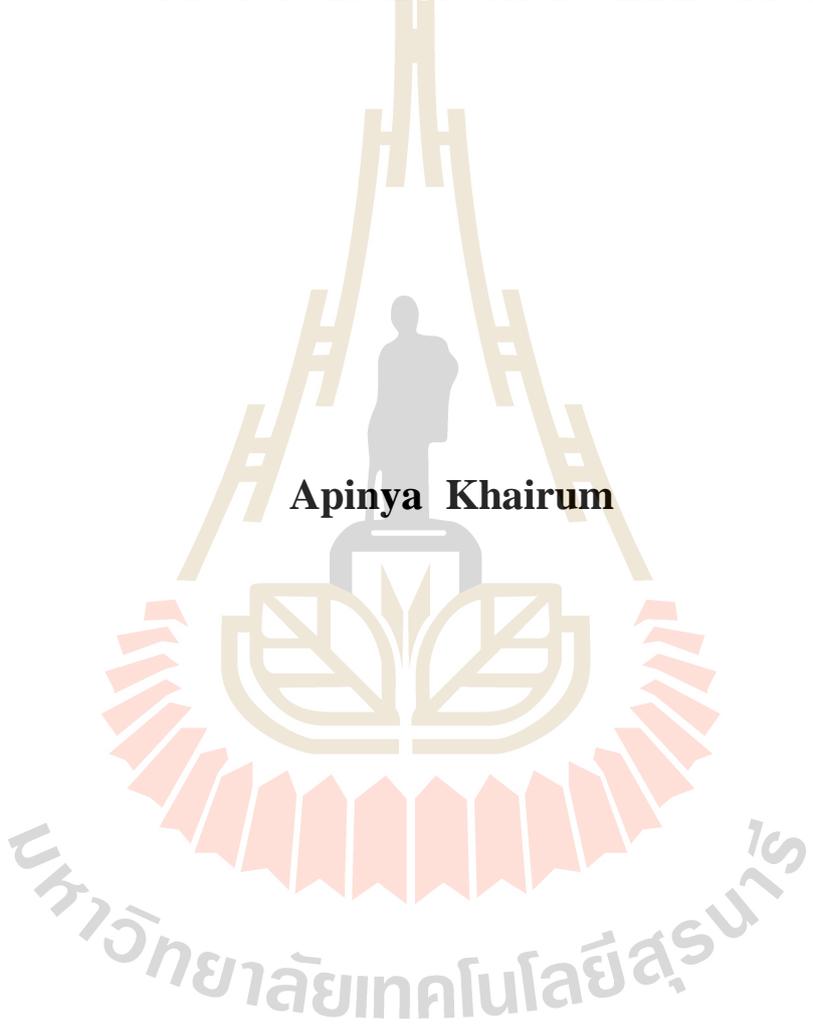# BREEDING FOR BLACK ROT RESISTANCE IN

Dendrobium 'Earsakul' VIA *IN VITRO*

MUTAGENESIS AND SELECTION

Apinya Khairum

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy in Crop Science

Suranaree University of Technology

Academic Year 2020

การปรับปรุงพันธุ์กล้วยไม้สกุลหวายพันธุ์เอียสกุลด้วยวิธีก่อกลายพันธุ์
และคัดเลือกในหลอดทดลองเพื่อให้ต้านทานโรคเน่าดำ

นางสาวอภิญญา ไขรัมย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

สาขาวิชาพืชศาสตร์

มหาวิทยาลัยเทคโนโลยีสุรนารี

ปีการศึกษา 2563

BREEDING FOR BLACK ROT RESISTANCE IN *Dendrobium*

'Earsakul' VIA *IN VITRO* MUTAGENESIS

AND SELECTION

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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อภิญญา ไขรัมย์ : การปรับปรุงพันธุ์กล้วยไม้สกุลหวายพันธุ์เอียสกุลด้วยวิธีก่อกลาย-พันธุ์และคัดเลือกในหลอดทดลองเพื่อให้ต้านทานโรคเน่าดำ (BREEDING FOR BLACK ROT RESISTANCE IN *Dendrobium* 'Earsakul' VIA *IN VITRO* MUTAGENESIS AND SELECTION) อาจารย์ที่ปรึกษา : ศาสตราจารย์ ดร.ปิยะดา อลิฌาณ์ ตันตสวัสดิ์, 185 หน้า.

การพัฒนากล้วยไม้สกุลหวายพันธุ์เอียสกุล (*Dendrobium* 'Earsakul') ที่มีความต้านทานต่อ โรคเน่าดำ มีความสำคัญต่อการผลิตกล้วยไม้แบบยั่งยืน งานวิจัยนี้มีวัตถุประสงค์เพื่อ (1) ประเมิน ลักษณะการเจริญเติบโตและการสร้างสปอร์ของเชื้อ *Phytophthora palmivora* บนอาหารเลี้ยงเชื้อที่ แตกต่างกัน และคัดเลือกไอโซเลตของเชื้อ *P. palmivora* ที่มีความรุนแรงในการก่อโรคสูงสุด เพื่อ นำไปใช้สำหรับการประเมินระดับความต้านทานโรคเบื้องต้นของกล้วยไม้สายพันธุ์กลายที่คาดว่า ต้านทานโรค (2) ปรับปรุงพันธุ์กล้วยไม้สกุลหวายพันธุ์เอียสกุลให้ต้านทานโรคเน่าดำด้วยวิธีก่อ-กลายพันธุ์และคัดเลือกในหลอดทดลอง (3) ประเมินระดับความต้านทานโรคเน่าดำของกล้วยไม้ สายพันธุ์กลายและสายพันธุ์ที่ไม่ได้ผ่านการก่อกลายพันธุ์ โดยใช้วิธีใบตัด (detached leaf assay) (4) ยืนยันการเปลี่ยนแปลงทางพันธุกรรมของสายพันธุ์กลายที่ต้านทานโรค โดยใช้การวิเคราะห์ด้วย เครื่องหมาย ISSR (5) ตรวจสอบปริมาณดีเอ็นเอและขนาดจีโนมโดยใช้เทคนิค flow cytometry และ ประเมินลักษณะทางสัณฐานวิทยาของต้นสายพันธุ์กลายที่ต้านทานโรคเน่าดำเปรียบเทียบกับต้นที่ ไม่ได้ผ่านการก่อกลายพันธุ์ และ (6) ศึกษาลักษณะการเปลี่ยนแปลงของรูปแบบโปรตีนรวมทั้ง กิจกรรมของเอนไซม์ β-1,3-glucanase และ chitinase ในต้นสายพันธุ์กลายและต้นที่ไม่ได้ผ่านการ ก่อกลายพันธุ์ ก่อนและหลังการเข้าทำลายของเชื้อ *P. palmivora* ผลการทดลอง พบว่า สูตรอาหาร เลี้ยงเชื้อที่ดีที่สุดสำหรับการเจริญของเส้นใยบนผิวอาหารของเชื้อ *P. palmivora* คือ pea sucrose agar (PSA) และ V8 agar (V8A) ในขณะที่สูตรอาหารที่พัฒนาใหม่ corn meal potato agar (CMPA) กระตุ้นสปอร์ของเชื้อ *P. palmivora* ได้เร็วกว่าสูตรอาหารอื่น ๆ จากการทดสอบความรุนแรงในการ ก่อโรคของเชื้อ *P. palmivora* ซึ่งถูกแยกเป็นสปอร์เดี่ยว ๆ จากจังหวัดนครราชสีมา พบว่า เชื้อ *P. palmivora* ไอโซเลต NK-53-9 มีความรุนแรงในการก่อโรคสูงสุด ซึ่งสามารถนำมาใช้ในการ ประเมินระดับความต้านทานโรคเน่าดำ หลังจากการคัดเลือกเพื่อต้านทานโรคเน่าดำในหลอด ทดลองจำนวน 3 รอบ ได้สายพันธุ์กลายที่มีศักยภาพในการต้านทานโรคจำนวน 50 สายพันธุ์ (22 สายพันธุ์ ได้จากการก่อกลายพันธุ์ด้วยสาร ethyl methanesulfonate (EMS) ระดับความเข้มข้น 1.4% (LD_{30}) และ 28 สายพันธุ์ ได้จาก 1.8% EMS (LD_{50})) หลังจากประเมินระดับความต้านทานโรคเน่า ดำของกล้วยไม้สายพันธุ์กลายและสายพันธุ์ที่ไม่ได้ผ่านการก่อกลายพันธุ์ โดยใช้วิธีใบตัดแล้ว พบว่า ได้สายพันธุ์กลายที่มีความต้านทานต่อโรคเน่าดำจำนวน 13 สายพันธุ์ ประกอบด้วยสายพันธุ์-

กลายที่มีระดับความต้านทานสูงจำนวน 4 สายพันธุ์ และต้านทานจำนวน 9 สายพันธุ์ ต้นสายพันธุ์-กลายเหล่านี้มีพันธุกรรมแตกต่างจากต้นที่ไม่ได้ผ่านการก่อกลายพันธุ์จากการวิเคราะห์ด้วยเครื่องหมาย ISSR สายพันธุ์กลายเหล่านี้และสายพันธุ์ที่ไม่ได้ผ่านการก่อกลายพันธุ์ มีจำนวนโครโมโซมเท่ากัน คือ 2n+4n+8n และหนึ่งในสายพันธุ์กลาย SUT17E18316 มีปริมาณดีเอ็นเอและขนาดจีโนมสูงสุด สายพันธุ์กลายที่ต้านทานโรคส่วนใหญ่มีลักษณะทางสัณฐานวิทยาบางลักษณะแตกต่างจากต้นที่ไม่ได้ผ่านการก่อกลายพันธุ์ โดยเฉพาะสายพันธุ์กลาย SUT13E18305 ซึ่งมีลักษณะที่โดดเด่นและอาจนำไปใช้ประโยชน์ทางการค้าได้ในอนาคต การเข้าทำลายของเชื้อ *P. palmivora* กระตุ้นให้กิจกรรมของเอนไซม์ β-1,3-glucanase และ chitinase เพิ่มขึ้นในสายพันธุ์กลายบางสาย-พันธุ์และสายพันธุ์ที่ไม่ได้ผ่านการก่อกลายพันธุ์ โดยเฉพาะสายพันธุ์ต้านทานสูง SUT13E18301 ที่แสดงการกระตุ้นสูงสุด SDS-PAGE พบแถบโปรตีนขนาด 15, 16, 39 และ 54 kDa ที่แสดงออกเพิ่มขึ้นเฉพาะในต้นสายพันธุ์กลายที่ต้านทานโรคเน่าดำบางสายพันธุ์ในระหว่างการเข้าทำลายของเชื้อ *P. palmivora* ซึ่งอาจนำมาใช้เป็นเครื่องหมายโปรตีนสำหรับกลไกการต้านทานโรคเน่าดำในกล้วยไม้ นอกจากนี้การทดสอบในระดับทั้งต้น (whole plant assay) พบว่า สายพันธุ์ต้านทานสูง SUT13E18305 แสดงการกระตุ้นเอนไซม์ β-1,3-glucanase และ chitinase ทั้งแบบเฉพาะที่และทั่วทั้งต้นสูงกว่าต้นสายพันธุ์กลายที่ต้านทานโรคเน่าดำอื่น ๆ และต้นที่ไม่ได้ผ่านการก่อกลายพันธุ์ แสดงถึงศักยภาพในการต้านทานต่อโรคเน่าดำ และ/หรือโรคอื่น ๆ ในการเข้าทำลายครั้งต่อมา ผลการศึกษาเหล่านี้มีประโยชน์ต่อการปรับปรุงพันธุ์กล้วยไม้เพื่อให้ต้านทานโรคเน่าดำ และสายพันธุ์-กลายบางสายพันธุ์ที่ได้จากการวิจัยครั้งนี้อาจนำไปใช้ประโยชน์ในการผลิตกล้วยไม้เชิงพาณิชย์หรือใช้เป็นแหล่งความต้านทานโรคเน่าดำใหม่ในอนาคต

สาขาวิชาเทคโนโลยีการผลิตพืช

ปีการศึกษา 2563

ลายมือชื่อนักศึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษา_____

APINYA KHAIRUM : BREEDING FOR BLACK ROT RESISTANCE IN

Dendrobium 'Earsakul' VIA *IN VITRO* MUTAGENESIS AND SELECTION.

THESIS ADVISOR : PROF. PIYADA ALISHA TANTASAWAT, Ph.D. 185 PP.

BLACK ROT/DENDROBIUM/ETHYL METHANSULFONATE SELECTION

The development of *Dendrobium* 'Earsakul' with improved resistance to black rot is essential for sustainable orchid production. The objectives of this research were to (1) evaluate different culture media for *Phytophthora palmivora* growth and sporulation, and select the most virulent *P. palmivora* isolate for preliminary evaluation of resistance levels of the potentially resistant lines in *D.* 'Earsakul', (2) breed *D.* 'Earsakul' for black rot resistance via *in vitro* mutagenesis and selection, (3) evaluate black rot resistance levels in *D.* 'Earsakul' mutants and non-mutagenized controls using detached leaf assay, (4) verify genetic changes of putative resistant mutants using ISSR analysis, (5) investigate DNA content and genome size using flow cytometry and evaluate morphological traits of black rot resistant mutants compared to non-mutagenized controls, and (6) characterize changes in protein profiles as well as β-1,3-glucanase and chitinase activities before and following *P. palmivora* infection in black rot resistant mutants and non-mutagenized controls. The results revealed that the best culture media for surface mycelial growth of *P. palmivora* were pea sucrose agar (PSA) and V8 agar (V8A), while corn meal potato agar (CMPA; a newly developed medium) induced sporulation earlier than other media. From pathogenicity test among single-spore *P. palmivora* isolates from Nakhon Ratchasima, it was found that NK-53-9 is the most virulent single-spore *P. palmivora* isolate which can be used for screening of black rot resistance. Fifty putative resistant mutants (22 putative mutants from 1.4% ethyl methanesulfonate (EMS) (LD_{30}) and 28 putative mutants from 1.8% EMS (LD_{50})) were obtained after 3 cycles of *in vitro*

selection for black rot resistance. Thirteen putative black rot resistant mutants, including four highly resistant and nine resistant putative mutants were identified after evaluation of black rot resistance levels using detached leaf assay. All of these black rot resistant putative mutants were genetically different from the non-mutagenized controls based on ISSR analysis. These *D.* 'Earsakul' mutants and non-mutagenized controls had the same chromosome number of 2n+4n+8n, and one of them SUT17E18316 had maximum DNA content and genome size. Most of the black rot resistant mutants were morphologically different on some characters from the non-mutagenized controls, particularly SUT13E18305 which possessed outstanding characters and may be useful for future commercialization. The infection of *P. palmivora* induced activities of both β-1,3-glucanase and chitinase activities in some mutants and non-mutagenized controls, particularly, a highly resistant mutant SUT13E18301 which exhibited the highest induction. Unique up-regulated protein bands of 15, 16, 39 and 54 kDa were identified by SDS-PAGE in some black rot resistant mutants during *P. palmivora* infection and may be used as protein markers for black rot resistance mechanisms in orchids. Moreover, whole plant assay demonstrated that SUT13E18305 had higher local and systemic β-1,3-glucanase and chitinase induction than other black rot resistant mutants and non-mutagenized control, suggesting its potential resistance against black rot and/or other diseases in subsequent infection. These findings are useful for breeding programs for black rot resistance in orchids. Some mutants obtained from this study may also be useful for commercial orchid production or used as new black rot resistance resource in the future.

