerous plant species. For example, Hui-Zhong et al. (2009) used 25 ISSR markers to assess the level of genetic diversity in *C. goeringii*. The results suggested that this marker is a powerful tool for cultivar identification and establishment of genetic relationships of 50 cultivars in *C. goeringii* with the polymorphism percentage of 93.75. Wannajindaporn et al. (2016) used 10 ISSR markers to evaluate genetic variability in in vitro NaN_3 induced *D.* 'Earsakul' mutants. They reviewed that this marker could characterize genetic profiles of 24 putative mutants compared with controls, and altered DNA profiles were found in 20 from 24 putative mutants (83.33%). Therefore, ISSR markers are likely be effective for evaluation of genetic variability and diversity of *Dendrobium* derived from somaclonal variation and induced mutation. In addition to characterization of *D.* 'Earsakul' mutants using cytogenetic and molecular markers, the evaluation of protein profiles may help identify the promising proteins involving resistance mechanisms to black rot in *Dendrobium*.

Pathogenesis-related (PR) proteins have a broad range of mechanisms to protect plants against attacks by pathogens like fungi, bacteria and viruses (Agrios, 1997; Ebrahim et al., 2011). The PR proteins are locally accumulated in the infected and adjacent cells in order to limit the spreading of plant pathogens as well as inhibit the infection (Ryals et al., 1996). Currently, PR-proteins are categorized into 17 different

families according to their property and function (Table 2.2). PR2 and PR3, which function similar as β-1,3-glucanases and chitinases, respectively, are two important defense enzymes in several plant species when confronting with pathogen infection (Van Loon and Van Strien, 1999). They can degrade pathogen cell wall, because chitin and β-1, 3-glucan are the major structural components of the cell walls of many pathogenic fungi. Both enzymes involve in direct defense by catalyzing pathogen cell wall lysis, or indirectly by releasing some molecules which are able to induce active defense. β-1,3-glucanases appear to be coordinately expressed along with chitinases after fungal infection (Ebrahim et al., 2011). Cohen et al. (1994) reported that β-aminobutyric acid protected tomato foliage against the late blight disease by inducing the accumulation of high levels of three proteins including P14a, β-1,3 glucanase, and chitinase involving in plant disease resistance. Qiu et al. (2004) reported that benzothiazole (BTH) treatment could increase resistance level to *P. palmivora* causing disease in papaya. This response resulted from the accumulation of β-1,3 glucanase and chitinase. In addition, Jayasankar et al. (2000) reported that anthracnose resistant lines from in vitro selection which derived from embryogenic grapevine culture had an up-regulated protein band about 36 kDa not found in nonselected control when identified using SDS-PAGE, which may be related to chitinase.

Table 2.2 Classification of pathogenesis-related proteins.

Family	Gene	Source	Host	Response	Reference
PR1	Antifungal	pepper	*N. tabacum*	Heavy metal and pathogen stress	Sarowar et al. (2005)
PR1	Antifungal	*Oryza sativa* cv. Nipponbare	*O. sativa*	*Magneporthe grisea* race 003	Mitsuhara et al. (2008)
PR1	Antifungal	*O. sativa*	*O. sativa*	Resistance to *Alternaria alternata*	Mitsuhara et al. (2008)
PR2	β-1,3-glucanase	Flax	Potato	*Fusarium culmorum* and *Fusarium oxysporum*	Wrobel-Kwiatkowska et al. (2004)
PR2	β-1,3-glucanase	Barley	Wheat	*Fusarium graminearum*	Mackintosh et al. (2007)
PR2	β-1,3-glucanase	Tobacco	Groudnut	*Cercospora arachidicola and Aspergillus flavus*	Sundaresha et al. (2010)
PR2	β-1,3-glucanase	*Pichia pastoris*	Arabidopsis	*Leptosphaeria maculans*	Oide et al. (2013)
PR2	β-1,3-glucanase	*Hevea brasiliensis*	*H. brasiliensis*	*Phytophthora palmivora*	Sunpao and Pornsuriya (2016)
PR2	endo-β-1,3(4)-glucanase	*Humicola insolens* Y1	*P. pastoris* GS115	Barley β-glucan and CMC-Na, birchwood xylan	Jinyang et al. (2017)
PR3	CHIT33, CHIT42	*Trichoderma harzianum*	*T. harzianum*	Biotic and abiotic stress	Cruz et al. (1992)
PR3	ChiA	*Pseudomonas* sp. BK1	*Escherichia coli*	*Pheidole dentata* and *Pyropia yezoensis*	Jang et al. (2005)
PR3	Chitinase	*Hordeum vulgare*	*Daucus carota*	*Alternaria radicola, Botrytis cinerea*	Jayaraj and Punja (2007)
PR3	Chitinase	*Momordica charantia*	*O. sativa*	*Magnaporthe grisea*	Li et al. (2009)
PR3	Chitinase	*N. tabacum*	*N. tabacum*	*Ralstonia solanacearum*	Tang et al. (2017)
PR4	Chitinase II	*O. sativa* L.	*O. sativa* L.	Drought stress and pathogen response	Wang et al. (2011)
PR4	Chitinase II	*Vitis pseudoreticulata*	*V. pseudoreticulata*	Powdery mildew	Dai et al. (2016)
PR4	Chitinase II	EuCHIT2 *Eucommia ulmoides*	*N. tabacum* cv. Xanthi	*Erysiphe cichoracearum* DC	Dong et al. (2017)
PR4	Chitinase classII	*Zjchi zoysiagrass.*	Zoysiagrass	*Rhizoctonia solani* AG2-2	Kang et al. (2017)
PR5	Thaumatin-like	*Prunus domestica*	*P. domestica*	Enhance resistance to fungal infection	El-kereamy et al. (2011)
PR5	Thaumatin-likeTaLr19TLP1	Barley	Wheat	*Puccinia triticina*	Yanjun et al. (2017)

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Table 2.2 Classification of pathogenesis-related proteins (Continued).

Family	Gene	Source	Host	Response	Reference
PR5	Thaumatin-like protein (VaTLP)	*V. amurensis*	*V. vinifera*	Downy mildew-resistant grapevine "Zuoshan-1	Rongrong et al. (2017)
PR5	Thaumatin-like protein (TLP29)	*V. vinifera* L.	*V. vinifera L.*	*Elsinoe ampelina*, *Erysiphe necator*	Xiaoxiao et al. (2017)
PR6	proteinase inhibitor	*Panax ginseng* Meyer	*P. ginseng* Meyer	Hormonal, heavy metals and abiotic stress	Myagmarjav et al. (2017)
PR10	Ribonuclease-like	*Capsicum annuum*	*C. annuum*	Ribonucleolytic activity against TMV	Park et al. (2004)
PR10	Ribonuclease-like	JIOaPR10	Rice	Biotic and abiotic stress	Wu et al. (2016)
PR12	Defensin	*Wasabia japonica*	Rice	*Magnaporthe grisea*	Kanzaki et al. (2002)
PR12	Defensin	Mungbean	*Pichia pastoris*	*Fusarium oxysporum*	Chen et al. (2004)
PR12	Defensin	*Brassica nigra*	Peanut	*Pheaoisariopsis personata and Cercospora arachidicola*	Anuradha et al. (2008)
PR12	BoDFN Defensin gene	*Brassica oleracea* var. *italica*	B. *oleracea* var. *italica*	Downy mildew	Jiang et al. (2012)
PR12	JcDef	*Jatropha curcas*	*N. tabacum*	Sheath blight disease resistance	Wang et al. (2017)
PR12	AtPDF1.1	*A. thaliana*	*A. thaliana*	*Pectobacterium carotovorum*	Hsiao et al. (2017)
PR12	VrPDF1	*Vigna radiata*	*V. radiata*	Weevils	Thao et al. (2017)
PR13	Thionin	Brassicaceae species	*Solanum tuberosum*	*B. cinerea*	Hoshikawa et al. (2012)
PR13	Thionin	Carrizo plant	Carrizo plant	Citrus canker	Hao and Stover, (2016)
PR13	Thionin	*A. thaliana*	*S. tuberosum*	*Fusarium* spp.	Hammad et al. (2017)
PR14	Lipid-transfer protein	*H. vulgare*	Plant nonspecific	Pathogen stress	Garcıa-Olmedo et al. (1995)
PR15	Oxalate oxidase	*H. vulgare*	Wheat	Powdery mildew	Zhang et al. (1995)
PR16	Oxidase-like	*H. vulgare*	Wheat	Powdery mildew	Wei et al. (1998)
PR17	Antifungal and antiviral	*N. tabacum*	Tobacco	Tobacco mosaic virus	Okushima et al. (2000)

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CHAPTER III
MATERIALS AND METHODS

3.1 Development of various culture media for stimulation of *D.* 'Earsakul' growth in vitro

3.1.1 Plant material and culture conditions

Plantlets of *D.* 'Earsakul' with height of approximately 2.4 - 2.7 cm were initially grown on Vacin and Went 0 medium; VW0 (Tantasawat et al., 2015) supplemented with the energy drink Ready® (1.2% (w/v) sucrose, 5.3% (w/v) taurine, 2.5% (v/v) concentrated fruit juice, 0.3% (w/v) caffeine and 0.1% (w/v) goji berry extract) at concentrations of 5, 10 and 15 mL/L; T.C. Pharmaceutical Industries Company Limited, Thailand, 6-benzylaminopurine (BAP) at concentrations of 1, 2 and 3 mg/L; Acros Organics, New Jersey, USA, V8® juice (44% (v/v) tomato juice and 33% (v/v) vegetable juice) at concentrations of 100, 200 and 300 mL/L; Campbell Soup Company, Camden, New Jersey, USA and shrimp paste (94% (w/v) shrimp paste, 5% (w/v) salt and 1% (w/v) sucrose) at concentrations of 2.5, 5.0 and 7.5 g/L; SGS (Thailand) Limited, Thailand. Plain VW0 medium was used as control. The pH of the culture media was adjusted to 5.0 before gelling with agar (0.7% w/v). All culture media were dispensed in volume of ca. 15 ml into glass bottles (4 oz.). The media were autoclaved at 121°C and 103.4 KPa pressure for 20 min. Roots were cut from plantlets under aseptic conditions, and the plantlets were randomly transferred to various media. Cultures were maintained at 25±2 °C during 16/8 h photoperiod. The following growth

parameters including fresh weight (g), height (cm), number of leaves, number of roots and total root length (cm) were measured and recorded at 0, 45 and 90 days.

3.1.2 Statistical analysis

The effects of culture media on stimulation of *D.* 'Earsakul' growth were statistically analyzed using the analysis of variance (ANOVA) and a completely randomized design with 15 replications. The means among all treatments were compared by Duncan's new multiple range test (DMRT) at $p<0.05$. All statistical analyses were performed using SPSS version 16.0 (Levesque, R. and SPSS Inc, 2006).

3.2 Evaluation of black rot resistance levels using detached leaf assay

3.2.1 Plant materials

PLBs derived from NaN_3 induction following by in vitro selection with *P. palmivora* culture filtrate for black rot resistance (Khairum et al., unpublished data) and nonmutagenized controls from clonal propagation were transferred to modified VW1 medium (Tantasawat et al., 2015) and were maintained at 25 ± 2 °C with 16 h photoperiod provided by cool-white fluorescent tubes at a photosynthetic photon flux density of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ until reaching the 4 to 6 leaf stage. Thirty five putative mutants (SUT13N01301, SUT13N01302, SUT13N01303, SUT17N01501, SUT17N05501, SUT17N05301, SUT17N05302, SUT17N05303, SUT17N05304, SUT17N05305, SUT17N05307, SUT17N05308, SUT17N05309, SUT17N05310, SUT17N05311, SUT17N05312, SUT17N05313, SUT17N05315, SUT17N05316, SUT17N05317, SUT17N05319, SUT17N05321, SUT17N05322, SUT17N05323, SUT17N05324, SUT17N05325, SUT17N05326, SUT17N05327, SUT17N05329, SUT17N05330, SUT17N05331, SUT17N05333, SUT17N05335, SUT17N05337 and SUT17N05338) were used for the experiments. In addition, five nonmutagenized controls (SUT16C001, SUT16C003, SUT16C007, SUT16C008 and SUT16C0012) were randomly selected from 0 mM NaN_3.

3.2.2 Pathogenicity tests

A 4-mm-diameter agar pieces containing mycelium of the most virulent *P. palmivora* isolate, NK-53-9 (Khairum et al., unpublished data) was collected from 5-days-old colonies grown on cereal meal potato agar (CMPA), transferred to sterile bottles, covered with 5 ml reverse osmosis water (ROW), and incubated overnight at 25 ºC in the dark (Khairum et al., 2016). Inoculums were adjusted to 10^6 zoospores/mL, and 3 µL of each suspension was inoculated on leaves of *D.* 'Earsakul' mutants after pin wounding though detached leaf assay (Khairum et al., 2016). While controls (no inoculum) were inoculated with ROW. All inoculated leaves were incubated under 25 ºC in the dark condition. Three replicates per *D.* 'Earsakul' mutants were made, and symptom development was observed at 3 and 5 days after inoculation. Scores were attributed according to the following scale (Nyasse et al. (1995) and Khairum et al. (2016); (Table 3.1) and were classified according to resistant levels with the following scale: highly resistant (0.00-0.49 scores), resistant (0.50-1.49 scores), moderately resistant (1.50-2.49 scores), moderately susceptible (2.50-3.49 scores) and susceptible (3.50-5.00 scores) as modified from Nyasse et al. (1995) and Khairum et al. (2016). The mutants that are highly resistant and resistant to black rot and nonmutagenized controls were used in experiment 3.3-3.6.