School of Crop production Technology   Student's Signature _Apinya Khairum_

Academic Year 2020   Advisor's Signature _Piyada Alisha Tantasawat_

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Apinya Khairum

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LIST OF ABBREVIATIONS

BSA   =   Bovine serum albumin

CMPA   =   Corn meal potato agar

CRD   =   Completely randomized design

DMRT   =   Duncan's new multiple range test

DNS   =   Dinitro salicylic acid

EMS   =   Ethyl methansulfonate

ISSR   =   Inter-simple sequence repeat

LD   =   Lethal dose

PDA   =   Potato dextrose agar

PSA   =   Pea sucrose agar

PSB   =   Pea sucrose broth

PLBs   =   Protocorm-like bodies

SDS-PAGE   =   Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis

V8A   =   V8 juice agar

VW1   =   Vacin and Went 1

CHAPTER I

INTRODUCTION

1.1 Rationale of the study

Orchids (Orchidaceae) are one of the flowering plant species, which are important for Thailand's economy due to their attractive flowers with colorful patterns that last longer. They are widely used for decoration and some of them can also be consumed as herbal medicines and food (Gutierrez, 2010). Nowadays, Thailand is one of the major tropical orchid exporters in the world. *Dendrobium*, *Mokara* and *Oncidium*, are the main species that are exported to other countries. Among these, *Dendrobium* is the most popular genus that accounted for approximately 80% of the total commercial production with the export values of 84.75 and 55.87 million US $ in 2019 and 2020, respectively (The Government Public Relations Department, 2018; Office of Agricultural Economic, 2021; Lerthiran, 2021). However, the global outbreak of COVID-19 is closing down national borders as countries around the world including the trading partners fight to contain the outbreak of this coronavirus, decreasing the export values of orchids around the world in 2020. But it was found that the export values of cut-flower and potted orchids in Thailand increased 2 and 5-folds, respectively from the ASEAN market, especially Vietnam and Myanmar (The Nation Thailand, 2021). However, the quantity and values of cut-flower and potted orchid exportation during 2016-2020 fluctuated (Office of Agricultural Economic, 2020). Hence, it appeared that many problems were encountered in orchid production

and commercialization, including competitive countries, labour management, and production factors such as water quality, transportation, and pests, especially diseases (Lerthiran, 2021).

Black rot or Phytophthora rot is caused by oomycete fungus *Phytophthora palmivora* Butl. It has been reported to cause diseases in various genera e.g., *Cattleya, Aerides, Ascocenda, Oncidium*, *Phalaenopsis*, and *Dendrobium* (Farr et al., 1989; Simone and Burnett, 1995; Erwin, and Ribeiro, 1996; Orlikowski and Szkuta, 2006; Cating et al., 2010). The symptoms of the disease are found on leaves as wet brown spots that turn to black and fall, respectively. This pathogen can spread throughout the rhizomes. Eventually, it can cause rapid plant death. It is widely found during the rainy season or in high humidity conditions (Aekarat, 2008; Cating et al., 2010). The application of fungicides is an easy and effective approach for orchid disease control, however, it is still limited by cost (Sritongin et al., 2017), and human health and the environment may also be harmed by these chemicals. Hence, new black rot resistant orchid varieties are highly desirable solution to these problems. The virulent isolates of *P. palmivora* are necessary to act as a selective agent for black rot resistance in a breeding program. However, its short-lived zoospores and sporangium (Narula and Meherotera, 1984) lead to the lack of success in isolating and culturing it in artificial medium. This problem makes it difficult to have an accurate diagnosis and conduct artificial inoculation using its reproductive structures. It was found that sporangia and mycelia play an important role in inoculation and development of infection (Palomar et al., 1999). Several media have been used to isolate and culture *Phytopthora* species e.g., potato dextrose agar (PDA), V8 agar (V8A) and pea sucrose agar (PSA) (Palomar et al., 1999; Jeffer, 2006; Savita et al., 2011), however, the effects of media are found

sufficient spores for selection process, it is necessary to aseptically culture the pathogen on suitable culture media supplemented with optimized essential elements.

Breeding for disease resistance in orchids through mutation and *in vitro* selection is a powerful and valuable approach that may be more efficient and practical than conventional breeding and genetic engineering/gene editing (Hammerschlag et al., 1995; Brar and Jain, 1998; Predieri, 2001). Their advantages include simple and rapid procedure, high rate of mutation, and cost-effective for *in vitro* clonal propagation of mutants (Alikamanoğlu, 2002). While conventional breeding may take a longer time to develop new resistance sources because no clear disease resistance source was found naturally. Meanwhile, genetic engineering/gene editing is costly and is not acceptable for commercialization in many countries including Thailand. It is well known that the mutation can be induced by physical mutagens (ionizing and non-ionizing agents such as gamma-rays (γ), X-rays (X), ultraviolet (UV) light and fast or thermal neutrons) and chemical mutagens (alkylating agents, acridine dyes, base analogues and others). The most frequen- tly used chemical mutagens include ethyl methanesulfonate (EMS), sodium azide (NaN$_3$), ethyl nitroso urea (ENU), and colchicine (Kodym and Afza, 2003; Kumar et al., 2015). EMS is an effective alkylating agent and commonly used for inducing point mutation in DNA. It produces transitions and transversions, resulting in gene mutations and chromosome aberrations, which may change or abolish protein functions, leading to morphological variation and varied nuclear DNA contents (van Harten, 1998; Khatri et al., 2005). As a result, it has been applied to improve new varieties with high yield or desirable traits i.e., producing phenotypic variation, disease resistance and salt tolerance in several plants (Krieg, 1963; Rao, 1977; Yudhvir, 1995;

Greene et al., 2003; Kodym and Afza, 2003; Kim et al., 2006; Till et al., 2007; Muangsorn and Te-chato, 2008; Lethin et al., 2020). Therefore, new alleles for disease resistance including black rot resistance may be acquired by *in vitro* EMS-induced mutation in orchids.

In vitro selection for disease resistance has been performed by extensive studies of about 30 plant species and 40 plant diseases (reviewed in Švabová and Lebeda, 2005). The cell-free culture filtrate (CF) or pure toxins and phytotoxin of the pathogens could be used as a selective agent for the selection of disease resistant plants, especially CF that can be easily incorporated into the culture media at appropriate concentrations (El-kazzaz et al., 2009; Dehgahi and Joniyas, 2016; Sayed et al., 2016; Kumar et al., 2017). For orchids, Dehgahi et al. (2016) selected *Fusarium proliferatum*-tolerant PLBs and assessed the effects of different concentrations of *F. proliferatum* CF (5-20%) *in vitro*. It was found that 2.7-folds increase in peroxidase (POD) activity observed in CF-treated PLBs as compared to untreated one. Furthermore, they also found that disease symptoms in all CF-treated leaflets were reduced compared to the control under the assessment of disease development. Similarly, EMS-derived mutants in the banana (*Musa paradisiaca* L.) cv. Puttable acquired from *in vitro* selection in MS medium supplemented with *F. oxysporum* f.sp. *cubense* (FOC) CFs (5-15%) showed a significant increase in some oxidative enzymes and pathogenesis-related (PR) proteins, reaching higher levels than control (Krishna et al., 2013).

Plants have evolved constitutive and inducible defense mechanisms against pathogen invasion. Constitutive defenses include thick cell walls, waxy epidermal cuticles, bark, and many other preformed barriers to inhibit the invasion of pathogens. Plant cells also detect the pathogen invasion and respond with inducible defenses

including production of toxic chemicals and pathogen-degrading enzymes, and deliberate cell suicide (Freeman and Beattie, 2008; Gupta et al., 2013). Systemic acquired resistance (SAR) is induced by most pathogens in distal uninfected plant tissues, leading to accumulation of salicylic acid (SA) and pathogenesis-related (PR) proteins (e.g., PR1, chitinase, glucanase), which then provides long-lasting and broad-spectrum resistance. Thus, SAR is a highly desirable mechanism of resistance in plants (Choudhary et al., 2007). PR proteins like ß-1,3-glucanase and chitinase are commonly encoded by multigenic families and have various functions in plants, including plant growth and development, wounding and defense responses. They were strongly induced when plants respond to infection by several pathogens (Leubner-Metzger et al., 1999; Neuhaus, 1999; van Loon, 1999; Wu and Bradford, 2003). Induced ß-1,3-glucanase and chitinase activities after fungal infection had been reported in lemon seedlings (Fanta et al., 2003). Similarly, Münch-Garthoff et al. (1997) reported that beta-1,3-glucanase and chitinase transcripts in resistant wheat were accumulated before the pathogen infects leaves through stomata. Consequently, infection led to induction of signaling between host plant and pathogen to form defense through activation of genes in the distal tissues approximately 16 hours before induction of hypersensitive response (HR).

Since *P. palmivora*'s cell walls compose mostly of β-glucan (ca. 90% dry weight) and small amount of chitin ($<1\%$), they can be degraded by various ß-glucanases and chitinases (Tokunaga and Bartnicki-Garcia, 1971; Hamid et al., 2013; Mélida et al., 2013). Anguelova-Merhar et al. (2001) reported that the constitutively expressed chitinase activity and induced β-1,3-glucanase activity were highly accumulated in leaf rust (*Puccinia recondita* f. sp. *tritici*) resistant wheat. Similarly,

Żur et al. (2013) found that resistant winter triticale showed higher chitinase activity than susceptible cultivar after *Microdochium nivale* infection. Moreover, the induction of glucanase activity which was increased in seaweed extract (SWE)-treated leaves could inhibit the growth of *P. palmivora* by hydrolyzing β-glucan in the cell wall (Khompatara et al., 2019). This chitinase and β-1,3-glucanase may play important roles in resistance mechanisms against *P. palmivora*.

Characterization of resistant mutants can be undertaken through several methods such as morphological, cytological and molecular marker analysis. Morphological characterization is based on visually direct observation of different characteristics in new mutant plants, including plant growth and architecture (i.e., flowering, maturity, shape, size), disease and pest resistance, and yield. These desirable characteristics observed are important for orchid production. Changes in morphological characteristics have been reported in mutants i.e., increased height, larger leaves, thicker roots, higher numbers of nodes, reduced node length, and three types of chimeras (sectorial, mericlinal and periclinal) (Muangsorn and Te-chato, 2008; Samala et al., 2014). However, this method may be insufficient because it is influenced by the plant growth stages and various environmental factors. Cytological evaluation based on flow cytometry has been used to estimate variability in quantitative traits as well as genome level and changes in nuclear and cytoplasmic organelle DNA (Larkin, 1998). It was also found that nuclear DNA content could be increased from 33 to 50% in colchicine treated orchid (mutants) when compared with non-mutagenized controls (Choopeng et al., 2019; Mohammadi et al., 2021). Thus, cytological analysis based on flow cytometry investigation is also useful for required to characterization of *Dendrobium* mutants.

In addition, molecular breeding techniques can be used to assist mutant identification. In orchids, several DNA markers e. g. , amplified fragment length polymorphism (AFLP), random amplified polymorphism DNA (RAPD), and inter simple sequence repeat (ISSR) have been used to characterize mutants and somaclonal variants (Xiang et al., 2003; Barakat et al., 2010; Khosravi et al., 2009). ISSR markers which are randomly distributed throughout the genome provide a highly informative multi- loci and discriminating information with good reproducibility (Thormann et al. , 1994; Zietkiewicz et al. , 1994; Karp et al. , 1997; Meudt and Clarke, 2007; Agarwal et al. , 2008; Costa et al. , 2016). ISSR markers have been used in various orchids (Parab et al., 2008; Qian et al, 2014). In *Dendrobium* 'Earsakul', Wannajindaporn et al. (2016) reported that ISSR markers are effective for determining genetic variation of *D.* 'Earsakul' mutants derived from *in vitro* mutagenesis using NaN_3, and are useful for characterization of putative black rot resistant *D.* 'Earsakul' mutants. Recently, Hualsawat (2019) also successfully identified genetic differences in black rot resistant *D.* 'Earsakul' mutants compared with non- mutagenized control by means of ISSR analysis. These findings demonstrate the evidence of deletion or duplication at ISSR primer binding sites during mutation and suggest the effectiveness of ISSR markers for revealing a number of mutants with limited genetic changes.

Biochemical/protein markers include isozymes, allozymes and other proteins (Haq et al., 2016). Protein profiling of germplasm have been widely and effectively used to propose the taxonomic and evolutionary aspects in several crops. This protein marker was successfully used to assess genetic diversity/ protein profile in rice (Dhawale et al., 2015), brassica (Jan et al., 2017), and fenugreek (Qadir et al., 2017) using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

It was also used to specify ca. 20-44 and 13-43 kDa bands of β-1,3-glucanase and chitinase, respectively, which were induced in several plants upon pathogen infection (Jayasankar et al., 2000; El-Hadary and Tayel, 2013; Pareek et al., 2014; Sinha et al., 2014). Moreover, it could identify fusarium wilt (*F. oxysporum*) resistant genotypes in faba bean (Mahmoud and Abd El-Fatah, 2020). Thus, the biochemical/protein markers may be used to identify black rot resistant mutants. These protein profiles can be used to characterize various putative black rot *D.* 'Earsakul' mutants. It is possible that some *D.* 'Earsakul' mutants which have high levels of PR proteins, β-1,3-glucanase and chitinase, may be able to inhibit the invasion of *P. palmivora*, the causal pathogen of black rot in orchids. Hypothetically, based on gene and chromosomal mutations, new resistance alleles that do not exist in nature may be acquired. In addition, other unique and distinguished characteristics (e.g., color, shape, flower size, inflorescence characteristics, height, leaf characteristics, growth) may also be found. These black rot resistant orchid mutants obtained from this study are highly desirable and may also help maintain commercial competitiveness with other countries. These findings are useful for commercial orchid production in the future.