Table 3.1 Score of symptom severity for detached leaf assay

Disease rating	Symptom severity for detached leaf assay
0	No symptom
1	Very small localized lesions
2	Yellow around lesions
3	Yellow around lesions with hyphae revealed
4	Brown lesions with hyphae expanded covered entire leaf surface
5	Brown lesions with hyphae covered entire leaf surface and outside area

Scores were modified from Nyasse et al. (1995) and Khairum et al. (2016)

3.3 Evaluation of genetic variability of in vitro NaN_3 induced *D.* 'Earsakul' mutants

3.3.1 DNA isolation

Fresh young leaves of resistant mutants and nonmutagenized controls were ground with liquid N_2 and were extracted using a modified cetyltrimethyl ammonium bromide (CTAB) method of Miaobin et al. (2009). DNA was quantified by spectrophotometry using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). The final concentration was adjusted to 40 ng/µL for polymerase chain reaction (PCR) analysis according to Wannajindaporn et al. (2014).

3.3.2 ISSR analysis

Sixteen ISSR primers homologous to microsatellite repeats and containing additional selective anchor nucleotides that were developed from the University of British Columbia were chosen for the analysis (Table 2). These primers with microsatellite repeats (AC, AG, AT, CA and GA) anchored at the 3'-end by 1-2 nucleotides were reported to be effective for identification of *D.* 'Earsakul' mutants (Wannajindaporn et al., 2014). Most of them have also been successfully used to determine the genetic diversity in wild *C. goeringii* (Orchidaceae) populations (Xiaohong et al., 2007). The remaining primers which included microsatellite repeats (GT, TC, TG and ACC) were also used. Each 20 µL of PCR mix contains 40 ng genomic DNA template, 1X buffer (75 mM Tris-HCl, pH 9.0, 50 mM KCl, 20 mM $(NH_4)_2SO_4$), 2.5 mM $MgCl_2$, 200 µM of each dNTP, 1U Geneaid DNA polymerase (Geneaid Biotech Ltd., Taipei, Taiwan), and 4 µM of each ISSR primer. The PCR mixes were subjected to amplification according to Wannajindaporn et al. (2014) with an initial denaturation at 94 °C for 5 min; 45 cycles of denaturing at 94 °C for 45 s,

annealing at 48-55.4 °C for 45 s (Table 3.2), extension at 72 °C for 90 s; and a final extension at 72 °C for 7 min in a T100^{TM} Thermal Cycler. The amplified products were revealed on 6% (w/v) denaturing polyacrylamide gel and were detected by silver nitrate according to Sambrook and Russell (2001). Molecular weights of the DNA bands were estimated using 100 bp DNA ladder (Invitrogen, USA) as standard.

Table 3.2 Primer sequences and annealing temperature for each ISSR primer used for the analysis of *D.* 'Earsakul' mutants and the controls.

Primers	Primer sequences	Annealing temperature (°C)	Primers	Primer sequences	Annealing temperature (°C)
ISSR 807	$(AT)_8T$	52.3	ISSR 840	$(GA)_8YT$	48.0
ISSR 809	$(AG)_8G$	52.0	ISSR 841	$(GA)_8YC$	54.0
ISSR 811	$(GA)_8C$	53.0	ISSR 851	$(GT)_8YG$	54.0
ISSR 812	$(GA)_8A$	53.0	ISSR 857	$(AC)_8YG$	54.0
ISSR 822	$(TC)_8A$	50.0	ISSR 860	$(TG)_8RA$	52.0
ISSR 827	$(AC)_8G$	53.0	ISSR 861	$(ACC)_6$	54.0
ISSR 834	$(AG)_8T$	55.4	ISSR 888	$BDB(CA)_7$	51.0
ISSR 836	$(AT)_8YT$	52.0	ISSR 890	$VHV(GT)_7$	51.0

Y = T, C; R = A, T; V =A, C, G; B = T, C, G; H = A, T, C; D = A, T, G

3.3.3 Data scoring, cluster, and principal coordinate analysis (PCoA)

The clearly amplified bands were coded as 0 or 1 for their absence or presence, respectively. Similarity coefficients between various putative mutants and a control, in a pairwise comparison, were computed using Jaccard's coefficient, and the resulting similarity matrix was further analyzed using the unweighted pair group method with arithmetic average (UPGMA) clustering algorithm. The computations were achieved by NTSYSpc version 2.2 (Rohlf, 2000). The goodness of fit of the putative mutants and

control to a specific cluster in the UPGMA cluster analysis was determined by the Mantel correlation test (Mantel, 1967). NTSYSpc version 2.2 was also be used to perform PCoA to show multiple dimensions of the distribution of the genotypes in a scatterplot (Keim et al., 1992). This multivariate approach was used to complement the information obtained from cluster analysis because it is more informative regarding distances among major groups (Tar'an et al., 2005).

3.4 Protein analysis

3.4.1 Protein extraction

Initially, 0.2 g mature leaves of black rot resistant mutants and nonmutagenized control which were inoculated with 10^6 zoospore/mL of *p. palmivora* at 0 to 5 days post inoculation were taken in pre-chilled pestle and mortar and were homogenized in chilled 1 mL of 0.1 M Tris HCl (pH 7), 0.1 M KCl, 1% Triton X-100, 1 mM Phenylmethylsulfonyl fluoride (PMSF), 1 µg/mL Leupeptin, 1 mM Ethylene-diaminetetraacetic acid (EDTA) and 3% Polyvinylpolypyrrolidone (PVPP). The extracts were centrifuged at 13,000 rpm for 30 min at 4 ^{o}C and were recovered as supernatants used for protein profiling. Protein concentrations of extracts were measured immediately and directly from the supernatants by dye binding assay as described by Bradford (Bradford, 1976). A standard curve of absorbance at 595 nm versus 10-40 µg/mL of bovine serum albumin (BSA) was drawn. From this curve, the amount of protein in samples was calculated and finally expressed as mg per g of leaves. The experiment was twice performed.

3.4.2 SDS-PAGE

The concentration of each sample was adjusted to 0.4 µg/ µL and was mixed with 4X loading dye (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1% β-

mercaptoethanol, 12.5 mM EDTA and 0.02% bromophenol blue). Then, these samples were heated in water bath at 95 ^{o}C for 5 min to denature the proteins. The protein samples were subjected to one dimensional SDS-PAGE in a gel slab of 1 mm thickness (4% (w/v) stacking gel and 12% separating gel). Electrophoresis was carried out in the discontinuous buffer system in a vertical electrophoresis apparatus according to the method of Laemmli (Laemmli, 1970). Twelve µL of each sample was loaded in the gel. Protein molecular weight marker (molecular weight range = 2-250 kDa) from Bio-Rad, USA, was used as standard. The gel was run at 100 V for 95 min at constant current mode. Then, the gel was stained with silver nitrate.

3.4.3 Analysis of gel documentation

All experiments were carried out with 3 replicates per treatment. Protein concentrations of extracts were measured immediately and were statistically analyzed by Duncan's new multiple range test (DMRT) at $p<0.05$. All statistical analyses were performed using SPSS version 16.0 (Levesque, R. and SPSS Inc, 2006). Gels were photographed and were scanned using Canon EOS 650D camera. Detailed analysis of protein band patterns in terms of band number and the determination of the molecular weight of each band was performed by Microsoft Excel.

3.5 Cytogenetic analysis

3.5.1 Nuclei extraction

Nuclei were extracted from the mature leaves of resistant mutants and nonmutagenized control for DNA content analysis. Extraction of nuclei and DNA staining was performed according to Pfosser et al. (1995). Approximately 20-30 mg of tissues were finely chopped using a sharp razor blade into 0.2-0.5 mm in 1 mL of 1 % (w/v) PVPP and 0.4 mL Quantum stain NA UV2 'component A' (extraction buffer)

and incubated for 1 min. After extraction, 0.4 mL of Quantum stain NA UV2 'component B' (staining reagent) was added immediately prior to filter through a 50 µm nylon mesh. After filtration, samples were incubated about 30 s and analyzed using flow cytometer (Meesawat et al., 2008).

3.5.2 Flow cytometric analysis

All experiments were carried out with 5 replicates per treatment. The stained nuclei were analyzed at a concentration of 10,000 nuclei per sample. Nuclei of resistant mutants and controls were measured by relative fluorescence of sample with Quantum analysis flow cytometer (Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand). The internal reference standard was *D. lindleyi* Steud. (2C = 2.40 pg $2C^{-1}$). During analysis the reference standard peak was positioned at channel 200 of relative fluorescence intensity for instrument calibration. The obtained histograms were computerized by software (CyPAD pantau). The sample 2C DNA content was calculated according to the formula below.

$$\text{2C DNA content} = \frac{\textit{Dendrobium} \text{ peak mean}}{\textit{D. lindleyi} \text{ peak mean}} \times \text{2C DNA of } \textit{D. lindleyi} \text{ Steud.}$$

The number of base pairs per haploid genome was calculated based on the equivalent of 1 pg DNA = 965 megabase pair (Bennett and Smith, 1976).

DNA content and genome size were statistically analyzed using the analysis of variance (ANOVA) and a completely randomized design. The means among all treatments were compared by Duncan's new multiple range test (DMRT) at $p<0.05$. All statistical analyses were performed using SPSS version 16.0 (Levesque and SPSS Inc., 2006).

3.6 Analysis of morphological characters

3.6.1 Measurements of morphological parameters

Morphological analysis of black rot resistant mutants and nonmutagenized controls were performed. The following morphological parameters were measured and recorded at 6 months: plant characteristics (plant height, numbers of nodes, and node length), leaf characteristics (numbers of leaves and leaf length), and root characteristics (numbers of roots and root length). To determine the plant height, the plantlets were removed from the media, thoroughly washed in distilled water and dried with paper before measuring plant height which was measured from the base to the top of the pseudobulbs. The numbers of nodes were measured by counting manually all the nodes of the pseudobulbs. The node length was measured by each node. The numbers of leaves and roots were manually counted. The root length and leaf length were measured from all of the roots/leaves from the base to the longest point.

3.6.2 Statistical analysis

Morphological analysis of resistant mutants and controls were statistically analyzed using the analysis of variance (ANOVA) and a completely randomized design (CRD) with 10 replications. The means among all treatments were compared by Duncan's new multiple range test (DMRT). All statistical analyses were performed using SPSS version 16.0 (Levesque and SPSS Ins., 2006).

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Development of various culture media for stimulation of *D.* 'Earsakul' growth in vitro

When the effects of the culture media on the growth and development of *D.* 'Earsakul' plantlets were evaluated based on various growth parameters (fresh weight, plant height, number of leaves, number of roots and total root length) at 0, 45 and 90 days, it was found as expected that all growth parameters of the plantlets at the initial stage (0 day) were not significantly different among various culture media.

After 45 days of the culture period, significant differences were observed on most growth parameters of the plantlets cultured on different media ($p<0.05$). The differences for all growth parameters were highly significant ($p<0.01$) at 90 days among the different media.

Fresh weight: At 45 days, plantlets that were cultured on 1 and 2 mg/L BAP had the highest total fresh weight, however, no significant difference was observed among these media and those supplemented with 5 and 10 mL/L Ready® and VW0 control. After 90 days of culture on different media, this parameter also differed significantly ($p<0.01$) among the culture media. *Dendrobium* plantlets had a tendency to grow and develop best when they were cultured on the medium supplemented with 1 mg/L BAP. The total fresh weight of plantlets grown on this medium was highest (1.8-fold higher than VW0 control), but it was not significantly different from those supplemented with

2 mg/L BAP and 10 mL/L Ready® (Figure 4.1A). The culture media supplemented with V8® and shrimp paste negatively affected the fresh weight of *D.* 'Earsakul' plantlets. Plantlets grown on these media were small and had yellowish-green pseudobulbs and leaves (Figure 4.2: H-M).

Plant height: At 45 days, plantlets grown on the culture media supplemented with 1 mg/L BAP and 10 mL/L Ready® showed highest plant height and this was significantly greater (1.2 and 1.3-fold) (p<0.05) than those plantlets which were cultured on VW0 (control). Nevertheless, no significant differences among these media and those supplemented with 2 mg/L BAP and 15 mL/L Ready® were observed. After 90 days of culture on different media, the highest plantlets were obtained in the medium supplemented with 1 mg/L BAP (1.5-fold higher than VW0 control), followed by 2 mg/L BAP, 10 and 15 mL/L Ready® respectively, which were significantly higher (p<0.05) than those grown on other media (1.2-fold higher than VW0 control) (Figure 4.1B). On the other hand, plantlets cultured on the medium supplemented with 300 mL/L V8® had the lowest height and this medium also caused yellowish-green pseudobulbs and leaves (Figure 4.2J).