1.2 The objectives

    1.2.1   To evaluate different culture media for *P. palmivora* growth and sporulation, and to select the most virulent *P. palmivora* isolate for preliminary evaluation of resistance levels of the potentially resistant lines in *D.* 'Earsakul'.

    1.2.2   To breed *D.* 'Earsakul' for black rot resistance via *in vitro* mutagenesis.

    1.2.3   To evaluate black rot resistance levels in *D.* 'Earsakul' putative mutants

and non-mutagenized controls using detached leaf assay in laboratory.

1.2.4 To verify genetic changes of putative resistant mutants using ISSR analysis.

1.2.5 To investigate DNA content and genome size using flow cytometry and morphological traits of black rot resistant mutants, compared to non-mutagenized controls.

1.2.6 To characterize the protein changes before and following *P. palmivora* infection in black rot resistant mutants and non-mutagenized controls.

1.3 Research hypotheses

1.3.1 Suitable culture media could be used to promote *P. palmivora* growth and sporulation.

1.3.2 The most virulent *P. palmivora* isolate could be used for preliminary screening of black rot resistance levels in *D.* 'Earsakul' mutants and non-mutagenized controls.

1.3.3 EMS chemical mutagen may induce chromosome breaks and other aberrations or generate new alleles involved with exotic features, including black rot resistance.

1.3.4 Putative resistant mutants acquired by *in vitro* selection using *P. palmivora* CFs will have higher black rot resistance levels at the laboratory and greenhouse levels.

1.3.5 The occurrence of genetic changes can be verified by ISSR analysis.

1.3.6 Black rot resistant mutants may have higher levels of PR proteins such as β-1,3 glucanase and chitinase than controls.

1.4   Scopes of research

1.4.1   *P. palmivora* isolates were cultured on five different culture media and were assessed for their growth and sporulation. Pathogenicity of five *P. palmivora* isolates were evaluated using detached leaf assay.

1.4.2   *D.* 'Earsakul' PLBs were mutagenized by EMS, which can induce random mutations in the genome, and then were *in vitro* selected for black rot resistance using *P. palmivora* CFs.

1.4.3   Putative *D.* 'Earsakul' mutants were evaluated for black rot resistance levels using detached leaf assay in laboratory.

1.4.4   Genetic changes of putative black rot resistant mutants were verified using ISSR analysis.

1.4.5   Black rot resistant mutants and controls were evaluated for their DNA content and genome size using flow cytometry as well as morphological changes.

1.4.6   Black rot resistant mutants and non- mutagenized controls were characterized for their biochemical changes following *P. palmivora* infection by evaluating protein patterns as well as β- 1,3- glucanase and chitinase activity levels.

1.5   References

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CHAPTER II

LITERATURE REVIEWS

2.1   General overview of *Dendrobium* orchids

Thailand is known as the 'Land of orchids'. Orchid consists of ca. 1,154 species and 168 genera. *Dendrobium* is one of the largest genera in the world, consisting of more than 1,100 species (Puchooa, 2004; Sittichattham, 2007). *D.* 'Earsakul' or *D.* Sonia is the mutant clone of Jo Daeng cultivar (*D.* Sonia 'Jo Daeng'), which possesses superior floral characters (shape, size, especially dark color petal), and provides higher price than the original clone (Piluek and Wongpiyasatid, nd).

2.2   Economic status of orchids

Dendrobium is accounted for approximately 80% of total orchid export from Thailand. The commercial production is widely used for decoration. In addition, it can also be consumed as herbal medicines and food (Gutierrez, 2010). Thailand is the major producer of tropical orchids which are exported to Japan, United States, Netherlands, China, India, Italy and ASEAN countries. The recorded incomes from 2015 to 2020 are shown in Table 2.1 (Office of Agricultural Economics, 2021). The largest orchid production area in Thailand is Nakhon Pathom, Samut Sakhon, Kanchanaburi, Nonthaburi and Ratchaburi provinces. Total harvested area and yield tended to decline from 2015 to 2020 as shown in Table 2.2 (Lerthiran, 2021; Office of Agricultural Economics, 2021). However, there still have been reductions in orchid production

largely due to high cost of production. The major constraints may stem from various production factors such as water quality, transportation, and pests. In addition, lack of genetic variability or absence of suitable varieties are also included.

Table 2.1 Export values of cut-flower and potted orchids from Thailand during 2015-2020.

	Cut-flower		Potted- orchid	
Year	Quantity (ton)	Export value (million US $)	Quantity (ton)	Export values (million US $)
2015	24,649.12	66.73	33,727.67	20.34
2016	25,880.16	73.95	30,165.47	18.58
2017	24,209.01	71.25	28,932.58	18.06
2018	23,716.96	73.30	32,773.03	17.18
2019	23,086.96	69.25	31,880.68	15.66
2020	21,871.53	43.97	19,526.90	11.91

Office of Agricultural Economics (2021)

Table 2.2 Harvested area, yield, and yield/rai of cut-flower in Thailand in 2015-2020.

Year	Harvested area (rai)	Yield (ton)	Yield per rai (kilogram; kg)
2015	22,285	50,030	2,245
2016	21,760	47,166	2,171
2017	21,334	47,137	2,285
2018	21,631	48,147	2,226
2019	21,521	48,794	2,267
2020	20,674	38,805	1,877

Office of Agricultural Economics (2021)

2.3 Diseases in orchid

Thailand is located in the tropical region where there is more rainfall and high humidity that encourages disease development and outbreak. There are many different diseases affecting orchid production i.e., flower rusty spot (*Curvularia eragrostidis*), yellow leaf spot (*Pseudocercospora dendrobii*), leaf spot (*Phyllostictina pyriformis*), anthracnose (*Collectotrichum* sp.), fusarium wilt (*Fusarium oxysporum*), soft rot (*Pseudomonas gladioli*), sooty mold (*Cladosporium* sp.), mosaic (Tobacco Mosaic Virus Orchid Strain: TMV-O and Cymbidium Mosaic Virus (*CyMV*) and black rot or Phytophthora rot.

Among these, black rot caused by oomycete fungus *Phytophthora palmivora* Butl. (Online 1) has been reported to be one of the destructive diseases in various orchid genera e.g., *Cattleya*, *Aerides*, *Ascocenda*, *Oncidium*, *Phalaenopsis*, including *Dendrobium* (Farr et al., 1989; Simone and Burnett, 1995; Erwin and Ribeiro, 1996; Orlikowski and Szkuta, 2006; Cating et al., 2010). The symptoms of the disease are found on leaves with wet brown spots that turn to black and fall, respectively. This pathogen can spread throughout the rhizomes. Eventually, it can cause rapid plant death. It is widely found during the rainy season or in high humidity conditions (Aekarat, 2008; Cating et al., 2010). The application of fungicides is an easy and effective approach for orchid disease control, however, it is still limited by cost (Sritongin et al., 2017), and human health and the environment may also be harmed by the chemicals. Therefore, new black rot resistant orchids cultivars are highly desirable solution to these problems.

The conditions such as temperature, humidity, nutrient preferences etc. are major abiotic factors impacting all levels of biological functions of plant pathogens. This can strongly affect epidemic development of the pathogen life cycle such as sporangia germination, zoospore, cyst formation and germ tube elongation (Mariette et

al., 2016). For *Phytophthora species,* both sporangia and zoospores are relatively short lived in infected leaf tissue. The pathogen seems to have a poor competitive saprophytic ability. In general, there is lack of success in isolating and growing *Phytophthora* sp. in an artificial medium (Narula and Meherotera, 1984; Padmaja et al., 2015). This problem makes it difficult to have an accurate diagnosis and conduct artificial inoculation using its reproductive structures of which its sporangia and mycelia play an important role in inoculation and development of infection (Palomar et al., 1999). The germination of sporangia and mycelia on an agar medium through artificial media with supplemented essential elements and compounds needed for their growth and other metabolic processes is necessary (Padmaja et al., 2015). V8 agar and broth are widely used as Jeffer (2006) revealed their effectiveness for *Phytophthora* and *Pythium* species. Similarly, V8A was also the best medium for growth and reproduction of *P. colocasiae* compared to potato dextrose agar (PDA) (Palomar et al., 1999). Likewise, Khairum et al. (2016) reported that V8A and pea sucrose agar (PSA) could induce the maximum mycelial growth of *P. palmivora* isolates from Nakhon Ratchasima. Moreover, corn meal potato agar (CMPA), a newly developed medium, could promote the earliest sporulation of *P. palmivora* isolates. Khairum et al. (2016) and Appiah et al. (2003) also revealed that growth characteristics of mycelium also depended on the types of media. Rosette, radiate, irregular, stellate, and cottony patterns were exhibited after *Phytophthora* species were cultured on different media.

2.4   Breeding in orchids

New orchid varieties with outstanding characteristics, exotic and higher qualities, especially disease resistance are helpful for commercial production. Several approaches can be used for orchid breeding; 1) conventional breeding, 2) genetic engineering/ gene editing, 3) molecular breeding and 4) mutation breeding. The information of these methods is given below.

2.4.1 Conventional breeding

Conventional breeding is the method which involves traditional hybridization to transmit useful traits into commercial varieties, and subsequent detection of novel varieties. The oldest natural hybrids of *P. intermedia* derived from crossing between *P. aphrodite* and *P. rosea* were first described in 1853, whereas the first artificial orchid hybrid *Calanthe* was recorded by Dominy in 1856. This artificial orchid hybrid was obtained from crossing between *C. masuca* and *C. furcata* (de Chandra et al., 2019). Disease resistant hybrids of orchids are generally created by a traditional crossing of different parents that possess disease resistant traits. Although this method is commonly used in orchids because it is easy, low-cost and convenient, a long life cycle and low germination rate in nature of orchids are the limiting factors for the propagation and improvement via conventional breeding. It may also be difficult to improve disease resistance in orchids by conventional breeding because no clear disease resistance source was found naturally, and it may take a long time (2-13 years) to develop new resistance sources (Teoh, 1986; Arditti, 1992; Kostenyuk et al., 1999; Kishor et al., 2006; Sim et al., 2007; Tang and Chen, 2007).

2.4.2 Genetic engineering/gene editing

Genetic engineering and tissue culture techniques are useful tools for introducing specific genes into plants. As a result, various desirable characteristics in plants are improved such as improved protein, the new color of flowers and insect and disease

resistance. In orchids, the first report on genetic transformation was made by Kuehnle and Sugii (1992). For disease resistance, *Phalaenopsis* PLBs were engineered with *CymMV* coat protein cDNA (CP) and was able to express dual (viral and bacterial) disease resistance traits and to exhibit enhanced resistance to *CymMV* and *E. carotovora* (Chan et al., 2005). Recently, genome editing (also called gene editing) is a group of technologies that give scientists opportunity to edit an organism's DNA, allowing genetic materials to be added, removed or altered at particular locations in the genome. A recent approach is known as CRISPR-Cas9, which is abbreviation for clustering regularly interspaced short palindromic repeats and CRISPR-associated protein 9. It is faster, cheaper, more accurate and efficient than other existing genome editing methods (Genetics Home Reference, 2020). CRISPR/Cas has been used to generate multiple mutants of *P. equestris* in *MADS* genes that encode DNA-binding proteins (Tong et al., 2019) which are highly expressed in floral organs and may be important for flower initiation and development (Lin et al., 2016). Although these methods are highly effective technologies that allow genetic changes without undesirable traits (Genetics Home Reference, 2020), they are costly and not acceptable for commercialization in many countries including Thailand.

2.4.3 Molecular plant breeding

Molecular markers which are regarded as steady landmarks play an important role in modern plant breeding, especially when they are tightly linked to any gene of interest. They have long been used by plant breeders to indirectly select promising plants or evaluate genetic variability of germplasms (Moose and Mumm, 2008; Randhawa et al., 2013; Grover and Sharma, 2016). Next-generation sequencing (NGS) technology is a recently advanced technique to generate abundant low-cost molecular markers through whole genome sequencing, and making them more useful for plant breeding

programs (Varshney et al., 2009; Metzker, 2010; Varshney et al., 2014). Marker-assisted selection (MAS) is an indirect selection process to select desirable traits (e.g. productivity, disease resistance, abiotic stress tolerance, and quality) based on any markers detecting the polymorphism or DNA/RNA variation among each genotype (Ribaut and Hoisington, 1998). Wu et al. (2017) used simple sequence repeat (SSR) to identify desirable genes responsible for flower color, flower shape, and resistance in *Phalaenopsis*, providing an important reference for genetic transformation of *Phalaenopsis* and the Orchidaceae.

2.4.4 Mutation breeding

Generally mutation includes spontaneous and induced mutations, and is well suited for breeding in orchids because many orchid species can be propagated easily, facilitating the production of mutant plants (Yamaguchi, 2018). The advantages of mutation include a high mutation rate, effective improvement of individual traits and shortening the breeding cycle. Therefore, this method has long been used to produce new orchids with unique phenotypic traits, higher content of medicinal ingredients and greater adaptability and resistance (Toker et al., 2007; de Chandra et al., 2019). Currently, polyploids from mutation breeding have been successfully obtained in many orchid species including *Cymbidium, Dendrobium, Oncidium* and *Phalaenopsis* (Li and An, 2009; Cui et al., 2010; Cheng, 2011; Wang et al., 2011, Zhang et al., 2011). For example, the tetraploid plants were obtained from colchicine-treated *Cymbidium* hybrid, which showed thicker leaves, roots, and rhizomes, a deeper stem color, and a slower growth rate (Yin et al., 2010). Moreover, the increased content of total polysaccharides, flavonoids, alkaloids and other major secondary metabolites were found in UV-B-irradiated *D. officinale* seedlings (Chen et al., 2020).

2.5   Causes and effects of mutation

Spontaneous mutations can occur due to replication errors or as a consequence of lesions introduced into DNA during normal cell growth at a relative low rate (Griffiths et al., 2000). While an artificially induced mutation causes a higher rate of mutation which is typically induced by physical mutagens (ionizing and non-ionizing agents such as gamma-rays (γ), X-rays (X), ultraviolet (UV) light and fast or thermal neutrons) and chemical mutagens (alkylating agents, acridine dyes, base analogues and others) including ethyl methanesulfonate (EMS), sodium azide (NaN$_3$), ethyl nitroso urea (ENU) and colchicine (Kodym and Afza, 2003; Kumar et al., 2015). These mutagens generally cause large-scale deletions, inversions or translocations of chromosomes, or generate point mutations (a type of mutation that causes a single change, insertion or deletion of the genetic material) in the DNA.