Number of leaves: At 45 days of the culture period, no significant differences among the culture media was observed for the number of leaves. However, *Dendrobium* plantlets cultured on different media began to exhibit significant differences in the number of leaves (p<0.01) after 90 days of culture. The highest number of leaves was achieved in plantlets cultured in the media containing 10 and 15 mL/L Ready®, but these were not significantly different from those grown on the media supplemented with 1 and 2 mg/L BAP, 5 g/L shrimp paste and VW0 as control (Figure 4.1C). By contrast, plantlets cultured on the media containing 2.5 and 7.5 g/L shrimp paste had fewer leaves with the lower leaves turning brown (Figure 4.2K and M).

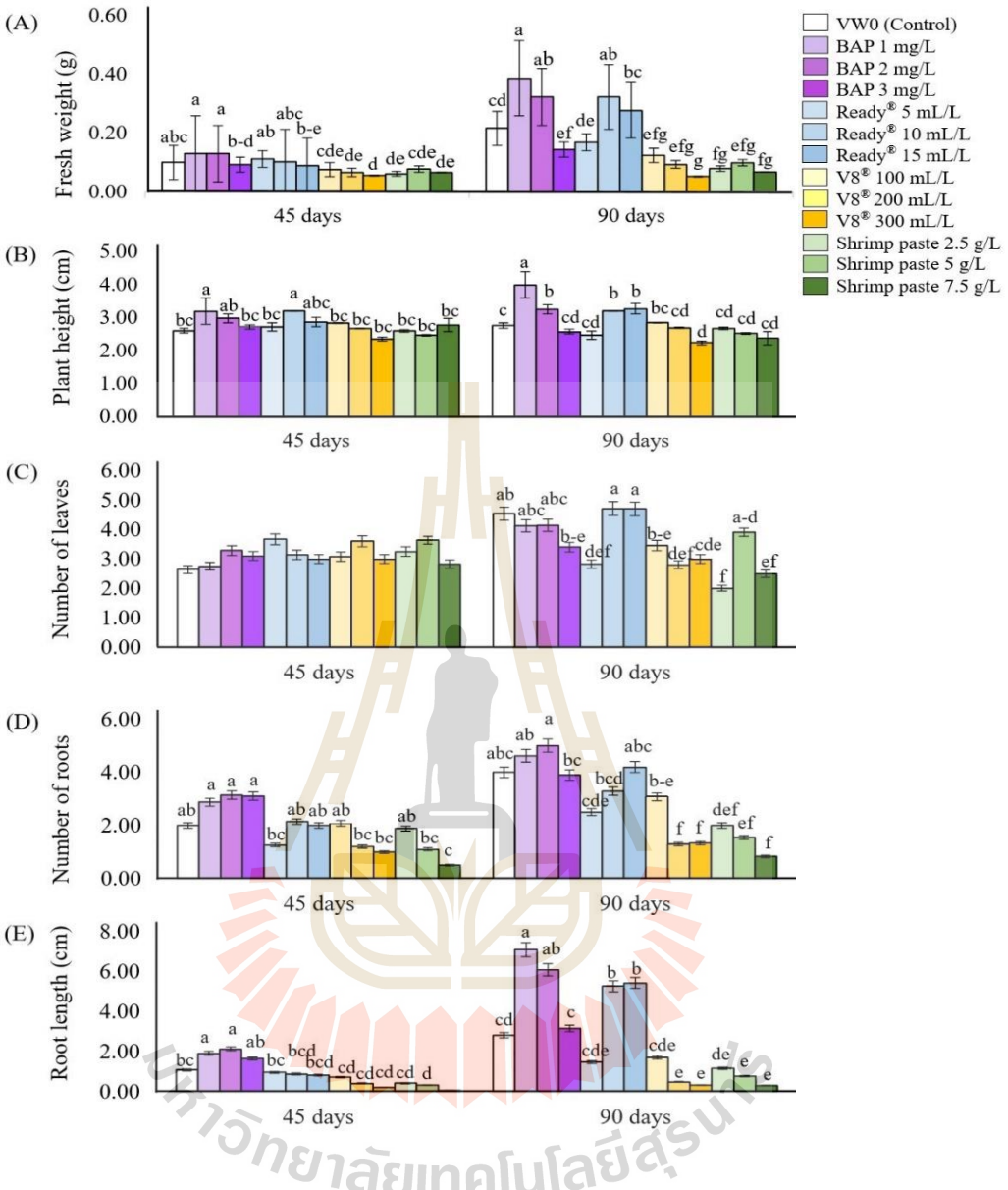


Figure 4.1 Effects of culture media on (A) fresh weight, (B) plant height, (C) number of leaves, (D) number of roots and (E) total root length of *D.* 'Earsakul' plantlets. Bars represent standard error (SE) of the mean. Different letters (a, b, c, d, e, f and g) show statistically significant differences (p<0.05) among the treatments (DMRT).

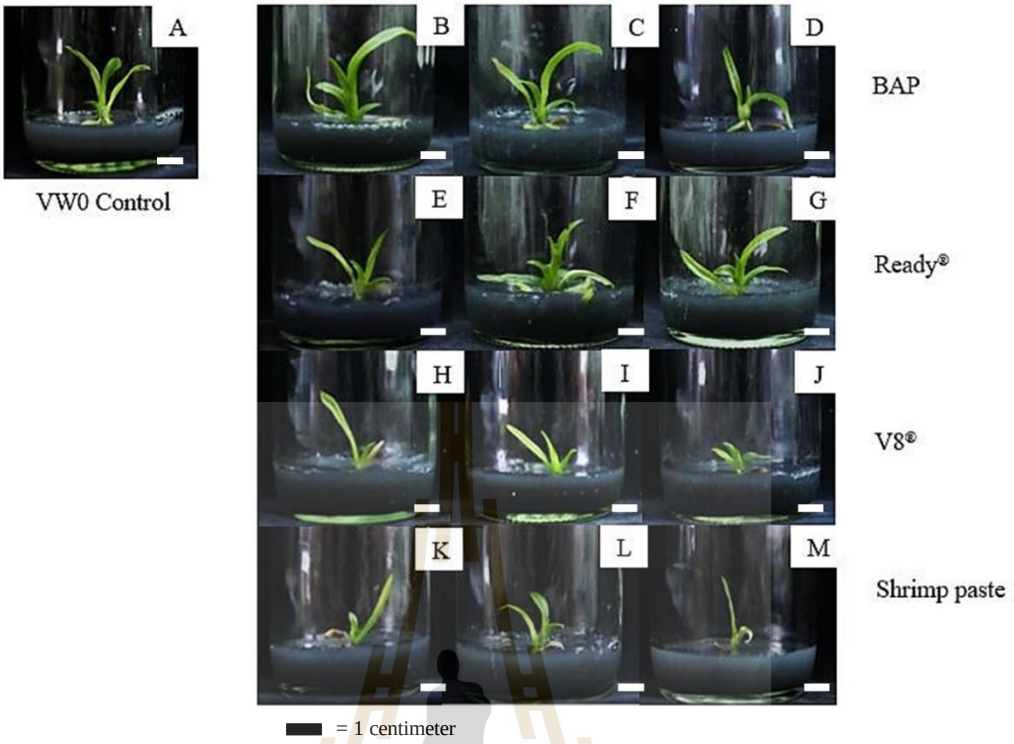


Figure 4.2 The effects of culture media on growth and development of *D.* 'Earsakul' in vitro at 90 days (A) VW0 control, (B) 1 mg/L BAP, (C) 2 mg/L BAP, (D) 3 mg/L BAP, (E) 5 mL/L Ready®, (F) 10 mL/L Ready®, (G) 15 mL/L Ready®, (H) 100 mL/L V8®, (I) 200 mL/L V8®, (J) 300 mL/L V8®, (K) 2.5 g/L shrimp paste, (L) 5 g/L shrimp paste and (M) 7.5 g/L shrimp paste.

Number of roots: At 45 days of the culture period, plantlets showed the highest number of roots when they were cultured in media supplemented with 1, 2 and 3 mg/L BAP. However, they were not significantly different from those cultured on VW0 control and many other media. After 90 days of culture on different media, plantlets cultured on the medium supplemented with 2 mg/L BAP tended to have the highest number of roots (Figure 4.1D), but with no significant difference from those cultured on media supplemented with 1 mg/L BAP, 15 mL/L Ready® and VW0 control. By contrast, culture media supplemented with shrimp paste and high concentrations of V8® adversely influenced root numbers. Plantlets grown on these media had the lowest numbers of roots (significantly lower than those cultured on VW0 control; $p<0.05$).

Total root length: At 45 days of the culture period, total root length of *D.* 'Earsakul' plantlets was highest in media supplemented with 1 and 2 mg/L BAP (1.8 and 2.0-fold significantly higher than VW0 control), but these were not significantly different from the medium supplemented with 3 mg/L BAP. After 90 days, the total root length of *D.* 'Earsakul' plantlets increased maximally when they were cultured on the medium supplemented with 1.0 mg/L BAP (2.5-fold significantly longer than VW0 control) but they were not significantly different from those cultured on 2 mg/L BAP (Figure 4.2). Media containing 200 and 300 mL/L V8® (Figure 4.2I and J) as well as 5 and 7.5 g/L shrimp paste (Figure 4.2L and M) significantly inhibited the root length of *D.* 'Earsakul' plantlets and also induced yellowish-green pseudobulbs and leaves.

When the results were taken together, it was observed that *D.* 'Earsakul' plantlets cultured on media supplemented with 1 mg/L BAP had the highest growth and were healthier and more vigorous (Figure 4.2B). These results are in agreement with previous studies in which it was reported that 1 mg/L BAP induced the growth of

orchids (*Vanda tessellate*, *Dendrobium* spp. and *Violet phalaenopsis*) (Rahman et al., 2009; Gnasekaran et al., 2010; Nambiar et al., 2012; Gansau et al., 2016). In addition, BAP promoted the expansion of leaves, roots and promoted chloroplast maturation (Chory et al., 1991; Chory et al., 1994; Spiro et al., 2004).

However, the increasing concentrations of BAP may induce programmed cell death (PCD) in plants by accelerating senescence, and causing slow and stunted growth (Carimi et al., 2004; Otroshy et al., 2013). In addition, the supplementation of 10 and 15 mL/L Ready® also produced relatively better growth of *D.* 'Earsakul' plantlets than those of V8® and shrimp paste and VW0 control. These may result from Ready® containing goji berry and mix berries juices that have high vitamin C and act as major redox buffers by regulating various physiological processes controlling growth and development (Hossain et al., 2018). In addition, it also contains 1.2% (w/v) sucrose, which is helpful for the equilibration of carbon source required to plant growth and development including signaling molecules that control gene expression related to metabolic processes, stress responses, and other processes including seed germination, floral transition, fruit ripening, embryogenesis, and senescence (Zhang and He et al., 2015). However, these culture media supplemented with Ready® had higher concentration of sucrose than other culture media which may cause stress that decreased plant growth and development. Chaikhiri and Chouychai (2017) reported that 10 mL/L Ready boot® was the most appropriate supplement for growth of *C. finlaysonianum* in vitro, especially with respect to shoot length, number of leaves, roots and fresh weight. By contrast, culture media supplemented with V8® and shrimp paste negatively affected *D.* 'Earsakul' plantlets in vitro, possibly due to the inappro-priate concentrations used, or the possible toxicity of some components. Therefore, *D.* 'Earsakul' plantlets grown on

these media were small and had yellowish-green pseudobulbs and leaves as well as root inhibition (Figure 4.2 H-M). However, previous reports showed that shrimp paste can accelerate ex vitro root formation in rose apple, *Ixora chinensis*, *Carmona retusa* (Vahl) Masam and *Duranta erecta* L. (Homjan et al., 2011; Chumpookam et al., 2015).