2.5.1   Chromosomal mutation

2.5.1.1 Structural aberrations usually occur during any errors in cell division. When the chromosome's structure is altered, it can be classified into four forms; deletions (the loss of genetic material), inversions (a chromosomal break rejoints to the correct chromosome but in an incorrect orientation), duplications (an extra copy of a region (or regions) in the DNA is produced) and translocations (a piece of one chromosome breaks off and attaches to the wrong chromosome) (Figure 2.1) (Elliott, 2018).
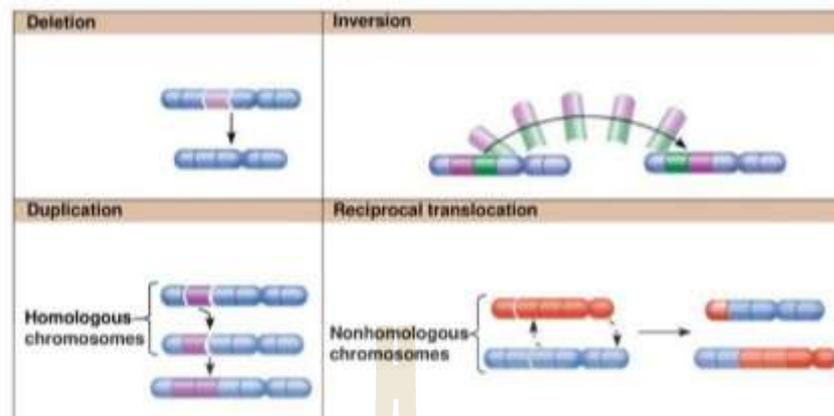
Figure 2.1   Changes in chromosome structure (Elliott, 2018)

          2.5.1.2 Numerical aberrations are caused by the alterations in the number of chromosomes in a cell. The change in the number of whole chromosomes is called heteroploidy, which produces phenotypic changes. Aneuploidy is a type of mutation that changes parts of a chromosome set, resulting in either the loss of one or more chromosomes or the addition of chromosomes, which is generally based on the number of chromosomes added or deleted. The aneuploidy variations are monosomic $(2n-1)$, trisomic $(2n+1)$, nullisomic $(2n-2)$, and disomic $(n+1)$. Euploidy a chromosomal variation that involves the entire set of chromosomes in a cell or an organism, which may be a single set or haploid/monoploidy (n) and two sets or diploid (2n). Meanwhile, polyploidy mutation is the consisting of more than two homologous chromosome sets, which has many levels such as triploidy $(2n=3x)$, tetraploidy $(2n=4x)$, hexaploidy $(2n=6x)$ and octaploidy $(2n=8x)$ (Figure 2.2) (Panawala, 2017; Montazerinezhad et al., 2020; BioExplorer, 2021)
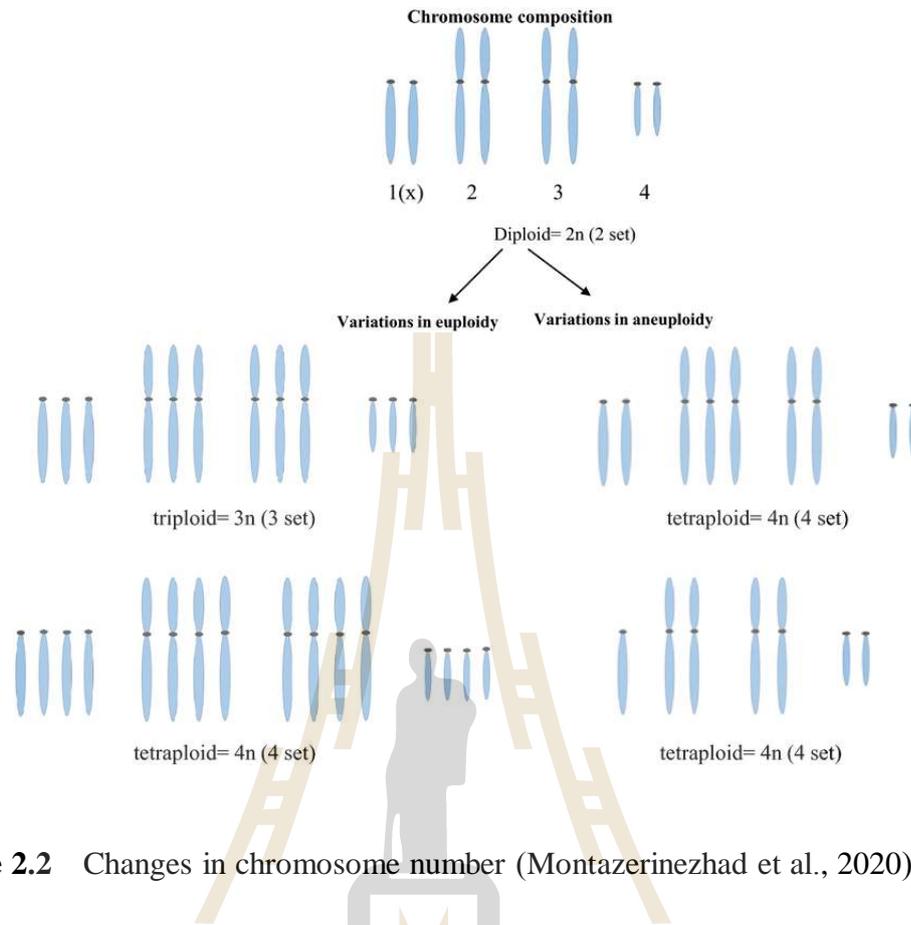
Figure 2.2   Changes in chromosome number (Montazerinezhad et al., 2020)

2.5.2   Gene mutation

2.5.2.1 Point mutation (based on the substitution of the base pairs), which involves alteration in a single base pair, and small deletions generally affect the function of only one gene. There are two types of point mutations: transition mutations and transversion mutations. Transitions are replacement of a purine base with another purine or replacement of a pyrimidine with another pyrimidine. Transversions are replacement of a purine with a pyrimidine or vice versa. Transition mutations are about ten times more common than transversions. Thus, an example of a transition mutation is a GC base pair that replaces a wild type (or naturally occurring) AT base pair. By contrast, transversion mutations occur when a purine base substitutes for a pyrimidine. A wild-type peptide sequence and the mRNA and DNA encoding are shown at the top

base, or vice versa, for example, when a TA or CG pair replaces the wild type AT pair and altered nucleotides and amino acid residues are also shown. Silent mutation is the change of single nucleotide base, but does not affect the amino acid sequence. Missense mutations lead to a change in a single amino acid in the encoded protein. Nonsense mutation is a nucleotide base change leading to the formation of a stop codon. This results in premature termination of translation, thereby generating a truncated protein (Figure 2.3) (Online 2).
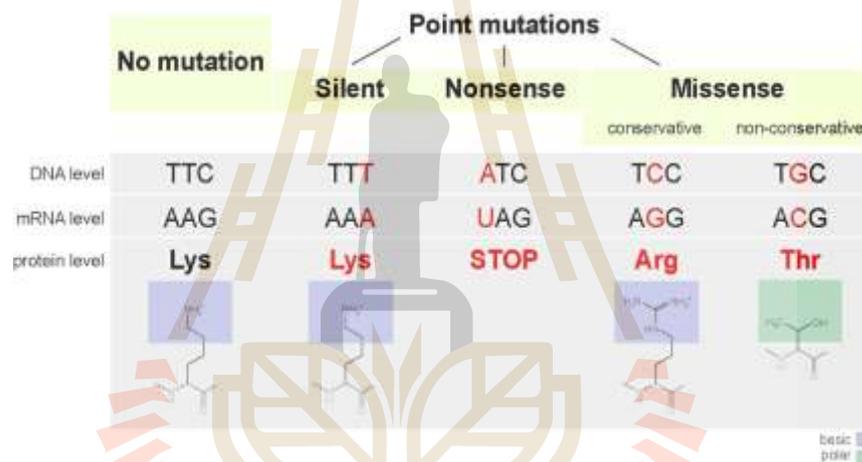
Figure 2.3 Types of point mutations to a codon (Online 2)

2.5.2.2 Frameshift mutations involve the addition or deletion of any number of nucleotides, causing a change in the reading frame. Consequently, completely unrelated amino acid residues are incorporated into the protein prior to encounter a stop codon (Figure 2.4) (Sapkota, 2021).
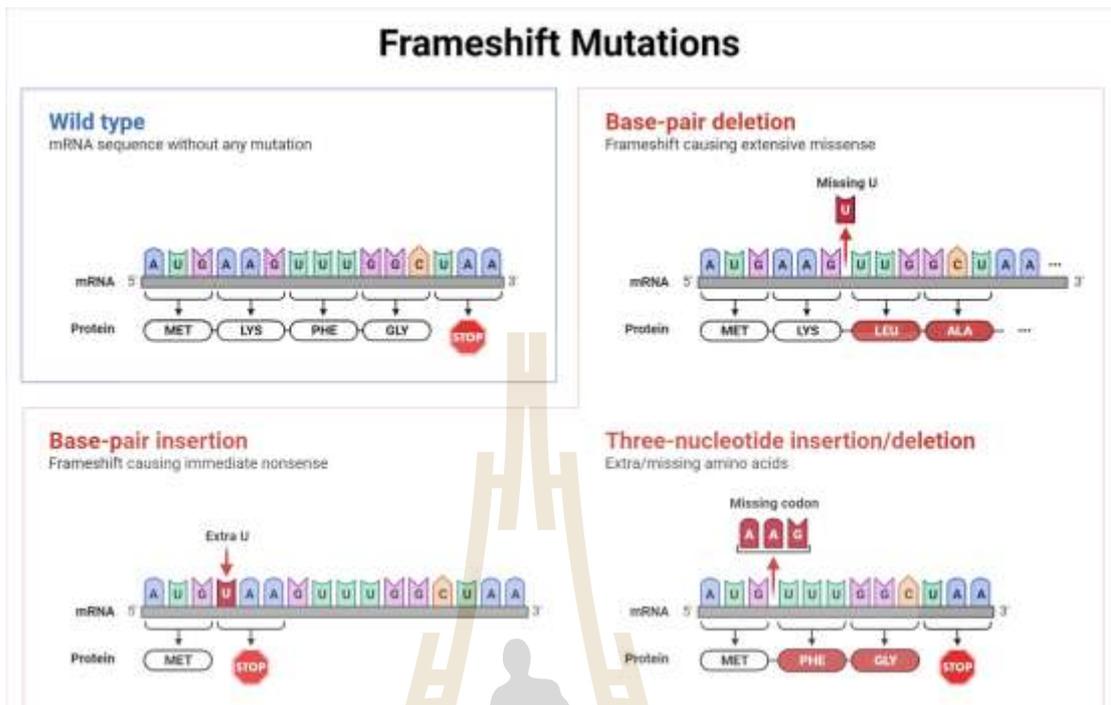
Figure 2.4   Frameshift mutations (Sapkota, 2021)**.**

2.6   EMS as effective mutagen for mutagenesis

Although there are a large number of chemical mutagens, only a small number has been used in plants. Alkylating agents has found large application in plant mutagenesis and breeding. These agents generate over 80% of the registered new mutant plant varieties reported in the IAEA database (http://mvgs.iaea.org/Search. aspx). Of these, three compounds are reported to be significant: EMS, 1-methyl-1-nitrosourea (MNU) and 1-ethyl-1-nitrosourea (ENU) which account for 64% of these varieties (Figure 2.5). EMS is an effective alkylating agent and commonly used as a chemical mutagen for inducing point mutation in DNA, which produces 99% transitions and 1% transversions (Krieg, 1963; Greene et al., 2003; Kodym and Afza, 2003; Kim et al., 2006; Till et al., 2007). EMS alkylating guanine at the oxygen on position 6 and

the O-4 position of thymine can lead to direct mispairing with thymine and guanine, respectively. The principal mutations detected are GC → AT transitions, indicating that the O-6 alkylation of guanine is most relevant to mutagenesis (Figure 2.6). It typically causes high frequency of gene mutations and low frequency of chromosome aberrations. The loss of a chromosome segment or deletion has also been reported in many plants, resulting in amino acid changes, which may change or abolish protein functions, leading to morphological variation and varied nuclear DNA contents (van Harten, 1998; Khatri et al., 2005). The frequencies of EMS induced mutations are estimated between 2-10 mutations/Mb (Till et al., 2007). EMS is commonly used mutagen to improve new varieties with high yield or desirable traits i.e., producing phenotypic variation, biotic and abiotic stress resistance in several plants (Table 2.3).

Figure 2.5   Relative number of released mutant varieties (direct and indirect) induced using the agents indicated. Ethyl methansulfonate (EMS), N-ethyl-N-nitrosourea (ENU), N-Methyl-N-nitrosourea (MNU), ethylenimine (EI) Dimethyl sulphate (DMS), diethyl sulphate (DES), Colchicine (Colch), NaN₃ (Sodium azide) (The Joint FAO/IAEA Programme) (van Harten, 1998).
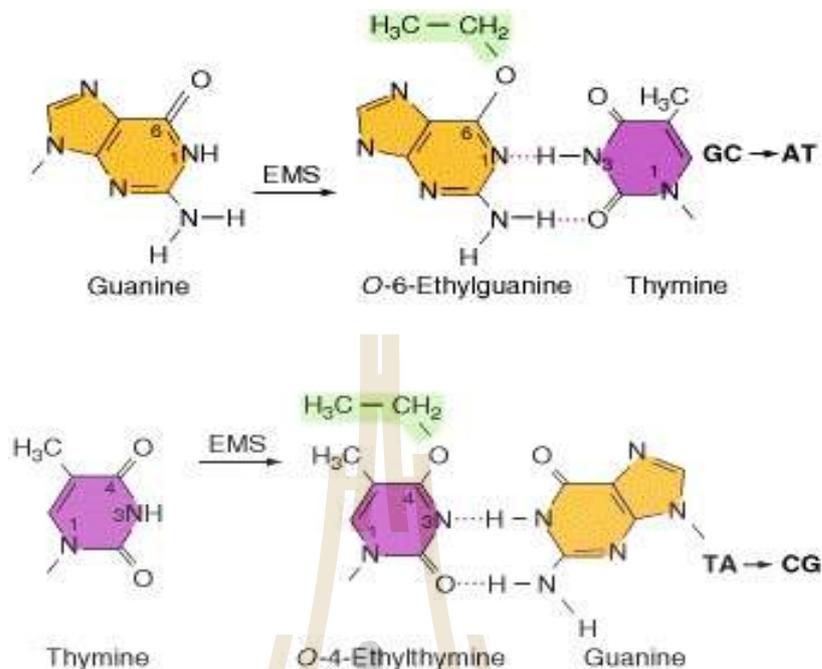
Figure 2.6 Induction of point mutations by EMS (Griffiths et al., 2000)

2.7 *In vitro* selection for disease resistance

In vitro culture of plant cells and tissues under controlled conditions offers a well-founded technology platform for the production of plant natural products. *In vitro* selection is used to screen a large number of plants or cells for a certain characteristic in a short period of time, leading to cost saving. This tool is suitable for breeding long-lived perennials as well as orchids. *In vitro* selection was used in several plants for improving various characteristics. For example, Verma et al. (2013) selected drought tolerant somaclonal lines of rice using *in vitro* selection on BM medium with supplemented polyethylene glycol 6,000 (PEG) at the concentrations of 30 to 70 g/L. While, Kang et al. (2012) identified salt-tolerant cell lines of *Ailanthus altissima* from callus derived protoplasts which were selected on Murashige-Skoog (MS) liquid medium incorporated with various concentrations of NaCl.