4.2 Evaluation of black rot resistance levels using detached leaf assay

Pathogenicity of *P. palmivora* isolate NK-53-9 was evaluated in 35 *D.* 'Earsakul' mutants induced by NaN_3 following by in vitro selection with *P. palmivora* culture filtrate and 5 nonmutagenized controls using detached leaf assay. When leaves of *D.* 'Earsakul' mutants and nonmutagenized controls were inoculated with a 10^6 zoospores/mL suspension, necrotic lesions and hyphae were observed from 3 days after inoculation. At 5 days after inoculation, severity of symptoms was highly significantly different among *D.* 'Earsakul' mutants and nonmutagenized controls ($p<0.01$). Two *D.* 'Earsakul' mutants, SUT17NO5304 and SUT17NO5308 were potentially highly resistant to *P. palmivora* isolate NK-53-9 because they did not develop any symptoms for the entire period of the experiment (Table 4.1). In addition, SUT17NO5305 and SUT17NO5501 which had severity scores of 0.67 and 1.33 score, respectively, were identified to be potentially resistant with very small localized lesions. In total, four from 35 *D.* 'Earsakul' mutants (11.4%) were resistant and highly resistant to black rot disease. While thirteen D. 'Earsakul' mutants (37.1%) were moderately resistant which had yellow halo around lesions with the score ranging from 1.50 to 2.49 and twelve *D.* 'Earsakul' mutants (34.3%) were moderately susceptible which had yellow halo around lesions and hyphae expanded with the score ranging from 2.50 to 3.49. However, six *D.* 'Earsakul' mutants (17.1%) were susceptible, having brown lesions with hyphae expanded and a severity score of 3.50-5.00. Of these mutants, SUT17NO5312 was the most susceptible to *P. palmivora* isolate NK-53-9 with severity score of 4.67, which was comparable to nonmutagenized controls and significantly higher than those of seven other *D.* 'Earsakul' mutants. The leaves of six mutants and all nonmutagenized controls had more brown lesions with hyphae covered entire leaf surface and outside

area and were classified as susceptible (Table 4.2). These results suggest that NaN_3 induced mutation followed by in vitro selection with *P. palmivora* culture filtrate was effective for generating black rot resistant mutants. All of nonmutagenized controls were susceptible to *P. palmivora* isolate NK-53-9 which were consistent with Khairum et al. (unpublished data). Detached leaf assay has been successfully used to identify black rot resistant *D.* 'Earsakul' mutants in previous work (Khairum et al., 2016). Four promising black rot resistant mutants (SUT17N05304, SUT17N05305, SUT17N05308, and SUT17N05501) were selected for further investigation of their genetic variability, chromosome variation, and protein profiles as well as morphological traits.

Table 4.1 Severity scores of symptoms at 0, 3 and 5 days after inoculation with *P. palmivora* isolate NK53-9 in 35 *D.* 'Earsakul' mutant lines and 5 non-mutagenized controls.

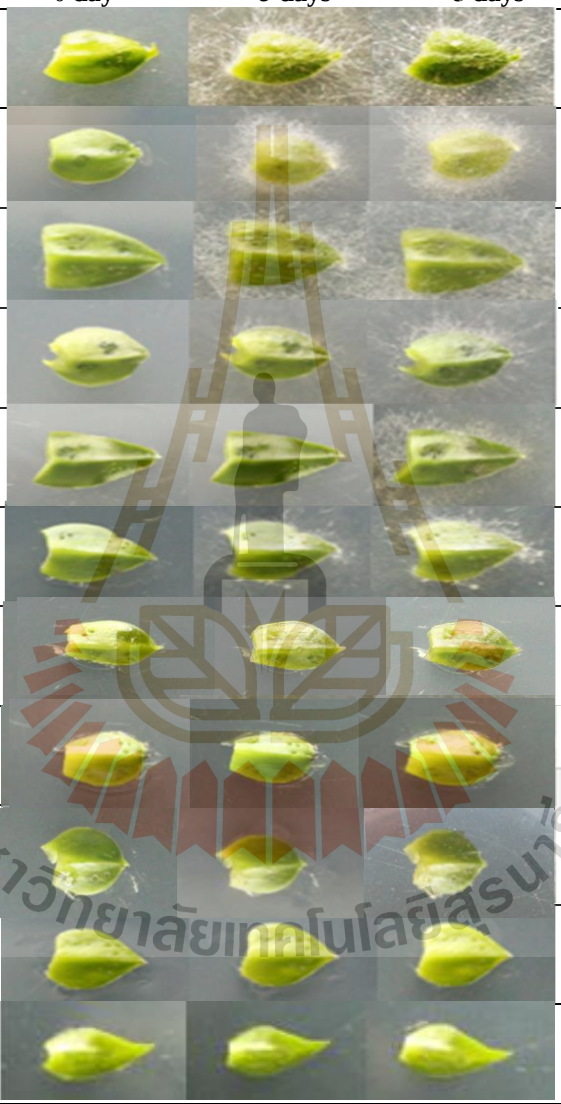
Lines	Severity score of symptoms			
	0 day	3 days	5 days	Disease response[/1]
SUT16C001[/2]	0.00 ± 0.00[/3]	4.33 ± 0.88 a	5.00 ± 0.58 a	Susceptible
SUT16C003[/2]	0.00 ± 0.00	3.67 ± 1.00 abc	4.00 ± 0.67 a-d	Susceptible
SUT16C007[/2]	0.00 ± 0.00	4.00 ± 0.33 ab	4.33 ± 0.00 abc	Susceptible
SUT16C008[/2]	0.00 ± 0.00	3.67 ± 0.67 abc	5.00 ± 0.00 a	Susceptible
SUT16C0012[/2]	0.00 ± 0.00	4.33 ± 1.33 a	5.00 ± 1.33 a	Susceptible
SUT13N01301	0.00 ± 0.00	1.00 ± 0.58 def	3.33 ± 0.19 a-d	Moderately susceptible
SUT13N01302	0.00 ± 0.00	1.67 ± 0.88 c-f	1.67 ± 0.88 c-f	Moderately resistant
SUT13N01303	0.00 ± 0.00	2.00 ± 1.00 b-f	2.00 ± 1.00 b-f	Moderately resistant
SUT17N01501	0.00 ± 0.00	1.00 ± 1.00 def	2.67 ± 1.33 a-e	Moderately susceptible
SUT17N05501	0.00 ± 0.00	1.00 ± 1.00 def	1.33 ± 1.33 def	Resistant
SUT17N05301	0.00 ± 0.00	1.00 ± 0.00 def	1.67 ± 0.54 c-f	Moderately resistant
SUT17N05302	0.00 ± 0.00	1.33 ± 0.88 c-f	2.00 ± 0.58 b-f	Moderately resistant
SUT17N05303	0.00 ± 0.00	2.00 ± 0.58 b-f	3.33 ± 0.88 a-d	Moderately susceptible
SUT17N05304	0.00 ± 0.00	0.00 ± 0.00 f	0.00 ± 0.00 e	Highly resistant
SUT17N05305	0.00 ± 0.00	0.33 ± 0.33 ef	0.67 ± 0.10 ef	Resistant
SUT17N05307	0.00 ± 0.00	0.00 ± 0.00 f	2.00 ± 0.00 b-f	Moderately resistant
SUT17N05308	0.00 ± 0.00	0.00 ± 0.00 f	0.00 ± 0.00 e	Highly resistant
SUT17N05309	0.00 ± 0.00	1.00 ± 1.00 def	2.33 ± 0.88 b-f	Moderately resistant
SUT17N05310	0.00 ± 0.00	2.00 ± 1.00 b-f	3.00 ± 1.00 a-d	Moderately susceptible
SUT17N05311	0.00 ± 0.00	0.67 ± 0.33 def	1.67 ± 0.33 c-f	Moderately resistant
SUT17N05312	0.00 ± 0.00	3.00 ± 0.33 a-d	4.67 ± 0.33 ab	Susceptible
SUT17N05313	0.00 ± 0.00	2.33 ± 0.67 a-f	4.00 ± 1.00 a-d	Susceptible
SUT17N05315	0.00 ± 0.00	1.67 ± 0.67 c-f	3.00 ± 1.00 a-e	Moderately susceptible
SUT17N05316	0.00 ± 0.00	2.33 ± 0.67 a-f	3.33 ± 0.33 a-d	Moderately susceptible
SUT17N05317	0.00 ± 0.00	2.00 ± 1.00 b-f	3.67 ± 1.33 a-d	Susceptible
SUT17N05319	0.00 ± 0.00	2.67 ± 0.33 a-e	4.00 ± 0.58 a-d	Susceptible
SUT17N05321	0.00 ± 0.00	2.33 ± 0.67 a-f	3.00 ± 1.00 a-d	Moderately susceptible
SUT17N05322	0.00 ± 0.00	2.00 ± 1.00 b-f	3.33 ± 0.67 a-d	Moderately susceptible
SUT17N05323	0.00 ± 0.00	1.00 ± 0.00 def	2.00 ± 1.00 b-f	Moderately resistant
SUT17N05324	0.00 ± 0.00	1.33 ± 0.88 c-f	2.00 ± 1.00 b-f	Moderately resistant
SUT17N05325	0.00 ± 0.00	0.33 ± 0.33 ef	3.00 ± 0.58 a-e	Moderately susceptible
SUT17N05326	0.00 ± 0.00	2.00 ± 1.00 b-f	3.00 ± 1.00 a-e	Moderately susceptible
SUT17N05327	0.00 ± 0.00	2.33 ± 0.67 a-f	3.00 ± 0.58 a-e	Moderately susceptible
SUT17N05329	0.00 ± 0.00	3.00 ± 1.00 a-d	3.67 ± 0.88 a-d	Susceptible
SUT17N05330	0.00 ± 0.00	2.00 ± 0.58 b-f	3.67 ± 1.20 a-d	Susceptible
SUT17N05331	0.00 ± 0.00	1.33 ± 0.33 c-f	2.00 ± 0.00 b-f	Moderately resistant
SUT17N05333	0.00 ± 0.00	1.00 ± 0.00 def	2.67 ± 0.33 a-e	Moderately susceptible
SUT17N05335	0.00 ± 0.00	2.00 ± 0.58 b-f	2.33 ± 0.88 b-f	Moderately resistant
SUT17N05337	0.00 ± 0.00	1.33 ± 0.88 c-f	2.00 ± 1.00 b-f	Moderately resistant
SUT17N05338	0.00 ± 0.00	1.33 ± 0.88 c-f	2.00 ± 0.58 b-f	Moderately resistant

[/1] = Disease response was observed at 5 days after inoculation. Scores were attributed according to the following scale: highly resistant (0.00-0.49 scores), resistant (0.50-1.49 scores), moderately resistant (1.50-2.49 scores), moderately susceptible (2.50-3.49 scores), and susceptible (3.50-5.00 scores), modified from Nyasse et al. (1995) and Khairum et al. (2016).

[/2] = Nonmutagenized controls

[/3]= Data are presented as means ± SE. Different letters show statistically significant differences among the treatments (DMRT).

Table 4.2 Severity of symptoms at 0, 3 and 5 days after inoculation with *P. palmivora* isolate NK53-9 in 35 *D.* 'Earsakul' mutant lines and 5 nonmutagenized controls.

Lines	Severity of symptoms			
	0 day	3 days	5 days	Disease response[/1]
SUT16C001[/2]				Susceptible
SUT16C003[/2]				Susceptible
SUT16C007[/2]				Susceptible
SUT16C008[/2]				Susceptible
SUT16C0012[/2]				Susceptible
SUT13N01301				Moderately susceptible
SUT13N01302				Moderately resistant
SUT13N01303				Moderately resistant
SUT17N01501				Moderately susceptible
SUT17N05501				Resistant
SUT17N05301				Moderately resistant

[/1] = Disease response was observed at 5 days after inoculation. Scores were attributed according to the following scale: highly resistant (0.00-0.49 scores), resistant (0.50-1.49 scores), moderately resistant (1.50-2.49 scores), moderately susceptible (2.50-3.49 scores), and susceptible (3.50-5.00 scores), modified from Nyasse et al. (1995) and Khairum et al. (2016).

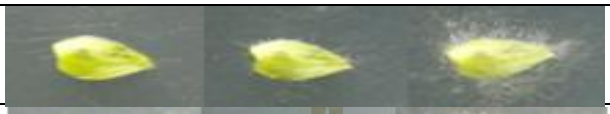
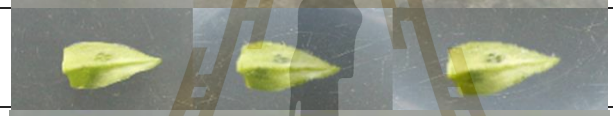
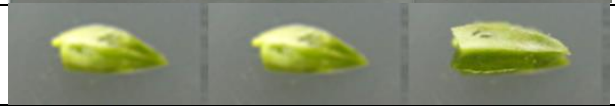
[/2] = Nonmutagenized controls

Table 4.2 Severity of symptoms at 0, 3 and 5 days after inoculation with *P. palmivora* isolate NK53-9 in 35 *D.* 'Earsakul' mutant lines and 5 nonmutagenized controls (Continued).

Lines	Severity of symptoms			
	0 day	3 days	5 days	Disease response[/1]
SUT17N05302				Moderately resistant
SUT17N05303				Moderately susceptible
SUT17N05304				Highly resistant
SUT17N05305				Resistant
SUT17N05307				Moderately resistant
SUT17N05308				Highly resistant
SUT17N05309				Moderately resistant
SUT17N05310				Moderately susceptible
SUT17N05311				Moderately resistant
SUT17N05312				Susceptible
SUT17N05313				Susceptible

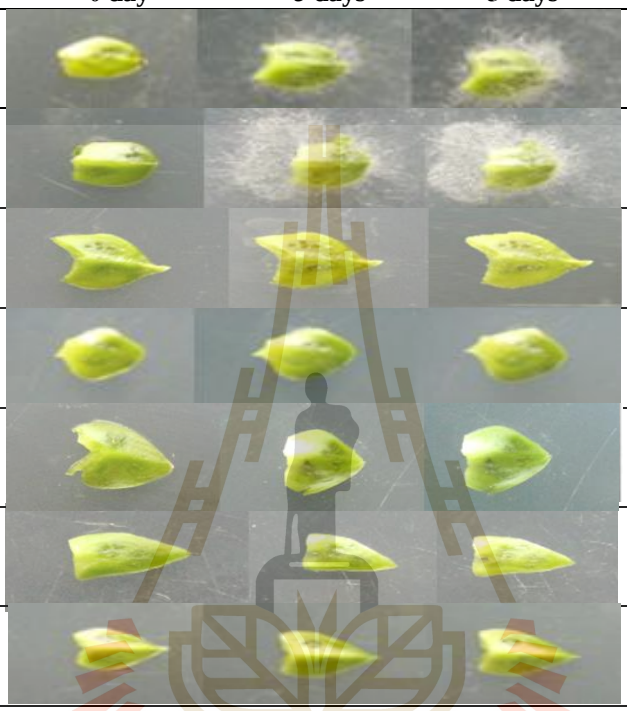
[/1] = Disease response was observed at 5 days after inoculation. Scores were attributed according to the following scale: highly resistant (0.00-0.49 scores), resistant (0.50-1.49 scores), moderately resistant (1.50-2.49 scores), moderately susceptible (2.50-3.49 scores), and susceptible (3.50-5.00 scores), modified from Nyasse et al. (1995) and Khairum et al. (2016).

Table 4.2 Severity of symptoms at 0, 3 and 5 days after inoculation with *P. palmivora* isolate NK53-9 in 35 *D.* 'Earsakul' mutant lines and 5 nonmutagenized controls (Continued).

Lines	Severity of symptoms			
	0 day	3 days	5 days	Disease response[/1]
SUT17N05315				Moderately susceptible
SUT17N05316				Moderately susceptible
SUT17N05317				Susceptible
SUT17N05319				Susceptible
SUT17N05321				Moderately susceptible
SUT17N05322				Moderately susceptible
SUT17N05323				Moderately resistant
SUT17N05324				Moderately resistant
SUT17N05325				Moderately susceptible
SUT17N05326				Moderately susceptible
SUT17N05327				Moderately susceptible

[/1] = Disease response was observed at 5 days after inoculation. Scores were attributed according to the following scale: highly resistant (0.00-0.49 scores), resistant (0.50-1.49 scores), moderately resistant (1.50-2.49 scores), moderately susceptible (2.50-3.49 scores), and susceptible (3.50-5.00 scores), modified from Nyasse et al. (1995) and Khairum et al. (2016).

Table 4.2 Severity of symptoms at 0, 3 and 5 days after inoculation with *P. palmivora* isolate NK53-9 in 35 *D.* 'Earsakul' mutant lines and 5 nonmutagenized controls (Continued).

Lines	Severity of symptoms			
	0 day	3 days	5 days	Disease response[/1]
SUT17N05329				Susceptible
SUT17N05330				Susceptible
SUT17N05331				Moderately resistant
SUT17N05333				Moderately susceptible
SUT17N05335				Moderately resistant
SUT17N05337				Moderately resistant
SUT17N05338				Moderately resistant

[/1] = Disease response was observed at 5 days after inoculation. Scores were attributed according to the following scale: highly resistant (0.00-0.49 scores), resistant (0.50-1.49 scores), moderately resistant (1.50-2.49 scores), moderately susceptible (2.50-3.49 scores), and susceptible (3.50-5.00 scores), modified from Nyasse et al. (1995) and Khairum et al. (2016).

4.3 Evaluation of genetic variation in black rot resistant mutants induced by NaN_3 using ISSR markers

Genetic variability of four *D.* 'Earsakul' black rot resistant mutants and three nonmutagenized controls was analyzed using 16 ISSR primers. Of these primers, a total of 225 clear and reproducible DNA bands were produced among all materials used (Table 4.3). The number of bands obtained were ranged from 8 (ISSR 861) to 23 (ISSR 841) bands per primer. Among the total bands, 51 bands from all primers were identified to be polymorphic (22.67%). A representative profile of ISSR amplification is shown in Figure 4.3. The size of all amplified bands was varied from 204 bp (ISSR 834) to 2000 bp (ISSR 807) (Table 4.3). Each ISSR primer generated an average of 14.06 bands per primer and 3.19 of these were polymorphic. The highest and lowest number of poly-morphic bands were obtained from ISSR 834 (9) and ISSR 812, 822, 861, 888 and 890 (1), respectively. The ISSR 834 exhibited the highest percentage polymorphism (50.00%), followed by ISSR 857 (45.45%), ISSR 827 (31.25%), ISSR 811 (28.57%), and ISSR 836 (28.57%), while the lowest percentage polymorphism was obtained from ISSR 812 (6.25%), with an average of 22.29% from all primers. This polymorphism percentage was comparable to that of Wannajindaporn et al. (2016) who found the percentage polymorphism of 34.81% in 24 *D.* 'Earsakul' mutants induced by NaN_3. The polymorphism may largely depend on the sequences of primers and backgrounds and number of materials being analyzed (Keim et al., 1992). In this study, all 4 resistant mutants exhibited the presence of genetic difference compared with nonmutagenized controls, particularly the six unique bands observed only in resistant mutants (2 unique bands from SUT17N05304, 2 unique bands from SUT17N05501, and 2 unique bands from SUT17N05305) (Table 4.4). In addition, SUT16C003 and

SUT16C007 (nonmuta-genized controls) were genetically different from SUT16C008. These results suggest the evidence of somaclonal variation in the absence of NaN_3 treatment during in vitro culture. Martínez-Estrada et al. (2017) reported that ISSR markers could detect the existence of somaclonal variation during micropropagation of sugarcane under temporary immersion systems. They found that the highest percentage of polymorphism and genetic distances (GD) of the subcultures 1, 9, and 10 were 10.1% and 0.0222 GD, 15.6% and 0.0181 GD, and 0.1% and 0.0181 GD, respectively. The molecular and statistical analysis showed that in vitro establishment and the number of subcultures are both factors that affected the frequency of somaclonal variation. The factors affected the frequency of somaclonal variation during the micropropagation may include e.g., types of tissues and explants, types and components of culture media, and duration, as well as methods of culture, that may possibly cause this variation through the alteration in DNA methylation patterns (Kaeppler et al., 2000; Rout et al., 2006; Leva et al., 2012; Wannajindaporn et al., 2016). On the other hand, the absence of ISSR fragments or the presence of additional ISSR fragments may result either from the loss/gain of primer binding sites as a result of changes in the nucleotide sequences of SSR motifs in each genotype thereby resulting in the polymorphism.

The polymorphic bands scored were used to construct a dendrogram based on cluster analysis using UPGMA. The Mantel test with a cophenetic correlation coefficient value of 0.99 (p<0.01) indicating that data in the similarity matrix were well represented by the dendrogram. The dendrogram showed 2 different clusters at the genetic similarity level of 0.73 (Figure 4.4). All three nonmutagenized controls (SUT16C003, SUT16C007, and SUT16C008) were classified as cluster I, while all black rot resistant mutants (SUT17N05304, SUT17N05305, SUT17N05308, and

SUT17N05501) were differently allocated in cluster II.

Jaccard's genetic similarity coefficients among the pairwise combinations of genotypes ranged from 0.722 to 0.990 with an average of 0.897. These nonmutagenized controls and the black rot resistant mutants also differed in their morphological characters e.g., number of leaves and number of roots. All plants in the nonmutagenized control group had high genetic similarity with an average of 0.974. In the resistant mutant group, the range of similarity coefficients between lines was 0.845 (SUT17N05305 and SUT17N05501) to 0.898 (SUT17N05304 and SUT17N05501) with an average of 0.867 (Table 4.5).

The genetic distance between the resistant mutants and nonmutagenized controls were 0.186 GD. These results demonstrate that NaN_3 has high potential for inducing mutations and will be useful for *Dendrobium* breeding program. The mechanism of NaN_3 is associated with the synthesis of an organic metabolite such as an amino acid analogue L-azidoalanine [N_3-CH_2-CH (-NH_2)-COOH] or L-cysteine, and O-acetyl- serine sulfhydrylase is simultaneously found in this process. This enzyme can catalyze the binding of azide (N^-_3) or sulfide (S^-_2) with O-acetylserine to produce L-azidoalanine (N_3-CH_2-CH (NH_2)-COOH) or L-cysteine (Klasterskii et al., 1976). This process occurred from NaN_3 induction can induce point mutation during DNA replication (Kredich, 1971; La and Momgold, 1987; Owais and Kleinhofs, 1988), causing the transversion such as purine replacing pyrimidine base or vice versa (A or G to T or C), which can lead to amino acid changes. This can result in a change in the function of proteins (Al-Qurainy and Khan, 2009; Wannajidaporn et al., 2016). As a consequence, it can affect the metabolisms related to plant growth and development (Al-Quariny and Khan, 2009; Gruszka and Szarejko, 2012; Wannajidaporn et al., 2016).

PCoA was used to identify multidimensional relationships that describe portions of the genetic variance in a data set. Three-dimensional plots of PCoA based on these 16 ISSR markers were generally consistent with the UPGMA cluster analysis with the three coordinates accounting for 58.9, 11.9, and 11.5% with a total of 82.3% of the total variance. PCoA could clearly separate the materials studied into 2 different groups (Figure 4.5), suggesting that it is effective to generate a good separation of these materials.

ISSR marker can efficiently identify genetic differences among the resistant mutants and nonmutagenized controls. It must be noted that the variation of ISSR loci observed may not necessarily reflect the variation of plant phenotypes because ISSR loci may be located in non-coding regions of genome where the change of DNA sequences are not limited by the maintenance of gene function, resulting in more rapid evolutionary change (Johns et al., 1997; Chaw et al., 2002). Using ISSR markers for assessment of genetic variability profiles of the resistant mutant and nonmutagenized control DNA proved to be useful for the improvement of *Dendrobium*.

Table 4.3 Primer sequences, annealing temperature, number of total scorable DNA bands, number of polymorphic DNA bands, percentages of polymorphism, and amplified band size for each ISSR primer used for the analysis of 4 resistant mutant lines and nonmutagenized controls.

Primers	Primer sequences	Annealing temperature (°C)	No. of total bands	No. of polymorphic bands	Polymorphism (%)	Amplified band size (bp)	PIC
ISSR 807	$(AG)_8T$	52.3	18	4	22.22	218-2,000	0.2449
ISSR 809	$(AG)_8G$	52.0	11	2	18.18	235-600	0.3673
ISSR 811	$(GA)_8C$	53.0	14	4	28.57	314-1,200	0.4286
ISSR 812	$(GA)_8A$	53.0	16	1	6.25	559-1,500	0.4082
ISSR 822	$(TC)_8A$	50.0	13	1	7.69	519-1,200	0.4898
ISSR 827	$(AC)_8G$	53.0	16	5	31.25	221-1,200	0.4082
ISSR 834	$(AG)_8YT$	58.5	18	9	50.00	204-600	0.4172
ISSR 836	$(AG)_8YA$	52.0	14	4	28.57	234-1,500	0.3265
ISSR 840	$(GA)_8YT$	48.0	12	3	25.00	360-1,000	0.3810
ISSR 841	$(GA)_8YC$	54.0	23	5	21.74	278-1,200	0.4490
ISSR 851	$(GT)_8YG$	54.0	11	3	27.27	235-500	0.4898
ISSR 857	$(AC)_8YG$	54.0	11	5	45.45	245-700	0.4898
ISSR 860	$(TG)_8RA$	52.0	11	2	18.18	236-1,200	0.4245
ISSR 861	$(ACC)_6$	54.0	8	1	12.50	260-1,000	0.2449
ISSR 888	$BDB(CA)_7$	51.0	15	1	6.67	242-800	0.4898
ISSR 890	$VHV(GT)_7$	51.0	14	1	7.14	333-1,000	0.4082
Total			225	51			
Average			14.06	3.19	22.29		0.4042

Y = T,C R = A,T V = A,C,G B = T,C,G H = A,T,C D = A,T,G N = A,T,C,G

Table 4.4 Presence (+) or absence (-) of polymorphic amplified product (s) from each primer used for molecular characterization of 4 resistant mutant lines and nonmutagenized controls.