Table 2.3 EMS mutagen used in several plants for mutagenesis

Crops	Explants	Dose (%)	Time of treatment (hr)	Effects	References
Phenotypic variation					
Pitaya (*Stnocereus* spp.)	Seeds	3.80	9.0	New bud with red tip and morphological variation of stem	Deng et al. (2020)
Fenugreeks (*Trigonella foenumgraecum* L.)	Seeds	0.30-0.60	3.0	Three types of chlorophyll mutants (albino, chlorina and viridis)	Kavina et al. (2020)
Tepary bean (*Phaseolus acutifolius*)	Seeds	0.50	1.0	Yield component improvements such as early maturity of pod yield	Thangwana et al. (2021)
Strawberry (*Fragariaxan anassa* Duch.)	Runner tips, shoot tips and leaf disc	0.10	1.5	Induced maximum number of flowers per plant	Bhat et al. (2017)
Banana (*Musa* spp.)	Shoot tip	2.63	2.0	LD_{50}, reduction of survival rate, growth and multiplication of shoot tips	Shirani et al. (2016)
Orchid (*D.*"Sonia")	PLBs	0.75	1.5	Bigger leaves, thicker roots, larger size of guard cells	Samala et al. (2014)
Orchid (*D. friedericksianum* Rchb.f)	PLBs	1.00	1.5	Three types of chimera (sectorial and mericlinal and periclinal)	Muangsorn and Te-chato (2008)
Biotic stress resistance					
Wheat (*Triticum aestivum* L.)	Seeds	0.80	2.0	Resistance to leaf and yellow rust	Hussain et al. (2018)
Potatoes (*Solanum tuberosum* L)	Shoot tips	0.20	0.3	*F. avenaceum* resistant potato with less symptoms	Arici et al. (2017)
Banana (*M. paradisiaca* (L.) cv. Puttabale)	Shoot buds	0.40	-	Higher enzyme activities (e.g. catalase, phenylalanine ammonia lyase (PAL), chitinase and β-1,3-glucanase) than control	Krishna et al. (2013)
Oat (*Avena sativa*)	Kernels	0.60	4.0	Increasing crown rust tolerance but gave inferior grain yields	Simons et al. (1979)
Abiotic stress resistance					
Strawberry (*F.* x *ananassa* Duth.)	Leaves	0.10	1.5	NaCl tolerance under NaCl stress (0 to 45 mM)	Abbas et al. (2018)
Sugarcane (*Saccharum officinarum* L.)	Calli	0.10	17.0	Drought tolerant mutant with better physiological adaptation under drought stress	Khalil et al. (2018)

In vitro selection for disease resistance has been performed by extensive studies of about 30 plant species and 40 plant diseases (reviewed in Švabová and Lebeda, 2005). In general, the selection of disease resistant plants is carried out using cell-free culture filtrates (CFs) or pure toxins and phytotoxins of the pathogens as the selective agents. Fungal or pathogen CFs has been extensively used for selection of host species. Toxins are compounds that are produced by pathogens and cause the symptom of disease. They constitute various chemicals e.g., peptide, glycoprotein, polysaccharide, fatty acid and terpenoid (Daub, 1984; Turner, 1984). Many reports have used toxic CFs and purified toxins for disease resistant selection (Thakur et al., 2002; Liu et al., 2005; Bajpai et al., 2007). CFs played an important role in host-pathogen interactions, and have been reported to cause necrosis on tobacco and rubber leave and *Dendrobium* PLBs (Churngchow and Rattarasarn, 2000; Khairum et al., 2018).

CFs can be easily incorporated into the culture media at appropriate concentrations to select several resistant plants (El-kazzaz et al., 2009; Dehgahi and Joniyas, 2016; Sayed et al., 2016; Kumar et al., 2017). There has been reports using CFs for *in vitro* selection for disease resistance in several plants (Table 2.4). By using CFs for EMS-derived mutant selection, Krishna et al. (2013) revealed that EMS-derived mutants in the banana (*Musa paradisiaca* L.) cv. Puttable derived from *in vitro* selection in MS medium supplemented with *F. oxysporum* f. sp. *cubense* (FOC) CFs (5-15%) showed a significant increase in some oxidative enzymes and pathogenesis-related (PR) proteins, reaching higher levels than control. In orchids, Dehgahi et al. (2016) selected *F. proliferatum*-tolerant PLBs and assessed the effects of different concentrations of *F. proliferatum* CF (5-20%) *in vitro*. It was found that CF-treated PLBs had 2.7-folds increased peroxidase (POD) activity when compared to untreated one. Furthermore, the

disease symptoms in all CF-treated leaflets were reduced compared to the control under the assessment of disease development.

Table 2.4 CFs from different pathogens were used for *in vitro* selection to obtain new resistant lines.

Plants	CFs	References
Orchid (*D.* "Sonia")	*F. proliferatum*	Dehgahi et al. (2016)
Carrot (*Daucus carota*)	*A. dauci*	Lecomte et al. (2014)
Banana (*M. paradisiaca* 'Puttabale')	*F. oxysporum* f.sp. cubense	Krishna et al. (2013)
Carnation (*Dianthus caryoplyllus* L.),	*F. oxysporum* f. sp. *dianthi*	Esmaiel et al. (2012)
Orange (*Citrus jambhiri* L.)	*P. parasitica*	Savita et al. (2011)
Sunflower (*Helianthus annuus* L.)	*A. helianthi*	Rao and Ramgoapl (2010)
Lilly (*Lilium* spp.)	*P. cactorum*	Sharmar et al. (2008)
Abaca (*M. textilis* Nee)	*F. oxysporum* f.sp. cubense	Purwati et al. (2007)
Grapevine (*Vitis vinifera* L.) 'Chardonnay'	*Elsinoe ampelina*	Jayasankar et al. (2000)

2.8   Plant defense mechanism

Plant defense can be classified generally as constitutive and induced defenses. Constitutive defense (pre-formed resistance) is always present in the plant, which includes many preformed barriers to inhibit the invasion of pathogens (e.g., cell walls, waxy epidermal cuticles, and bark). An induced defense is a temporary defense that

targets against an area of the plant where it is attacked or injured, and can also be activated in the distal uninfected portions (e. g. , production of toxic chemicals, pathogen-degrading enzymes, and deliberate cell suicide) (Freeman and Beattie, 2008; Gupta et al., 2013; Online 3). The resistance of plants is normally controlled by several genes for example, Wu (2017) found two genes, *OnFd* and *OnFNR*, which had significant effects on soft rot in *Onciduim*, implicating their important roles in resistance against soft rot. Gou (2018) used Agrobacterium- mediated transformation to transfer the *PR1* gene into PLBs of *Oncidium* for transient expression, and the transformed plants exhibited higher resistance to the disease. Constitutive resistance and innate immunity mechanisms conferring by multiple *R* genes provide non- host pathogen resistance and moderate- host pathogen resistance, respectively. While induced resistance mechanism conferring by one or few *R* genes provides specific-host pathogen resistance (Table 2.5).

Table 2.5 Summary of resistance in plants against pathogens.

Resistance type	Constitutive resistance	Innate immunity	Specific resistance
	Preformed	Induced	Induced
Spectrum of protection	Very broad	Broad	Narrow
Number of genes determining specificity	Many genes	Many genes	One or very few genes
Specific recognition of invaders	None	Moderate	High degree

Parinthawong (2016)

Since *P. palmivora*' s cell walls compose mostly of β- glucan (ca. 90% dry weight) and small amount of chitin (<1%), they can be degraded by various ß-glucanases and chitinases (Tokunaga and Bartnicki-Garcia, 1971; Hamid et al., 2013; Mélida et al., 2013). Both β-glucan and chitin have been found to act as elicitors. A wide variety of *Phytophthora* spp. elicitors (Table 2. 6) (microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs)) are recognized by pattern-recognition receptors (PRRs) in plants called MAMP- or PAMP-triggered immunity (PTI) (Naveed et al., 2020), followed by the activation of defense responses including influx of Ca^{2+} in the cytosol, reactive oxygen species (ROS) burst, nitric oxide production, hypersensitive response (HR), activation of mitogen-activated protein kinase (MAPK) cascades, callose deposition, stomatal closure, phytoalexins biosynthesis, defense hormone biosynthesis, transcriptional induction of a large suite of defense- related genes, accumulation of antimicrobial secondary metabolites and systemic acquired resistance (SAR) (Capasso et al., 2001; Baillieul et al., 2003; Malik et al., 2020; Naveed et al., 2020). SAR is an induced secondary resistance response after HR, leading to the expression of PR-proteins which play a direct defensive role against pathogenic agents in tissues distal to the infection sites. SAR provides broad spectrum and long-lasting resistance to secondary infections throughout the plant using a long distance signaling mechanism that involves salicylic acid (SA) or its derivative (methyl salicylate) as the key signal (Beckers and Sopel, 2006; Vlot et al., 2008; Durrant and Dong, 2009; Gao et al., 2015).

PR proteins were firstly discovered from tobacco mosaic virus infected leaves in tobacco (*Nicotiana tabacum*) and then have been detected in several plants of different species (van Loon and van Kammen, 1970). They exhibit various biochemical

characteristics which are requisite when the plant is under pathogenic infections or any undesired stresses (van Loon, 1985). PR proteins are generally proteins with molecular weight in the range of 5 to 100 kDa, and are grouped according to sequence similarity and biological activity. At least 14 families of PR proteins are recognized. Examples of well-known PR proteins are shown in Table 2.7 including PR1 proteins (anti-oomycete and antifungal; 15-17 kDa), PR2 (β-1,3-glucanases; 30-41 kDa), PR3, PR4, PR8 and PR11 (chitinases; 13-46 kDa), PR5 (thaumatin-like proteins; 16-26 kDa), PR6 (proteinase-inhibitors; 8-22 kDa) and PR9 (peroxidases; 50-70 kDa) etc. (van Loon and van Strien, 1999; Agrios, 2005; Ebrahim et al., 2011). PR proteins are typically either acidic or basic form which have been classified according to their isoelectric points (pI). The acidic and basic forms of PR proteins are chiefly secreted to the extracellular space and are transported to the vacuole by a signal located at the C-terminus, respectively (Buchel and Linthorst, 1999; van Loon and van Strien, 1999). PR proteins are usually found to be localized in plant organs including leaves, stems, roots, and flowers, however, these PR proteins are found with maximum abundance in the leaves (Sinha et al., 2014).

ß-1,3-glucanase and chitinase are two important hydrolytic enzymes that are abundant in many plant species after infection by different types of pathogens. ß-1,3-glucanase and chitinase are commonly encoded by multigenic families and have various functions in plants, including plant growth and development, wounding, and defense responses. They are strongly induced when plants respond to infect by several pathogens (Leubner-Metzger et al., 1999; Neuhaus, 1999; van Loon, 1999; Wu and Bradford, 2003). These ß-1,3-glucanase and chitinase are one of the pre-formed constitutive and induced defense proteins which may have different isoforms, varying

in pI. Many studies have been reported that the accumulation of chitinase and glucanase affected fungal viability and promoted plant immunity responses (El-Hadary and Tayel, 2013; Kumar et al., 2018a; Pusztahelyi, 2018). Induced ß-1,3-glucanase and chitinase activities after fungal infection had been reported in lemon seedlings (Fanta et al., 2003). Anguelova-Merhar et al. (2001) reported that constitutively expressed chitinase activity and induced β-1,3-glucanase activity were significantly observed in leaf rust (*Puccinia recondita* f. sp. *tritici*) resistant wheat. Żur et al. (2013) also found that higher chitinase activity was markedly induced in cold-induced snow mould resistance in the Polish cultivars of winter triticale. Moreover, Khompatara et al. (2019) reported that seaweed extract (SWE) could enhance rubber tree resistance against *P. palmivora* infection. The defense enzymes including catalase, POD, and β-1,3-glucanase were induced, and there had been the accumulation of secondary metabolites (scopoletin; Scp) and SA).