ISSR	Nonmutagenized controls			Black rot resistant mutants			
	SUT16 C003	SUT16 C007	SUT16 C008	SUT17N0 5304	SUT17N0 5305	SUT17N0 5308	SUT17N0 5501
807_{218}[/1]	-	-	-	+	-	-	-
807_{251}	-	-	-	-	-	-	+
807_{516}	-	-	-	-	-	-	+
807_{647}	+	+	+	+	+	+	-
809_{235}	+	+	+	+	+	-	+
809_{315}	+	+	+	-	+	-	-
811_{314}	+	+	+	-	-	-	+
811_{321}	+	+	+	+	+	-	-
811_{324}	+	+	+	-	+	+	-
811_{559}	+	+	+	+	+	-	-
812_{559}	+	+	+	+	+	-	-
822_{519}	+	+	+	-	-	-	-
827_{221}	+	+	+	-	-	-	-
827_{234}	+	+	+	-	+	+	-
827_{243}	+	+	+	+	+	+	-
827_{251}	+	+	+	-	-	-	-
827_{452}	+	+	+	+	-	-	+
834_{204}	-	-	-	+	-	+	+
834_{229}	+	+	+	+	-	+	-
834_{239}	+	+	+	+	-	-	-
834_{256}	-	-	+	+	+	+	+
834_{261}	+	+	+	-	-	-	+
834_{268}	+	+	+	-	-	-	-
834_{371}	-	-	+	+	-	-	-
834_{393}	+	+	-	-	-	-	-
834_{400}	+	+	+	-	-	-	-
836_{234}	-	-	-	-	+	+	-
836_{236}	-	-	-	-	-	+	+
836_{315}	+	+	+	+	-	+	+
836_{318}	+	+	+	+	-	+	+
840_{360}	+	+	+	-	-	-	-
840_{425}	+	+	+	-	-	-	-
840_{630}	+	+	+	-	+	+	-
841_{278}	+	+	+	+	-	+	-
841_{384}	-	-	-	-	+	+	+

[/1] The number preceding the subscript (size of ISSR marker in bp) refers to the primer used to generate the marker.

Table 4.4 Presence (+) or absence (-) of polymorphic amplified product (s) from each primer used for molecular characterization of 4 resistant mutant lines and nonmutagenized controls (Continued).

ISSR	Nonmutagenized controls			Black rot resistant mutants			
	SUT16 C003	SUT16 C007	SUT16 C008	SUT17N0 5304	SUT17N0 5305	SUT17N0 5308	SUT17N0 5501
841_{392}	-	-	-	+	+	+	+
841_{428}	+	+	+	+	-	-	-
841_{457}	-	-	-	+	+	+	+
851_{235}	-	-	-	+	+	+	+
851_{275}	-	-	-	-	+	+	+
857_{318}	+	+	+	-	-	-	-
857_{245}	+	+	+	-	-	-	-
857_{249}	+	+	+	-	-	-	-
857_{258}	-	-	-	+	-	-	-
857_{270}	+	+	+	-	+	-	-
857_{388}	-	-	-	+	+	-	-
860_{236}	+	+	+	+	+	+	-
860_{241}	-	-	-	-	+	-	-
861_{260}	-	-	-	-	+	-	-
888_{242}	+	+	+	-	-	-	-
890_{333}	-	-	-	-	-	+	+

/1 The number preceding the subscript (size of ISSR marker in bp) refers to the primer used to generate the marker.

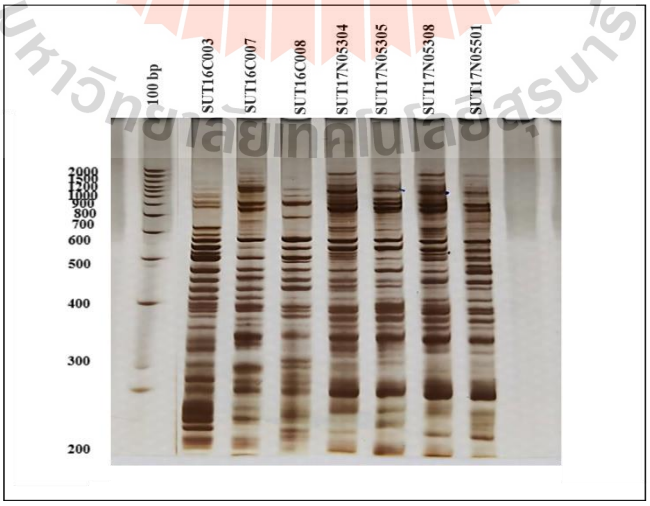


Figure 4.3 ISSR banding patterns amplified using ISSR 841 primer of black rot resistant mutant lines and nonmutagenized controls.

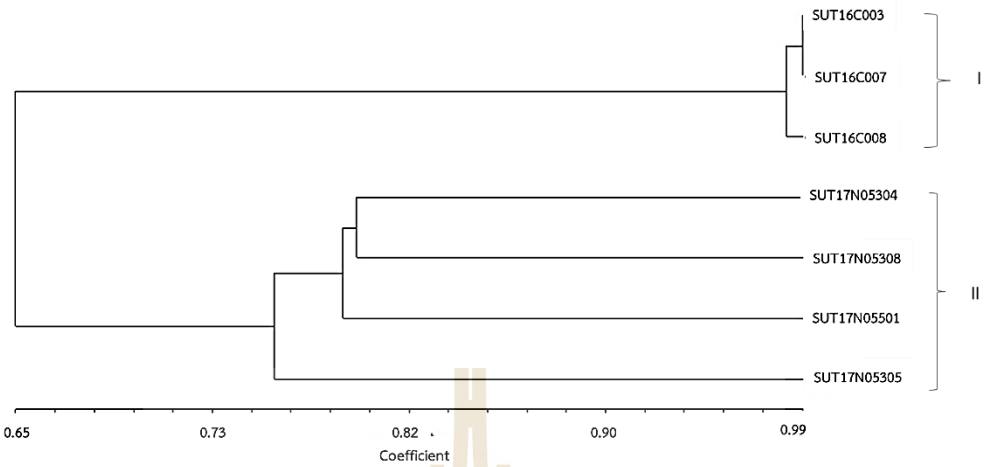


Figure 4.4 ISSR marker derived dendrogram of black rot resistant mutants, and nonmutagenized controls.

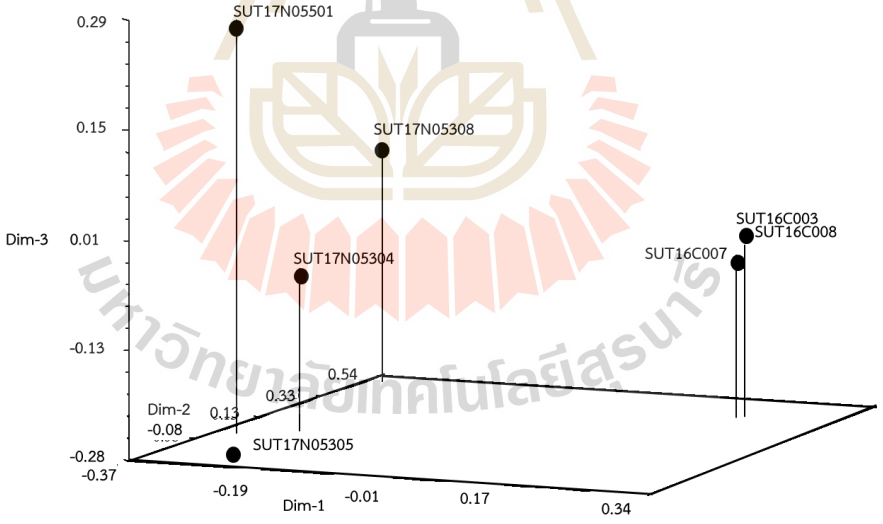


Figure 4.5 ISSR derived three-dimensional plot based on the first three principal coordinates from a principal coordinate analysis of black rot resistant mutants and nonmutagenized controls.

Table 4.5 Similarity matrix of black rot resistant mutant lines and nonmutagenized controls.

Mutant lines /controls	Nonmutagenized controls			Black rot resistant mutants			
	SUT16 C003	SUT16 C007	SUT16 C008	SUT17N0 5304	SUT17N0 5305	SUT17N0 5308	SUT17N0 5501
SUT16C003	1.000						
SUT16C007	0.961	1.000					
SUT16C008	0.990	0.971	1.000				
SUT17N5304	0.764	0.786	0.775	1.000			
SUT17N5305	0.796	0.800	0.807	0.864	1.000		
SUT17N5308	0.781	0.804	0.792	0.870	0.867	1.000	
SUT17N5501	0.722	0.764	0.734	0.898	0.845	0.867	1.000
Average	0.859	0.854	0.822	0.908	0.904	0.934	1.000

4.4 Characterization of protein changes following infection in black rot resistant mutants and nonmutagenized controls

Evaluation of total protein changes of black rot resistant mutants and nonmutagenized controls following the *P. palmivora* inoculation from 0-5 days was carried out. Leaves of nonmutagenized controls developed the very small localized lesions at the infection sites and hyphae were also found on leaves after 1 day of spore inoculation, while very small localized lesions were found on 4 black rot resistant mutants after 3 days. Lesion development on black rot resistant mutants were very slow and minimal symptoms were observed after 5 days and even after several days. By contrast, nonmutagenized controls changed to brown lesions (rot) with hyphae covered over the leaf surface and surrounding area after 5 days. The infected leaves were collected every day after inoculation and extracted to reveal their total proteins. At 0 day (uninoculation), it was found that black rot resistant mutant (SUT17N05501) had the significantly highest total proteins (18.89 µg/mg fresh weight) compared with nonmutagenized controls and other resistant mutants ($p<0.05$). While another black rot resistant mutant (SUT17N05305) had the lowest total protein (10.25 µg/mg fresh weight) but not significant from most resistant mutants and nonmutagenized controls (Table 4.6). The total proteins from 1 day to 5 days after inoculation were not significantly different from those observed on 0 day (uninoculation). However, all black rot resistant mutants and nonmutagenized controls tended to have the decrease in total proteins, suggesting that pathogen infection may influence total protein changes. These could be substantiated by Datta et al. (1999) who reported that decrease in protein content in susceptible cultivars may stem from degradation of the plant proteins by the proteolytic enzymes secreted by the virulent pathogens or may result from the changes

in the metabolic activity due to plant interactions. However, Koç et al. (2011) reported that total proteins in resistant pepper (*Capsicum annuum* L.) cultivars showing small lesions of disease were gradually increased from 2 to 6 days after *Phytophthora capsici* Leon inoculation while susceptible cultivars showing brownish lesions and rot after 6 days inoculation had lower total protein accumulation than resistant cultivars. These results suggest that total protein changes may be specific to plant pathogen interactions.

However, when considering protein profiles of these materials using SDS-PAGE analysis with calibrating protein markers (2-250 kDa), two clearly up-regulated protein bands of ca. 13 and 39 kDa MW were observed. One up-regulated protein band of 39 kDa, which was expressed in all black rot resistant mutants was detected in leaves at 3-5 days after inoculation. This protein band was also observed in all nonmutagenized controls but at 4-5 days after inoculation (Figure 4.6). In addition, 13 kDa protein was uniquely presented only in two of the black rot resistant mutants (SUT17N05304 and SUT17N05305), suggesting that the resistance mechanisms of four resistant mutants may differ (Figure 4.6). These up-regulated proteins may be related to PR-proteins. Plants have developed various mechanisms to defend themselves against pathogens which include the production of low molecular weight secondary metabolites, proteins and peptides having antifungal activities (Singh et al., 2014). These results are in agreement with previous studies, reporting the induction of β-1,3-glucanases which are referred to as “pathogenesis-related protein 2” (PR2) having molecular mass in the range of 33 to 44 kDa (Hong and Meng, 2004, Saikia et al., 2005). In addition, most chitinases having molecular mass in the range of 15 kDa to 43 kDa were also found when plants response to disease infection (Golshani et al., 2015). It is possible that the 13 kDa protein found in this study was chitinase and the 39 kDa protein may be

chitinase or β-1,3-glucanase, but further analysis was needed to verify this hypothesis. Van Sluyter et al. (2005) compared the chitinase activities in rust resistant Chardonnay and Cabernet Sauvignon euvitis with rust resistant *Vitis rotundifolia* cv. Fry. They found that Cabernet Sauvignon and Chardonnay extracts displayed an up-regulation of chitinase isoforms of similar molecular weights (39 and 26 kDa) compared with Fry. In addition, Chardonnay consistently expressed 36, 34, and 31 kDa isoforms, which were likely unique. Fry isoform appeared to be the same molecular weight (30 kDa) as the corresponding Cabernet Sauvignon isoform, but not the 31 kDa Chardonnay isoform. Changes of chitinase activities of two species of grape suggest that they have evolved different strategies for defense mechanisms both physically and biochemically. Jayasankar et al. (2000) reported that protein profiles from SDS-PAGE of resistant lines derived from embryogenic grapevine cultures had an up-regulated protein band about 36 kDa, which may be related to chitinase and it could not be identified in nonselected control. These resistant *Dendrobium* mutants will be further evaluated for black rot resistance at the greenhouse level and the alteration of their enzyme activities related to resistance mechanisms will also be analyzed. In addition, proteomic changes of these resistant mutants compared to nonmutagenized control will be studied to identify proteins that may be related to defense mechanisms. This knowledge will be highly beneficial for *Dendrobium* breeding to improve black rot resistance in the future.

Table 4.6 Total proteins of resistant mutant lines and controls following the *P. palmivora* inoculation after 0-5 days.