Table 2.6 *Phytophthora* spp. elicitors and associated PTI components

Name	Pathogen group carrying PAMP	Chemical nature	Cognate PRR	PAMP perception model	Signaling	References
Elicitins	Unique to oomycete genera; *Phytophthora* and *Pythium*	Protein	ELR (Reported for Cryptogein, INF1 and ParA1)	BAK1/SERK3 dependent (Reported for INF1 and ParA1)	SA (Cryptogein), JA and ET (INF1), Ca and MAPK (Cryptogein and INF1)	Kamoun et al. (1998); Lebrun-Garcia et al. (1998); Kawamura et al. (2009); Amelot et al. (2011); Du et al. (2015); Peng et al. (2015); Derevnina et al. (2016)
OPEL	Oomycetes	Protein	Unknown	Unknown	SA	Chang et al. (2015)
Pep-13	*Phytophthora* spp.	Protein/peptide	Unknown	BAK1/SERK3 independent	SA and JA, Ca and MAPK	Nürnberger et al. (1994); Blume et. (2000); Halim et al. (2009); Wang et al. (2019)
β-glucans	Oomycetes and fungi	Carbohydrate	Unknown	Unknown	SA	Kopp et al. (1989); Klarzynski et al. (2000); Fesel and Zuccaro (2016)
Eicosapolyenoic acids (EPs)	Oomycetes, primitive fungi and nematodes	Lipids	Unknown	Unknown	JA	Preisig and Kuć (1985); Savchenko et al. (2010)
Elicitors with dual, PAMP and effector status						
XEG-GH12	Fungi and oomycetes	Protein-CWDEs	Unknown	BAK1/SERK3 independent	Unknown	Ma et al. (2015; 2017)
CBEL-CBM1	Fungi and oomycetes	Protein-CWDEs	Unknown	BAK1/SERK3 independent	SA, JA and ET	Khatib et al. (2004); Larroque et al. (2013)
Nlp20-NLPs	Bacteria, fungi and oomycetes	Protein	RLP23	BAK1/SERK3 independent	SA	Böhm et al. (2014); Albert et al. (2015)

Naveed et al. (2020)

Table 2.7 PR proteins

Family	Type	Biochemical Properties	Molecular size range (kDa)
PR-1	Tobacco PR-1a	Antifungal	15-17
PR-2	Tobacco PR-2	β-1,3-glucanase	30-41
PR-3	Tobacco P, Q	Chitinase type I,II,IV,V,VI,VII	35-46
PR-4	Tobacco 'R'	Chitinase type I,II	13-14
PR-5	Tobacco S	Thaumatin-like	16-26
PR-6	Tomato Inhibitor I	Proteinase-inhibitor	8-22
PR-7	Tomato P69	Endoproteinase	69
PR-8	Cucumber chitinase	Chitinase type III	30-35
PR-9	Tobacco 'lignin forming peroxidase'	Peroxidase	50-70
PR-10	Parsley 'PR1'	Ribonuclease like	18-19
PR-11	Tobacco 'class V' chitinase	Chitinase, type I	40
PR-12	Radish Rs- AFP3	Defensin	5
PR-13	Arabidopsis THI2.1	Thionin	5-7
PR-14	Barley LTP4	Lipid-transfer protein	9
PR-15	Barley OxOa (germin)Oxalate oxidase	Oxalate oxidase	22-25
PR-16	Barley OxOLP	Oxalate oxidase-like	100 (hexamer)
PR-17	Tobacco PRp27	Unknown	Not known

van Loon and van Strien (1999), van Loon et al. (2006) and Ebrahim et al. (2011)

2.9   Characterization of mutant plants

The characterization of mutants can be performed by several methods such as morphological observation, cytogenetic and molecular marker analysis.

2.9.1   Morphological characteristics

Morphological characteristics based on direct observation is widely used to characterize mutant plants. Changes in morphological characteristics were observed in mutants i.e., increased height, larger leaves, thicker roots, higher numbers of nodes, reduced node length, and three types of chimeras (sectorial, mericlinal and periclinal) (Muangsorn and Te-chato, 2008; Samala et al. 2014). For more details, Samala et al. (2014) found that EMS-treated *D*. Sonia PLBs at 0.75% for 90 minutes showed the highest growth rate, bigger leaves, and thicker roots. In addition, these mutants also showed the larger size of guard cells and lower stomatal density than control. Similarly, Wannajindaporn et al. (2016) also reported that multiple morphological alterations and negative effects on growth were induced by 0.5 mM NaN_3, while 0.1 mM gave thicker leaves and shorter roots in *D*. 'Earsakul'.

2.9.2   Cytogenetic analysis

Cytogenetic analysis using flow cytometry can be used to estimate DNA content and genomic size as well as for determination of DNA ploidy level and cell cycle analysis (Galbraith, 2004; Shapiro, 2003; Bennett and Leitch, 2005). Genetic variation was enhanced by inducing mutations at the gene, chromosome and genome levels in both nuclear and cytoplasmic organelle DNA (Larkin, 1998). Flow cytometry enable measurement of these variations in mutants. Jones et al. (1998) found that DNA content of 26 orchid genera ranged from 1.53 to 4.23 pg $2C^{-1}$. It was also found that nuclear DNA content could be increased from 33 to 50% in colchicine-treated orchid mutants

based on flow cytometry when compared to non-mutagenized controls (Choopeng et al., 2019; Mohammadi et al., 2021). In addition, Lapjit and Teng (2017) reported that the changes in profiles of flow cytometric histograms were found in several regenerated plants after *Erycina pulsilla* PLBs were treated with 0.1% and 0.2% EMS. Moreover, Hualsawat (2019) reported that all of the black rot resistant *D.* 'Earsakul' mutants were identified to be mixoploid (2n+4x+8n) and their DNA content and genome size were higher than those of all non-mutagenized controls.

2.9.3 Molecular markers

Biochemical markers such as isozymes and allozymes are a molecular tool that can be used for genetic characterization. Protein markers are based on the changes in sequence of amino acids in a protein molecule (Tanksley and Orton 1983, Smith, 1986, Soltis and Soltis, 1990; Kumar et al., 2018b). In orchids, Wang et al. (2017) conducted a proteomic analysis on self-pollen or cross-pollen treated pistils derived from *D. chrysanthum* to investigate the early proteomic response between self- and cross-pollination using two-dimensional electrophoresis to identify differentially expressed proteins (DEP). A total of 54 DEP spots were identified and classified into different functional categories (metabolic process, response to stimulus, biosynthetic process, protein folding and transport). Similarly, Xu et al. (2015) found that three proteins including resistance protein, NBS-LRR type resistance protein, and disease resistance protein were upregulated in the roots of *D. officinale* after induction *Mycena dendrobii*. Moreover, Krishna et al. (2013) reported that total phenols, PAL, oxidative enzymes (POD, polyphenol oxidase (PPO), catalase), and PR proteins (β-1,3-glucanase and chitinase) of EMS-treated banana were higher than the control. These enzyme activities were used as biochemical markers to predict tolerance and resistance in EMS-treated

banana to FOC. Protein markers can also be used to assess genetic diversity/protein profiles using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis in several plants i.e., rice (Dhawale et al., 2015), brassica (Jan et al., 2017), and fenugreek (Qadir et al., 2017). It could specify ca. 20-44 and 13-43 kDa bands of β-1,3-glucanase and chitinase, respectively, which were induced in several plants upon pathogen infection i.e., grapevine, tomato, banana, moth bean and faba bean (Jayasankar et al., 2000; El-Hadary and Tayel, 2013; Krishna et al., 2013; Pareek et al., 2014; Sinha et al., 2014; Mahmoud and Abd El-Fatah, 2020). Thus, the biochemical/ protein markers may be used to identify and characterize various putative black rot resistant *D.* 'Earsakul' mutants.

DNA molecular markers are nucleotide sequences which can be used to detect polymorphism between alleles of a gene for a particular sequence of DNA or different genotypes. These fragments are linked with a definite location within the genome and may be detected by using certain molecular technology. Different types of DNA markers have been developed and successfully applied in breeding programs in various crops (Nadeem et al., 2018). DNA markers are being used for the acceleration of plant selection through MAS (Lateef et al., 2015). Many different types of DNA molecular markers have been utilized in orchids e.g., random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), amplified fragment length polymorphism (AFLP), start codon targeted (SCoT), target region amplification polymorphism (TRAP) (Xiang et al., 2003; Bhattacharyya et al., 2013; Wannajindaporn et al., 2014; Feng et al., 2015; Wannajindaporn et al., 2016; Tantasawat et al., 2017; Hualsawat, 2019; Yuhanna et al., 2021). ISSR makers involve the use of microsatellite sequences as primers to detect multi-locus polymorphisms throughout the genome. They provide

discriminating power with good reproducibility (Thormann et al., 1994; Zietkiewicz et al., 1994; Karp et al., 1997; Meudt and Clarke, 2007; Agarwal et al., 2008; Costa et al., 2016). ISSR markers have been used in various orchids (Parab et al., 2008; Qian et al, 2014). Wannajindaporn et al. (2016) used ISSR markers to evaluate the genetic profiles of 24 putative *D.* 'Earsakul' mutants derived from *in vitro* mutagenesis using NaN_3. The results showed that 83.33% altered DNA profiles were found among putative *D.* 'Earsakul' mutants and 63 polymorphic bands were produced from a total of 181 bands (34.81%). Similarly, Tantasawat et al. (2017) estimated the genetic variability among putative black rot resistant *D.* 'Earsakul' and non-mutagenized controls using ISSR analysis. A total of 114 amplified ISSR fragments was generated from seven ISSR markers, and 53 of which were polymorphic (46.5%). All eight putative mutants showed altered genetic profiles compared to controls and were identified as mutants. Moreover, five ISSR markers were significantly associated with black rot resistance. Recently, Hualsawat (2019) revealed that black rot resistant *D.* 'Earsakul' mutants were genetically different from non-mutagenized controls by means of 16 ISSR markers. These results suggested that ISSR markers are effective for determining genetic variation and are useful for the characterization of putative black rot resistant *D.* 'Earsakul' mutants.

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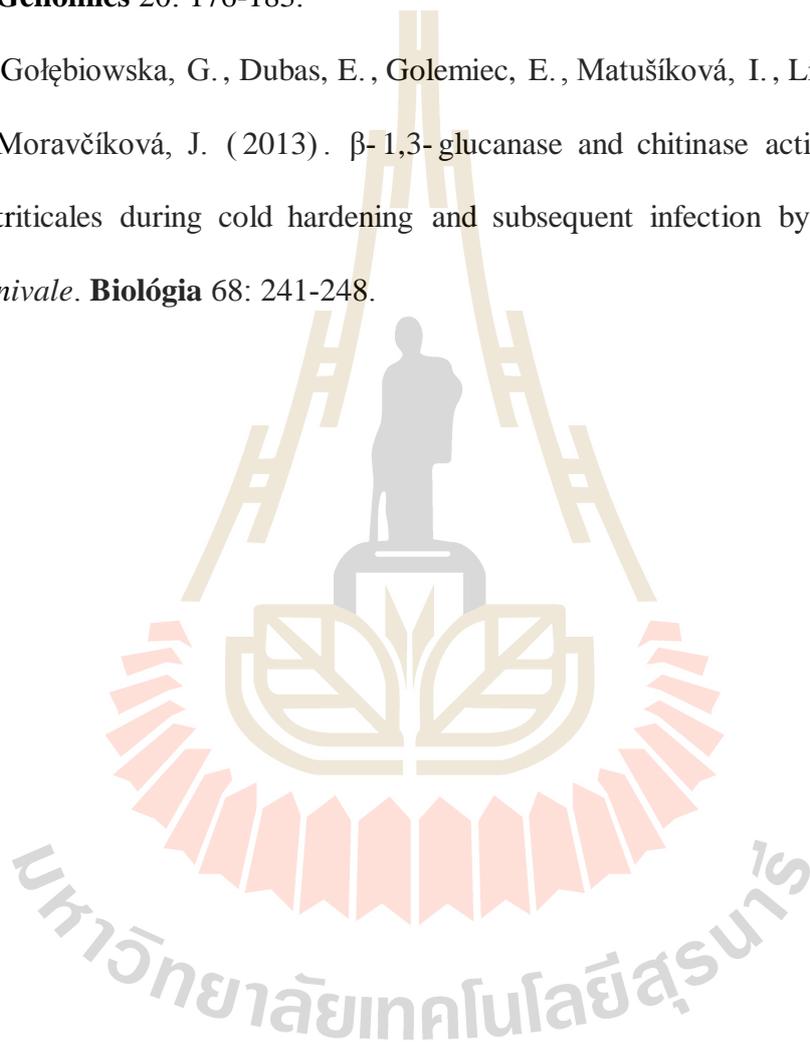
CHAPTER III

CULTURAL CHARACTERISTICS AND

PATHOGENICITY ANALYSIS OF *Phytophthora palmivora*,

CAUSAL PATHOGEN OF BLACK ROT IN ORCHIDS

3.1   Abstract

Phytophthora palmivora is the causal pathogen of black rot, one of the major diseases affecting orchids. To evaluate its variability, *P. palmivora* was isolated from infected orchid leaves in Thailand using PARBPH selective medium. It was purified again by the modified baiting method, and was cultured on Rose Bengal medium. Single-spore isolates were obtained and 5 representatives were cultured on potato dextrose agar (PDA), pea sucrose agar (PSA), cereal meal potato agar (CMPA; a newly developed medium) and V8 juice agar (V8A) for cultural characterization. It was found that the different culture media affected growth, appearance, and sporulation. The best culture media for surface mycelial growth were PSA and V8A, while CMPA induced sporulation earlier than other media. Pathogenicity analysis using 3 *Dendrobium* 'Earsakul' lines (a non-mutagenized control and 2 mutants) and 5 single-spore *P. palmivora* isolates showed that NK-53-9 was the most virulent isolate, which is useful for future screening of black rot resistance. A *Dendrobium* mutant, SUT13E18301 was resistant to all isolates, suggesting its usefulness as a resistance source in future breeding program.

3.2 Introduction

Orchid is one of the largest families of flowering plants in the world comprising of ca. 28,000 species and more than 700 different genera (Christenhusz and Byng, 1992). Its wide range of different characteristics in shapes, sizes and colors has made orchid the plant for all types of interests and sought for by collectors. Therefore, it is undoubtedly recognized as an economically important ornamental in the international floriculture industry, both as cut flowers and potted plants (Arditi, 1992; Kuehnle, 2007). Among various orchid genera, *Dendrobium* orchids have become increasingly popular (Kuehnle, 2007). And they are also the major cut-flower orchid export for Thailand, however, they usually face with numerous disease problems. Several species of *Phytophthora* have been reported to cause economic damage on orchids worldwide. Black rot (*Phytophthora palmivora*) is an exotic, polyphagous pathogen that has been reported worldwide on different hosts, mainly causing root and crown rot diseases. It has become widespread in Asia and Europe, where it has been reported on several ornamental plants including orchids (Uchida, 1994; Pane et al., 2006; Cacciola et al., 2008). *P. palmivora* is also recorded as a foliar pathogen of cocoa (Wharton and Turner, 1960) and macadamia (Bazan De Segura, 1970). The incidence of these diseases has increased during the past few years due to both polyphagy and means of dissemination. And because it is a thermophilic species, the hot environmental conditions such as in Thailand are favorable for its development (Cacciola et al., 2008). The pathogen can spread throughout the rhizomes, causing rapid plant death (Orlikowski and Szkuta, 2006; Cating et al., 2010). The objectives of this work were to evaluate culture media

for *P. palmivora* and to select *P. palmivora* that was the most virulent isolate to preliminarily evaluate resistance levels of the potentially resistant lines in *Dendrobium* 'Earsakul'.

3.3 Materials and Methods

3.3.1 Pathogen isolation

Isolates of *P. palmivora* from Nakhon Ratchasima Province, Thailand were collected from leaves of susceptible orchid varieties infected by the black rot disease in 2010. They were washed under running tap water, surface-sterilized in 70% (v/v) ethyl alcohol for 5-10 seconds then dried on filter paper. Approximately 2-4 mm-wide tissues were cut from the edge of lesions and placed on potato dextrose agar (PDA) amended with 10 mg/L pimaricin, 200 mg/L ampicilin, 10 mg/L rifampicin, 10 mg/L benomyl, 25 mg/L pentachloronitrobenzene and 50 mg/L hymexazol (PARBPH) (Jeffers and Martin, 1986). Inoculated plates were incubated at 25ºC in the dark and examined within 2-3 days. *P. palmivora* isolates were obtained by subculturing the hyphal tips onto PDA, were purified again by the modified baiting method, and were cultured on Rose Bengal medium (Jarvis, 1973). Single-spore isolates were obtained from individual fungal colonies.

3.3.2 Cultural characterization

Five single-spore *P. palmivora* isolates (NK-53-5, NK-53-6, NK-53-7, NK-53-9 and NK-59-11) were characterized. A 4-mm-diameter agar disk of each isolate was obtained by cutting with a sterile cork borer, and placed onto 4 different culture media: (1) PDA (20% (w/v) potato, 2% (w/v) D-glucose, 2% (w/v) agar); (2) pea sucrose agar (PSA; 12.5% (w/v) pea, 1% (w/v) sucrose, 1.5% (w/v) agar); (3) corn meal potato agar

(CMPA; 5% (w/v) oat, 5% (w/v) corn grit, 5% (w/v) rice bran, 5% (w/v) potato, 1% (w/v) D-glucose, 4% (w/v) potato dextrose agar and 0.3% agar) and (4) V8 juice agar (V8A; (20% (v/v) V8 juice, 0.3% (w/v) calcium carbonate ($CaCO_3$) and 1.5% (w/v) agar) in 9-cm-diameter Petri dishes and incubated at 25ºC in the dark. Five replicates per isolate were made, and the following observations were made at 3, 7, 14 and 21 days after plating on the medium: (1) colony size (area = ¶ (width/2) (length/2)) and colony appearance (colonies were characterized based on surface characteristics [rosette, radiate, stellate and irregular]) (Ilieva et al., 1998; Appiah et al., 2003; Phytophthora database, 2016). Sporulation was observed daily by cutting mycelial piece and examined microscopically.

3.3.3 Pathogenicity tests

Agar pieces containing mycelium of 5 single-spore *P. palmivora* isolates were collected from 5-day-old colonies grown on CMPA, were transferred to sterile bottles, were covered with sterile reverse osmosis water (ROW), and were incubated overnight at 25ºC in the dark (Daniel and Guest, 2006). Inoculums were adjusted to 10^6 zoospores/mL, and 3 µL drops of each suspension were inoculated on leaves of 3 *Dendrobium* lines (a non-mutagenized control; SUT13C003 and two mutants; SUT13E18301 and SUT13E18303 from ethyl methanesulfonate (EMS)-mutagenized plants) after pin wounding in a detached leaf assay (Ann, 2000a). While control (no inoculum) was inoculated with ROW. All inoculated leaves were incubated under 25ºC in the dark. Four replicates per *Dendrobium* line were made, and symptom development was observed at 3 and 5 days after inoculation; scores were attributed according to the following scale: 0, no symptom; 1, very small localized lesions; 2, yellow around lesions; 3, yellow around lesions, hyphae was revealed; 4, brown lesions, hyphae was

expanded; 5, brown lesions, hyphae was expanded outside the area covered (Nyasse et al., 1995).

3.3.4 Data analysis

Data was analyzed using analysis of variance (ANOVA) of colony size and $(X+1)^{1/2}$ transformed severity of symptom score. Mean comparison was performed by Duncan's multiple range test (DMRT) to evaluate the differences in the ability of culture media to promote mycelial growth and severity of symptoms using SPSS version 14.0 (Levesque and SPSS, 2006).

3.4 Results

Five single-spore isolates of *P. palmivora* (NK-53-5, NK-53-6, NK-53-7, NK-53-9 and NK-59-11) which were grown on various media (PDA, PSA, CMPA, and V8A) were characterized after 3-21 days. It was shown that the effects of isolates were highly significant ($p < 0.01$) on colony size at 3, 7 and 14 days. At 3 days the most rapidly grown isolate was NK-53-6 with a colony size of 19.27 cm^2, which was significantly higher than other isolates. Whereas NK-59-11 and NK-53-9 had the smallest colony size at 3 and 7 days after culture, respectively. But after being cultured for 14 days, all isolates reached similar colony size except for NK-53-7. And colonies of all isolates grew to edges of Petri dishes at 21 days after culture (Table 3.1).

When these isolates were grown on PDA, PSA, CMPA, and V8A, it was found that different culture media affected colony size of all *P. palmivora* isolates significantly ($p < 0.01$) at 3, 7 and 14 days. Overall, the best media for promoting colony size were PSA and V8A for all culture periods up to 14 days. At 3 and 7 days after culture, the colony sizes on PSA and V8A were 21.68, 20.38 and 60.00 and 61.81

cm^2, respectively, which were significantly higher than those of other media, particularly PDA. Although the colony sizes on PSA and V8A were not significantly different from that on CMPA at 14 days after culture, their colony size was still 1.2-fold significantly larger than that on PDA (Table 3.2).

Table 3.1 Effects of single-spore isolates on colony size of *P. palmivora* at 3, 7, 14 and 21 days after culture.

Isolates	Colony size (cm^2)			
	3 days	7 days	14 days	21 days
NK-53-5	14.02 ± 1.99 b [1]	49.15 ± 3.79 ab	63.64 ± 3.26 a	63.64 ± 3.26
NK-53-6	19.27 ± 1.72 a	53.06 ± 3.94 a	62.97 ± 0.67 a	63.64 ± 3.26
NK-53-7	15.23 ± 1.87 b	44.58 ± 4.89 bc	55.37 ± 3.62 b	63.64 ± 3.26
NK-53-9	12.64 ± 1.73 c	40.81 ± 4.07 c	62.48 ± 1.07 a	63.64 ± 3.35
NK-59-11	10.24 ± 1.44 d	46.54 ± 4.54 b	61.30 ± 1.46 a	63.64 ± 3.26
F-test	**	**	**	ns [2]
CV (%)	18.37	19.27	9.45	0.00

[1] Means ± SE in the same column with different letters are significantly different ($p < 0.05$) based on DMRT.

[2] ns=not significant

A highly significant interaction ($p < 0.01$) on colony size was observed among single-spore isolates of *P. palmivora* grown on different culture media. When both factors were evaluated at 3 days after culture, a maximum colony size was obtained in NK-53-7 when cultured on PSA (25.03 cm^2), which was not significantly different from those of NK-53-6 cultured on V8A and PSA, NK-53-5 cultured on V8A, and NK-53-9 cultured on PSA. And after being cultured for 7 days, most isolates which were cultured on PSA and V8A, and some isolates which were cultured on CMPA had grown

to significantly larger colony size than those cultured on PDA. This tendency was observed until 14 days after culture in some isolates (Table 3.3). Each single-spore isolate of *P. palmivora* had effectively grown on different culture media, which were depended on culture time. The highest colony sizes were obtained in most of single-spore isolates of *P. palmivora* when cultured on PSA and V8A for 3 days. At 7 days after culture, most single-spore isolates of *P. palmivora* which were cultured on PSA, V8A, and CMPA gave a larger colony size than on PDA. At 14 days after culture, all culture media gave maximum colony size when most single-spore isolates of *P. palmivora* were cultured, except NK-53-7 and NK-53-11.

Table 3.2 Effects of culture media on colony size of *P. palmivora* at 3, 7, 14 and 21 days after culture.

Isolates	Colony size (cm^2)			
	3 days	**7 days**	**14 days**	**21 days**
PDA	5.74 ± 0.52 c [1]	21.74 ± 1.82 c	54.40 ± 3.02 b	63.64 ± 4.35
PSA	21.68 ± 0.86 a	60.00 ± 1.60 a	63.64 ± 4.35 a	63.64 ± 4.35
CMPA	9.18 ± 1.26 b	43.88 ± 3.03 b	62.95 ± 0.70 a	63.64 ± 4.44
V8A	20.38 ± 0.84 a	61.81 ± 1.29 a	63.64 ± 4.35 a	63.64 ± 4.35
F-test	**	**	**	ns [2]
CV (%)	18.37	19.27	9.45	0.00

[1] Means $\pm$ SE in the same column with different letters are significantly different ($p < 0.05$) based on DMRT.

[2] ns=not significant

Growth characteristics of mycelium depended on the media used. Among the 4 media, it was found that a radiate morphology was observed when all isolates were cultured on PSA, a rosette morphology was found on PDA and CMPA. By contrast,

V8A showed irregular colony morphology. All isolates were white and aerial mycelium was found on most media. The highest aerial mycelium formation was found on PDA and CMPA, while PSA and V8A induced poor aerial mycelium formation (Figure 3.1). Moreover, all isolates which were cultured on CMPA medium had earlier sporulation than other media within 2 days after culture. While sporulation was induced by PSA and PDA, and V8A media at 3 and 4 days after culture, respectively.

Table 3.3 Colony size of single-spore *P. palmivora* isolates cultured on different culture media at different culture periods.

Isolates	Media	Colony size (cm^2)		
		3 days	7 days	14 days
NK-53-5	PDA	4.39 ± 0.25 gh [1]	24.03 ± 1.56 efg	63.64 ± 3.55 a
	PSA	20.76 ± 1.11 bc	63.64 ± 3.55 a	63.64 ± 3.55 a
	CMPA	6.92 ± 0.45 fg	45.29 ± 2.03 cd	63.64 ± 3.55 a
	V8A	24.01 ± 1.13 ab	63.64 ± 3.55 a	63.64 ± 3.55 a
NK-53-6	PDA	8.25 ± 0.29 f	25.17 ± 4.38 efg	60.97 ± 2.68 ab
	PSA	24.43 ± 2.77 a	59.80 ± 3.67 ab	63.64 ± 3.55 a
	CMPA	19.65 ± 1.08 cd	63.64 ± 3.55 a	63.64 ± 3.55 a
	V8A	24.75 ± 1.59 a	63.64 ± 3.55 a	63.64 ± 3.55 a
NK-53-7	PDA	8.37 ± 1.37 f	22.57 ± 7.70 fg	33.91 ± 9.08 c
	PSA	25.03 ± 0.87 a	63.08 ± 0.57 a	63.64 ± 3.55 a
	CMPA	7.52 ± 2.56 fg	34.48 ± 9.03 de	60.30 ± 3.34 ab
	V8A	20.00 ± 0.86 cd	58.20 ± 5.44 ab	63.64 ± 3.55 a
NK-53-9	PDA	4.87 ± 0.26 fgh	21.38 ± 1.61 fg	59.23 ± 4.02 ab
	PSA	21.39 ± 1.05 abc	49.82 ± 5.33 bc	63.64 ± 3.55 a
	CMPA	5.08 ± 0.97 fgh	29.93 ± 3.73 ef	63.64 ± 3.55 a
	V8A	17.14 ± 0.79 de	59.93 ± 3.71 ab	63.64 ± 3.55 a
NK-59-11	PDA	2.83 ± 0.17 h	15.57 ± 1.53 h	54.26 ± 4.91 b
	PSA	16.77 ± 0.50 de	63.64 ± 3.55 a	63.64 ± 3.55 a
	CMPA	5.36 ± 0.29 fgh	43.30 ± 0.97 cd	63.64 ± 3.55 a
	V8A	16.00 ± 0.64 e	63.64 ± 3.55 a	63.64 ± 3.55 a
	F-test	**	**	**
	CV (%)	18.37	19.27	9.45

[1] Means ± SE in the same column with different letters are significantly different ($p < 0.05$) based on DMRT.
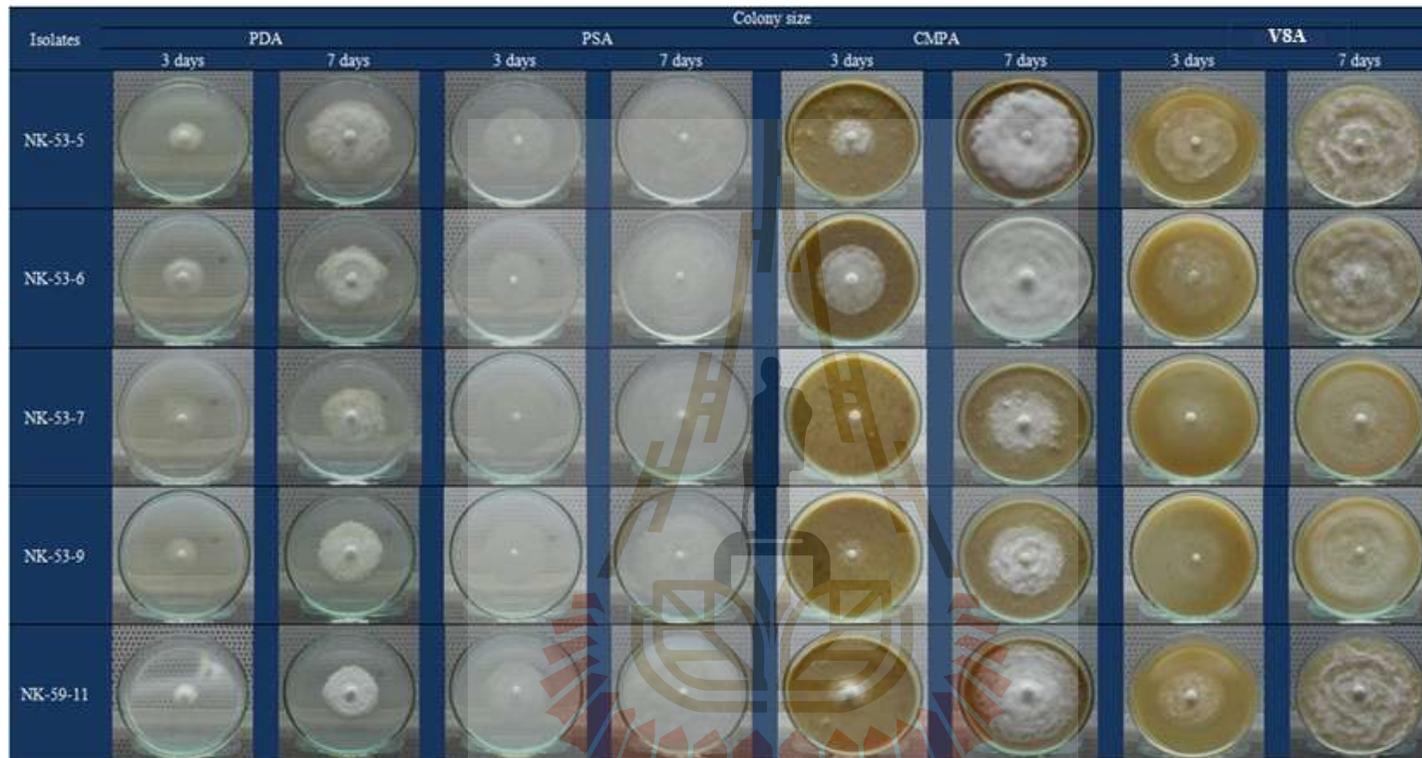
Figure 3.1 Colony morphology of single-spore *P. palmivora* isolates on PDA, PSA, CMPA and V8A at 3 and 7 days after culture

at 25°C in the dark.