Lines	Total proteins following infection (µg/mg fresh weight)					
	0 day	**1 day**	**2 days**	**3 days**	**4 days**	**5 days**
SUT16C003	15.47 ± 1.15 b [/1]	13.54 ± 1.25	13.45 ± 0.75	8.78 ± 0.70	10.08 ± 2.88	9.01 ± 3.94
SUT16C007	12.34 ± 1.67 bc	16.19 ± 1.10	15.54 ± 1.33	9.77 ± 2.30	14.34 ± 2.20	9.91 ± 4.88
SUT16C008	12.74 ± 2.07 bc	10.80 ± 0.89	12.47 ± 1.40	9.20 ± 1.29	9.42 ± 1.93	9.37 ± 1.59
SUT17N05304	12.34 ± 2.24 bc	12.21 ± 0.77	11.68 ± 0.78	8.94 ± 2.18	9.01 ± 1.80	5.92 ± 0.51
SUT17N05305	10.25 ± 1.19 c	11.41 ± 1.39	8.79 ± 0.94	6.24 ± 1.20	8.10 ± 2.30	8.03 ± 0.14
SUT17N05308	12.49 ± 1.05 bc	15.34 ± 0.92	14.24 ± 1.51	10.84 ± 1.68	12.45 ± 1.80	6.90 ± 0.99
SUT17N05501	18.89 ± 3.13 a	15.59 ± 2.33	13.87 ± 3.13	12.90 ± 1.63	15.45 ± 4.28	14.48 ± 4.10
F-test	*	ns	ns	ns	ns	ns

[/1] = Data are presented as means ± SE. Different letters show statistically significant differences among the treatments (DMRT).
ns = Nonsignificant and * = significant (p<0.05)

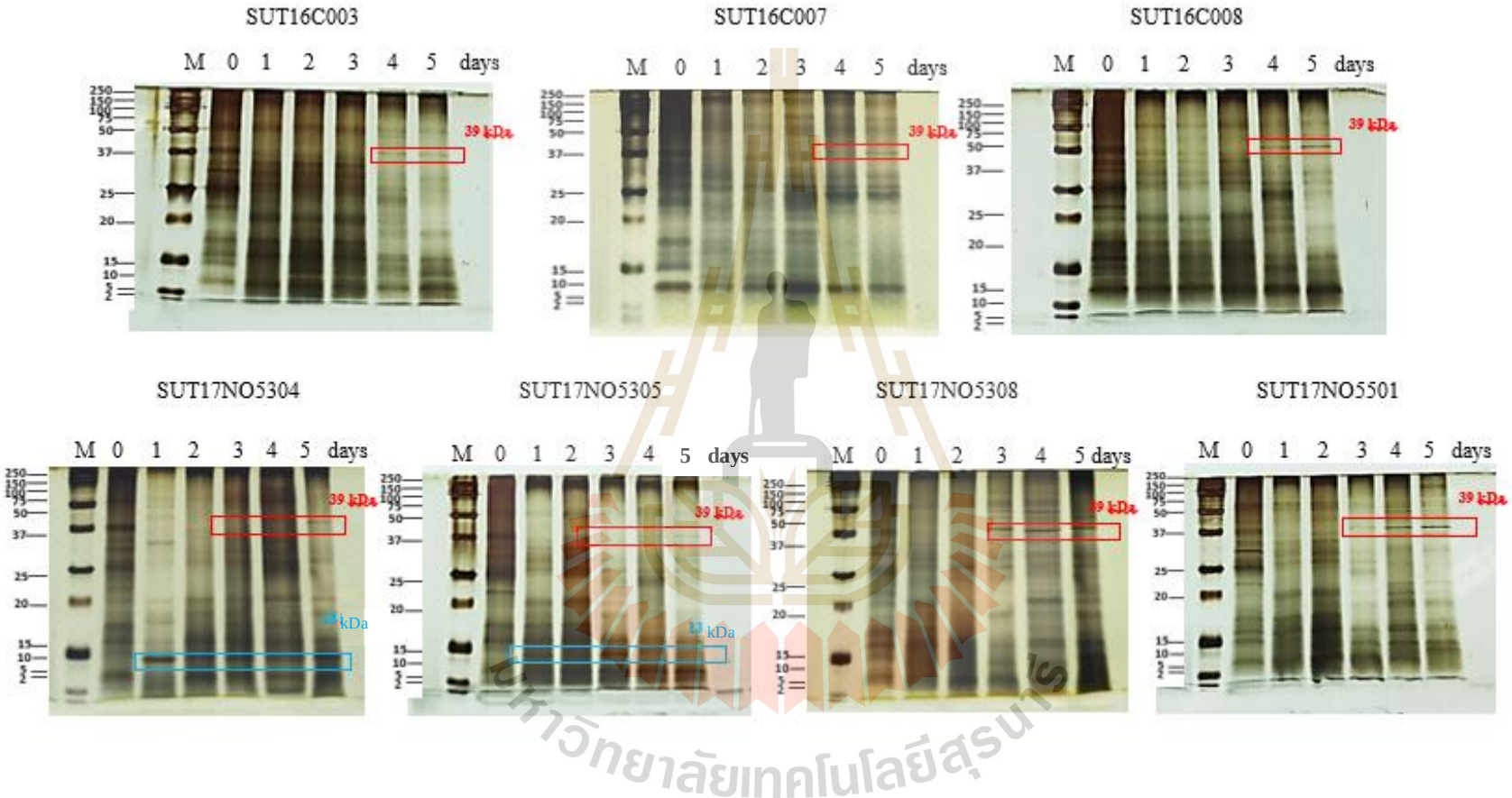


Figure 4.6 Protein changes of black rot resistant mutants and nonmutagenized controls at 0-5 days following the *P. palmivora* inoculation using SDS-PAGE.

4.5 Investigation of chromosomal variation of black rot resistant *D.* 'Earsakul' mutants using flow cytometry

The four black rot resistant mutants (SUT17N05304, SUT17N05305, SUT17N05308, and SUT17N05501) and three controls (SUT16C003, SUT16C007, and SUT16C008) were also investigated for their chromosome number, DNA content and genome size using flow cytometry. Flow cytometry analysis revealed that a peak at channel 200 (2n) was related to the G0/G1 of diploid plants, while the peaks at about 400 (4n) and 800 (8n) were tetraploid and octoploid, respectively. All black rot resistant mutants and nonmutagenezied controls possessed all peaks of 200, 400 and 800, indicating the presence of diploid, tetraploid and octoploid cells, called mixoploid (2n+4n+8n). However, their DNA content and genome size were significantly different (p<0.01). The mean DNA content and genome size of black rot resistant mutants (3.77 to 3.90pg $2C^{-1}$ and 3,640 to 3,764 Mbp) were significantly higher than those of nonmutagenized controls (3.41 to 3.49 pg $2C^{-1}$ and 3,344 to 3,389 Mbp. Among these, the black rot resistant mutants (SUT17N05305 and SUT17N05501) had the highest DNA content (3.90 pg $2C^{-1}$) and genome size (3,762 and 3,764 Mbp, respectively), but with no significant difference from the remaining black rot resistant mutants (SUT17N05304 and SUT17N05308) (Table 4.7 and Figure 4.7). These results are consistent with previous study of Jones et al (1998) who reported that nuclear DNA content of 26 orchids genera ranged from 1.53 to 4.23 pg $2C^{-1}$ nuclei for *D. cruentum* and *D. spectabile* using flow cytometry.

Flow cytometry can be a very useful method for rapid detection with high accuracy and can also be employed to detect structural chromosome changes and DNA content as well as ploidy level variation (Meesawat et al., 2008). It should be noted that

the genome size of these materials studied may depend on DNA content. This is consistent with the results of Meesawat et al. (2008) who reported that genome size of *D. crumenatum* was increased due to DNA content. The increasing/decreasing DNA content and genome size may result from multiple point mutation occurring in DNA or RNA in which nucleotide bases are added or changed or duplication/deletion of chromosome. In addition, the mutation may cause cell division abnormalities in the phase of cell cycle, especially in the G1/G0 phase, which result in higher DNA content and genome size in mutants than nonmutagenized plants (Jones et al., 1998). Yenchon and Te-chato (2014) reported that this method could characterize the polyploidy induction of *D. formosum* by colchicine at different concentrations (0, 0.05, 0.10, 0.15, and 0.20%) for 24 or 48 h. The results showed that treatment with 0.20% colchicine for 48 h gave the highest tetraploid plantlets (75%). Chromosome observation revealed that tetraploid plants had chromosome number of 2n=4x=76 whereas diploid plants had 2n=2x=38. This evidence was also confirmed by flow cytometry. Choopeng et al (2019) also used this method to study the effect of colchicine on survival rate and ploidy level of hydrid between *D. santana* and *D. friedericksianum* orchid. They found that plants treated with 0.05-0.1% colchicine for 24-72 h had more than 50% increase in DNA content compared with control plants.

The results obtained from this study confirmed the usefulness of flow cytometry as an efficient method for screening DNA content and genome size in black rot resistant mutants and nonmutagenized controls.

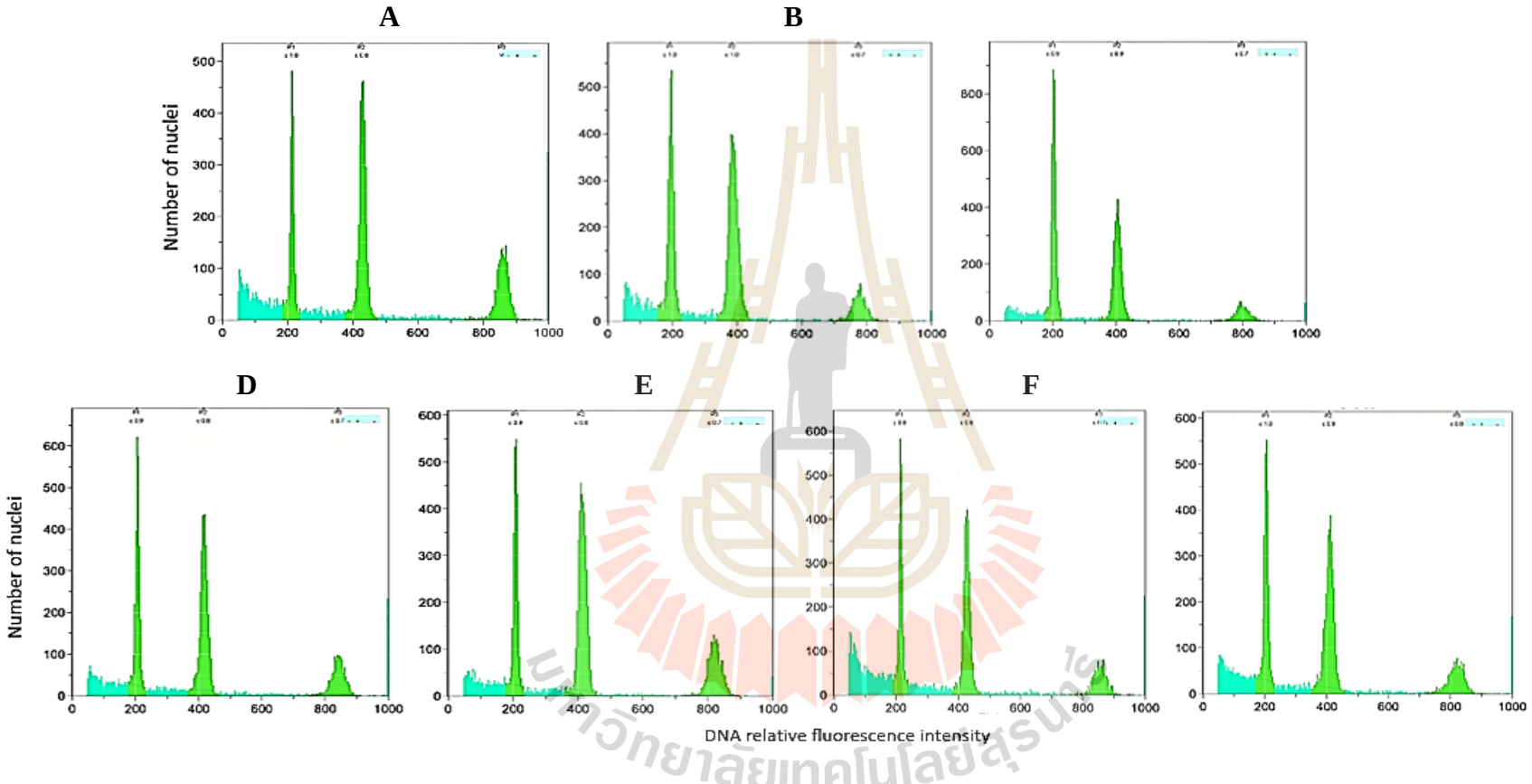


Figure 4.7 Flow cytometry histogram of nonmutagenized controls (A-C) and resistant mutants (D-G). A) SUT16C003 B) SUT16C007 C) SUT16C008 D) SUT17N05304 E) SUT17N05305 F) SUT17N05308 G) SUT17N05501.

Table 4.7 DNA content and genome size with standard error in picograms and mega base pairs for black rot resistant mutants and nonmutagenized controls.

Lines	DNA content (pg)	Genome size (Mbp)[/2]
SUT16C003	3.41 ± 0.17 b[/1]	3,389 b
SUT16C007	3.47 ± 0.01 b	3,344 b
SUT16C008	3.49 ± 0.07 b	3,372 b
SUT17N05304	3.77 ± 0.04 a	3,640 a
SUT17N05305	3.90 ± 0.04 a	3,762 a
SUT17N05308	3.79 ± 0.05 a	3,660 a
SUT17N05501	3.90 ± 0.02 a	3,764 a
F-test	**	**

[/1] Data are presented as means. Means in the same column with different letters are significantly different among lines (DMRT) at $p<0.05$.
[/2] Megabase pairs per haploid genome (Mbp 1 C^{-1} nuclei) calculated based on the equivalent of 1 pg = 965 Mbp (Meesawat et al., 2008)
** = Highly significant ($p<0.01$)

4.6 Characterization of morphological traits of black rot resistant mutants and nonmutagenized controls

Morphological analysis was performed with 4 black rot resistant mutants and 3 nonmutagenized controls. All plants were evaluated based on various parameters e.g., plant characteristics (plant height, numbers of nodes, and node length), leaf characteristics (numbers of leaves, leaf length and width), and root characteristics (numbers of roots and root length). These following morphological parameters were measured at 6 months after culture. The results revealed that most parameters including length of nodes, number of leaves, length of leaves, length of roots, and number of roots were highly significantly different ($p<0.01$), except number of nodes and width of leaves which showed significant difference ($p<0.05$). While plant height was not significantly different among all plants (Table 4.8 and Figure 4.8).

Plant height: All resistant mutants and nonmutagenized controls were not significantly different in plant height. Plant height varied from 2.60 cm (SUT16C003) to 3.53 cm (SUT17N05308). However, the resistant mutant SUT17N05308 (3.53 cm) tended to have the highest plant height, followed by SUT16C007 (3.30 cm). While SUT16C003 showed the lowest plant height (2.60 cm) when compared with the remaining lines. Although resistant mutants had significantly higher average number of nodes, they had lower average length of nodes compared with nonmutagenized controls, thereby resulting in the same height.

Number of nodes: All resistant mutants had higher number of nodes which varied from 3.8 nodes (SUT17N05304) to 4.30 nodes (SUT17N05501). SUT17N05501 showed the highest number of nodes (4.30 nodes), followed by SUT17N05308 (4.00 nodes). However, number of nodes of other resistant mutants (SUT17N05304 and

SUT17N05305) were not significantly different from all nonmutagenized controls. The nonmutagenized control SUT16C003 had the lowest number of nodes (1.90 nodes).

Length of node: SUT17C007 showed the highest length of node (0.42 cm) but not significantly different from SUT16C003 (0.31 cm) and SUT16C008 (0.33 cm). While resistant mutant SUT17N05501 had the lowest node length (0.21 cm.) but not significantly different with other resistant mutants, SUT17N05305 (0.24 cm), SUT17N05304 (0.26 cm), and SUT17N05308 (0.28 cm). Three of these (SUT17N05304, SUT17N05305, and SUT17N05308) showed no significant difference in length of nodes from SUT16C003 and SUT16C008.

Number of leaves: SUT17N05305 showed the highest number of leaves (6.20) but not significantly different from SUT17N05308 (5.90) and SUT17N05304 (5.70). However, their numbers of leaves were significantly higher than SUT17N05501 and all nonmutagenized controls. The lowest number of leaves was revealed in SUT16C003 (3.20 leaves).

Length of leaves: the highest length of leaves was found in all of the nonmutagenized controls (SUT16C008 (1.11 cm), followed by SUT16C007 (0.94 cm) and SUT16C003 (0.93 cm)). However, these were not significantly different from SUT17N05304 (0.88 cm) and SUT17N05308 (0.86 cm). By contrast, SUT17N05305 and SUT17N05501 had significantly shorter leaf length than those of nonmutagenized controls.

Width of leaves: SUT17N05501 showed the highest width of leaves (0.49 cm), but it was not significantly different from those of other resistant mutants and all nonmutagenized controls. In addition, all of the resistant mutants had visibly thicker leaves which were darker green than nonmutagenized controls.

Number of roots: SUT17N05305 had the highest number of roots (6.10 roots), which was significantly higher than those of others. It was followed by SUT17N05308 (4.30 roots) and SUT17N05304 (3.70 roots) but not significantly different from SUT16C007 (3.10 roots). While SUT16C003 had the lowest number of roots (2.30 roots) but not significantly different from SUT16C007 (3.10 roots), SUT16C008 (2.40 roots) and SUT17N05501 (2.70 roots).

Length of roots: SUT17N05304 had maximal mean root length (1.23 cm) followed by SUT16C007 (1.13) and SUT17N05501 (1.05 cm). By contrast, SUT16C003, SUT16C008 and SUT17N05308 had significantly shorter roots than other lines (0.36, 0.41, and 0.48 cm, respectively).

When the results were taken together, it should be noted that the resistant mutants had different morphological traits compared with the nonmutagenized controls including number of nodes (SUT17N05308 and SUT17N05501), number of leaves (SUT17N05304, SUT17N05305, and SUT17N05308) and number of roots (SUT17N05305). While nonmutagenized controls had longer leaf length than resistant mutants except SUT17N05304 and SUT17N05308. These results, demonstrate that NaN_3 is an effective mutagen that can induce genetic alteration through point mutation, especially transitions on other chromosomal changes, resulting in the changes of amino acid arrangement that may change protein function and/or morphological characters.

These results are consistent with Ilbas et al. (2005) who reported the effects of different concentrations of NaN_3 (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mmol/L) and different treatment time (3 and 4 h) on the morphological and cytogenetic characteristics of barley (*Hordeum vulgare* L.) seedlings. It was found that the increase in concentrations of NaN_3 affected germination rates, reduced length of the roots and leaves, as well as

coleoptile length. Wannajindaporn et al. (2016) used 0.1 mM (LD_{30}) and 0.5 mM (LD_{50}) NaN_3 to induce mutation in *D.* 'Earsakul'. The results revealed that 0.1 mM and 0.5 mM of NaN_3 contributed to an increase in morphological variability e.g., reduced plant height and node length, the higher number of node, induced shorter and thicker as well as visibly thicker and darker green leaves. Srivastava and chetri, (2015) studied the effects of NaN_3 on the plant growth and yield characteristics in wheat (*Triticum aestivum* L. em. Thell.). It was found that NaN_3 reduced root and shoot length as well as seed germination percentages.

Alteration of morphological traits may result from genetic changes as a result of mutation and/or physiological changes induced by NaN_3. The morphological alterations of these mutants will also be evaluated at the flowering stage and evaluated for black rot resistance levels at the greenhouse level in the future.

Table 4.8 Morphological characterization of resistant mutants and nonmutagenized controls at 6 months**.**

Lines	Morphological characters[/1]							
	Height (cm)	**Number of nodes**	**Length of nodes (cm)**	**Number of leaves**	**Length of leaves (cm)**	**Width of leaves (cm)**	**Number of roots**	**Length of roots (cm)**
SUT16C003	2.60 ± 0.12	1.90 ± 0.18 b[/1]	0.31 ± 0.03 ab	3.20 ± 0.25 c	0.93 ± 0.07 a	0.46 ± 0.03 a	2.30 ± 0.58 c	0.36 ± 0.10 c
SUT16C007	3.30 ± 0.22	2.90 ± 0.46 b	0.42 ± 0.05 a	3.90 ± 0.46 bc	0.94 ± 0.13 a	0.48 ± 0.05 a	3.10 ± 0.35 bc	1.13 ± 0.20 a
SUT16C008	3.01 ± 0.13	3.30 ± 0.65 b	0.33 ± 0.04 ab	4.00 ± 0.47 bc	1.11 ± 0.10 a	0.46 ± 0.03 a	2.40 ± 0.73 c	0.41 ± 0.10 c
SUT17N05304	2.98 ± 0.12	3.80 ± 0.47 ab	0.26 ± 0.02 bc	5.70 ± 0.52 a	0.88 ± 0.07 ab	0.45 ± 0.03 ab	3.70 ± 0.60 bc	1.23 ± 0.17 a
SUT17N05305	2.90 ± 0.18	3.90 ± 0.41 ab	0.24 ± 0.03 bc	6.20 ± 0.53 a	0.74 ± 0.04 b	0.46 ± 0.03 a	6.10 ± 0.95 a	0.87 ± 0.18 b
SUT17N05308	3.53 ± 0.35	4.00 ± 0.73 a	0.28 ± 0.01 bc	5.90 ± 0.84 a	0.86 ± 0.05 ab	0.44 ± 0.03 ab	4.30 ± 0.92 b	0.48 ± 0.10 c
SUT17N05501	3.02 ± 0.50	4.30 ± 0.90 a	0.21 ± 0.02 c	5.10 ± 0.86 bc	0.78 ± 0.03 b	0.49 ± 0.04 a	2.70 ± 0.76 c	1.05 ± 0.59 ab
F-test	ns	*	**	**	**	*	**	**

[/1] Data are presented as means. Means in the same column with different letters are significantly different among lines (DMRT) at $p<0.05$.
ns = Nonsignificant, * = significant ($p<0.05$), and ** = highly significant ($p<0.01$)

Figure 4.8 Morphological traits of resistant mutants and nonmutagenized controls at 6 months.

CHAPTER V
CONCLUSION

In Thailand, *Dendrobium* is a cut-flower that is important to commercial production but its micropropagation is limited due to slow growth. Our results suggest that the culture medium VW0 supplemented with 1 mg/L BAP was the most appropriate culture medium for stimulation of *D.* 'Earsakul' plantlet growth in vitro. Black rot caused by *P. palmivora* is a serious destructive disease that severely cause yield reduction. Therefore, breeding for new orchid varieties with resistance to black rot can be helpful. Our study evaluated the response to black rot in 35 *D.* 'Earsakul' mutants induced by NaN_3 followed by in vitro selection with *P. palmivora* culture filtrate compared with nonmutagenized controls using *P. palmivora* isolates NK-53-9 through detached leaf assay. It was found that two *D.* 'Earsakul' mutants, SUT17N05304 and SUT17N05308 were potentially highly resistant to *P. palmivora* isolate NK-53-9. In addition, SUT17N05305 and SUT17N05501 were identified to be potentially resistant. In addition, using 16 ISSR markers, these four resistant mutants (SUT17N05304, SUT17N05305, SUT17N05308, and SUT17N05501) were genetically different from non-mutagenized controls (SUT16C003, SUT16C007, and SUT16C008) with genetic dissimilarity of 0.186 GD. While protein profiles of these black rot resistant mutants and nonmutagenized controls following the *P. palmivora* inoculation from 0-5 days revealed two clearly up-regulated protein bands of ca. 13 and 39 kDa MW. One up-regulated protein of 39 kDa, which was expressed in all black rot resistant mutants was detected in leaves at 3-5 days after inoculation, while this protein was observed in all

nonmutagenized controls at 4-5 days after inoculation. In addition, a protein with molecular weight of 13 kDa was uniquely induced only in two of the black rot resistant mutants (SUT17N05304 and SUT17N05305), suggesting that the resistance mechanisms of four resistant mutants may differ. These induced proteins may be related to PR-proteins. Flow cytometry showed that all black rot resistant mutants and nonmutagenized controls were identified to have diploid, tetraploid and octaploid cells, called mixoploid (2n+4n+8n). In addition, the DNA content and genome size of black rot resistant mutants (3.77 to 3.90 pg $2C^{-1}$ and 3,640 to 3,764 Mbp) were higher than those of nonmutagenized controls (3.41 to 3.49 pg $2C^{-1}$ and 3,344 to 3,389 Mbp). Morphological characterization of black rot resistant mutants and nonmutagenized control demonstrated that the resistant mutants differed in several morphological traits compared with the nonmutagenized controls including number of nodes, number of leaves and number of roots.

This study demonstrates that NaN_3 is a powerful mutagen which may cause point mutation or deletions in the nucleotide sequences of gene structure. It can change the sequences of promoter region that may lead to modified gene expression. In addition, duplication may also occur and results in an increase in the amount of DNA content of black rot resistant mutants. It is expected that the recently developed resistant mutants induced by NaN_3 will be beneficial for the future development of *D.* 'Earsakul' with black rot resistance.

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BIOGRAPHY

Ms. Sasitorn Hualsawat was born on February 25, 1994 at Surin, Thailand. She graduated with the Bachelor of Science Degree Program in Crop Production Technology, Suranaree University of Technology in 2016. Then, in the same year, she decided to further study for master degree in School of Crop Production Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. During her study, she received scholarship from external grants and scholarships for graduate students of research and development of institute of research and development SUT to support her tuition and fee. Her research topic was characterization of sodium azide induced black rot resistant *D.* 'Earsakul' mutants under supervision of Professor Dr. Piyada Alisha Tantasawat. The results from some part of this study have been presented in the 5th International Conference on Agricultural and Biological Sciences (ABS 2019), July 22-25, 2019, Macau, China (Poster presentation in "Identification of suitable culture media for stimulation of *D.* 'Earsakul' growth).