Pathogenicity of 5 single-spore *P. palmivora* isolates (NK-53-5, NK-53-6, NK-53-7, NK-53-9 and NK-59-11) and ROW (control) were evaluated on 3 *Dendrobium* lines (SUT13C003, SUT13E18301 and SUT13E18303) by a detached leaf assay. *Dendrobium* leaves started to show necrotic lesions at 2 days after inoculation and the hyphae initially covered them at 3 days after inoculation. When all isolates were inoculated on *Dendrobium* leaves with a 10^6 zoospores/mL suspension, necrotic lesions and hyphae were formed differently among isolates ($p < 0.01$). After inoculation for 3 days, NK-53-9 was the most virulent isolate with an average severity score of 1.67, which was not significantly different from NK-53-6 (1.00). ROW (control) did not show any symptoms for the entire period of experiment (Table 3.4).

The effects of *Dendrobium* lines on severity of symptoms were highly significant ($p < 0.01$). After inoculation for 3 days, a non-mutagenized control SUT13C003 was the most susceptible with average severity score of 1.75, which was significantly higher than those of other lines. In addition, we found that one of the mutant, SUT13E18301 had no symptom on its leaves throughout the experiment (Table 3.5).

The interactions between single-spore isolates and *Dendrobium* lines were highly significant ($p < 0.01$). The highest severity of symptoms on *Dendrobium* leaves was observed when a non-mutagenized control SUT13C003 was inoculated with NK-53-9 at 3 days after inoculation (5.00). In addition, we also found moderate severity of symptoms on SUT13C003 leaves when it was inoculated with NK-53-6 and NK-53-5 (3.00 and 2.50, respectively). However, no symptom was observed when it was inoculated with NK-53-7 and NK-59-11. By contrast, all *Dendrobium* leaves that were inoculated with control (ROW), remained unchanged throughout the experiment. The

mutant, SUT13E18303 could resist some *P. palmivora* isolates with no symptom and showed very low severity of symptoms when inoculated with NK-53-7 and NK-59-11 (1.00). In addition, it was found that SUT13E18301 was potentially resistant to all isolates of *P. palmivora* with no symptom observed when infected with any isolates (Table 3.6). Three of five single-spore *P. palmivora* isolates had efficiently infected non-mutagenized control SUT13C003, especially NK-53-9, which was the most virulent isolate with the highest severity score and followed by NK-53-6 and NK-53-5, respectively. While the mutant SUT13E18301 was resistant to all single-spore *P. palmivora* isolates and the mutant SUT13E18303 showed very low severity of symptoms when inoculated with NK-53-7 and NK-59-11, which may have resulted from genetic variation induced by EMS. These preliminary results suggest that the single-spore *P. palmivora* isolates and plant genotypes affected the severity of symptoms. Therefore, an evaluation of resistance levels should be assessed with more than one isolate.

Table 3.4 Effects of single-spore isolates of *P. palmivora* on severity of symptoms of *Dendrobium* leaves by a detached leaf assay at 3 days after inoculation.

Isolates	Severity of symptoms [1]
ROW (control) [2]	0.00 ± 0.00 c [3]
NK-53-5	0.83 ± 0.56 b
NK-53-6	1.00 ± 0.43 ab
NK-53-7	0.33 ± 0.22 bc
NK-53-9	1.67 ± 0.71 a
NK-59-11	0.33 ± 0.22 bc
F-test	**
CV (%)	19.96

[1] Severity scale: 0, no symptom; 1, very small localized lesions; 2, yellow around lesions; 3, yellow around lesions, hyphae was revealed; 4, brown lesions, hyphae was expanded; 5, brown lesions, hyphae was expanded outside the area covered (Nyasse et al., 1995).

[2] ROW: reverse osmosis water

[3] Means $\pm$ SE in the same column with different letters are significantly different ($p < 0.05$) based on DMRT.

Table 3. 5 Effects of *Dendrobium* lines on severity of symptoms at 3 days after inoculation with *P. palmivora*.

Lines	Severity of symptoms [1]
SUT13C003 (control)	1.75 ± 0.45 a [2]
SUT13E18301	0.00 ± 0.00 b
SUT13E18303	0.33 ± 0.16 b
F-test	**
CV (%)	19.96

[1] Severity scale: 0, no symptom; 1, very small localized lesions; 2, yellow around lesions; 3, yellow around lesions, hyphae was revealed; 4, brown lesions, hyphae was expanded; 5, brown lesions, hyphae was expanded outside the area covered (Nyasse et al., 1995).

[2] Means $\pm$ SE in the same column with different letters are significantly different ($p < 0.05$) based on DMRT.

Table 3.6 Severity of symptoms on different *Dendrobium* lines after inoculation with different single-spore isolates of *P. palmivora*.

Lines	Isolates	Severity of symptoms [1]
	ROW (control) [2]	0.00 ± 0.00 d [3]
	NK-53-5	2.50 ± 1.44 bc
	NK-53-6	3.00 ± 0.00 b
SUT13C003 (control)	NK-53-7	0.00 ± 0.00 d
	NK-53-9	5.00 ± 0.00 a
	NK-59-11	0.00 ± 0.00 d
	ROW (control)	0.00 ± 0.00 d
	NK-53-5	0.00 ± 0.00 d
	NK-53-6	0.00 ± 0.00 d
SUT13E18301	NK-53-7	0.00 ± 0.00 d
	NK-53-9	0.00 ± 0.00 d
	NK-59-11	0.00 ± 0.00 d
	ROW (control)	0.00 ± 0.00 d
	NK-53-5	0.00 ± 0.00 d
	NK-53-6	0.00 ± 0.00 d
SUT13E18303	NK-53-7	1.00 ± 0.58 cd
	NK-53-9	0.00 ± 0.00 d
	NK-59-11	1.00 ± 0.58 cd
	F-test	**
	CV (%)	19.96

[1] Severity scale: 0, no symptoms; 1, very small localized lesions; 2, yellow around lesions; 3, yellow around lesions, hyphae was revealed; 4, brown lesions, hyphae was expanded; 5, brown lesions, hyphae was expanded outside the area covered (Nyasse et al., 1995).

[2] ROW: reverse osmosis water

[3] Means ± SE in the same column with different letters are significantly different ($p < 0.05$) based on DMRT.

3.5 Discussion

Cultural characteristics of single-spore *P. palmivora* isolates from Nakhon Ratchasima, Thailand were affected by different media used. All isolates exhibited maximum mycelial growth when they were cultured on PSA and V8A. While the lower induction on mycelial growth of some isolates was observed on CMPA and PDA which induced the lowest growth in all isolates. Therefore, optimal culture media for mycelial growth of all *P. palmivora* isolates were PSA and V8A. While CMPA, a newly developed medium could promote the earliest sporulation. Similarly, V8 agar and broth have been reported as excellent growth media for species of *Phytophthora* and *Pythium* (Jeffer, 2006). V8A was also the best medium for growth and reproduction of *P. colocasiae* giving 83.47 mm of mycelial growth while PDA gave a mycelial growth of only 51.46 mm (Palomar et al., 1999).

In addition, carrot agar also promoted a maximum radial growth of *P. colocasiae* (Padmaja et al., 2015). PSA, and PSB (agar-free) have been used for culturing *P. parasitica*, and PSB was successfully used to obtain CF for *in vitro* selection of *Citrus jambhiri* for resistance to *Phytophthora* species (Savita et al., 2011). Likewise, supplementing other legumes in soybean agar medium was also superior to V8A for supporting growth rates and reproduction of sporangia of *Phytophthora* and *Pythium* species (Guo and Ko, 1993). The colony characteristics of *Phytophthora* species; appearance, rates and manner of growth, amount of sporulation, and sporangia sizes appear to depend on media types (Appiah et al., 2003). *Phytophthora* species usually grow best on media that contain thiamine, a suitable carbohydrate source (sucrose), organic additives (potato, oat, pea, etc.), nitrogen sources, inorganic salts and minor elements (Erwin and Ribeiro, 1996). In addition, growth characteristics of mycelium

also depended on the types of media used. While our results showed rosette, radiate and irregular morphology of *P. palmivora* when isolates were cultured on PDA and CMPA, PSA, and V8A, respectively, *Phytophthora* species from cocoa (*Theobroma cacao*, L.) exhibited stellate, cottony and cottony, and rosette with slight stellate morphology for *P. palmivora*, *P. megakarya* and *P. capsici* on V8A, respectively (Appiah et al., 2003). Moreover, *P. arenaria* isolates produced colonies with a radiate morphology on V8A and CA and radiate patterns on MEA and PDA, while some isolates also produced irregular colony morphologies (Phytophthora database, 2016). The variation in colony patterns depended on the frequency, angle and extent of hyphal branching, and emphasized that certain *Phytophthora* species had distinctive colony patterns that persisted under a variety of cultural conditions (Appiah et al., 2003).

When the three *Dendrobium* lines were inoculated with five isolates of *P. palmivora* in a detached leaf assay (10^6 zoospores/mL), NK-53-9, NK-53-6 and NK-53-5 appeared to be virulent on SUT13C003 (control), and NK-53-9 was found to elicit the highest severity of symptoms. The symptoms observed were as found on orchid leaves naturally. Similarly, when curcuma was inoculated with 10^6 zoospores/mL of *P. palmivora*, disease symptoms were similar to those appeared in natural conditions (Ann, 2000b). The severity of symptoms also largely depended on plant genotypes. When rubber (*Hevea brasiliensis*) leaves were inoculated with $5x10^6$ spores/mL of *P. palmivora*, the BPM-24 (resistant) line was found to be the most resistant with small lesion size while the RRIM600 (susceptible) line showed opposite observation (Churngchow and Rattarasarn, 2001). We also found the different severity of symptoms among various *Dendrobium* lines. Interestingly, SUT13E18301 (a *Dendrobium* mutant) was resistant to all isolates of *P. palmivora* evaluated in this study and showed significantly

lower severity than control. Similarly, transgenic orange that contained PR-5-type proteins with antifungal activity against several classes of fungi and oomycetes showed higher tolerant to *Phytophthora* species than the control (Fagoaga et al., 2001).

3.6 Conclusion

These results suggest that PSA and V8A are the optimal media for promoting mycelial growth and CMPA, a newly developed medium, can induce the earliest sporulation in *P. palmivora*. We obtained the most virulent isolate (NK-53-9) to be used as an efficient screening agent for black rot resistance in *Dendrobium*. In addition, a *Dendrobium* 'Earsakul' mutant, SUT13E18301, was found to be resistant to all isolates. These finding will be useful for the development of black rot resistant *Dendrobium* cultivars in the future.

3.7 References

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CHAPTER IV

IN VITRO MUTAGENESIS AND SELECTION FOR BLACK ROT RESISTANCE IN *Dendrobium* 'Earsakul', AND CHARACTERIZATION OF BLACK ROT RESISTANT MUTANTS

4.1   Abstract

The objectives of this study were (i) to breed *D.* 'Earsakul' for black rot resistance via *in vitro* mutagenesis and selection, (ii) to evaluate black rot resistance levels in *D.* 'Earsakul' mutants and controls using detached leaf assay, (iii) to verify genetic changes of putative resistant mutants using inter simple sequence repeat (ISSR) analysis and (iv) to investigate DNA content and genome size using flow cytometry and morphological traits in black rot resistant mutants. Ethyl methanesulfonate (EMS)-mutagenized PLBs obtained by 1.4% (LD_{30}) and 1.8% (LD_{50}) EMS concentrations and non-mutagenized PLBs were selected for black rot resistance using *in vitro* selection with PSB medium supplemented with 0, 30 and 50% (first 2 cycles) and 0, 40 and 60% (3rd cycle) of *P. palmivora* culture filtrates (CFs). After 3 cycles, we obtained 50 putative resistant mutants, and 42 of these were used for black rot resistance level evaluation by detached leaf assay at the laboratory level using *P. palmivora* isolate NK-53-9. We obtained 13 black rot resistant *D.* 'Earsakul' putative mutants, including four highly resistant putative mutants namely SUT17E14303, SUT13E18301, SUT13E18305

and SUT17E18311, and nine resistant putative mutants namely SUT13E18303, SUT13E18304, SUT17E18303, SUT17E18313, SUT17E18316, SUT17E18322, SUT16E18502, SUT16E18503 and SUT16E18505. Less disease symptoms were also exhibited on leaves of black rot resistant putative mutants when compared to a non-mutagenized control after evaluating the stability of resistance against two additional *P. palmivora* isolates. These black rot resistant *D.* 'Earsakul' putative mutants were further evaluated for genetic variation using ISSR markers and also investigated for nuclear DNA content and genome size using flow cytometry. The results showed that all black rot resistant putative mutants were genetically different from the non-mutagenized controls and were confirmed as mutants. Ten highly resistant and resistant mutants and three non-mutagenized controls were found to possess the same chromosome number of 2n+4n+8n. Moreover, a mutant SUT17E18316 exhibiting maximum DNA content and genome size was identified. Morphological characterization revealed that most of the black rot resistant mutants were morphologically different on some characters from non-mutagenized lines such as plant height and number of roots etc. Particularly, a highly resistant mutant SUT13E18305 which possessed outstanding characters may be useful for future commercialization.

Keywords: Black rot, ethyl methanesulfonate, genome size, *in vitro* selection, morphological trait, nuclear DNA content, orchid.

4.2 Introduction

The genus *Dendrobium* was identified by Olaf Swartz in 1799 AD (Puchooa, 2004). It is the third-largest genus in the family of Orchidaceae. Thailand is one of the

major tropical orchid exporters in the world. The main orchid species that are exported in Thailand include *Dendrobium, Mokara* and *Oncidium,* respectively (The Government Public Relations Department, 2018; Lerthiran, 2020). However, many problems were encountered with labour management and production factors such as water quality, transportation, disease and pests, especially the water mold, *Phytophthora palmivora*, causing black rot disease. It is one of the major diseases of various orchid genera including *Dendrobium, Oncidium, Paphiopedilum, Phalaenopsis, Rhynchostylis* etc. After infection, small black lesions are initially shown on roots or basal pseudobulbs, subsequently the black lesions completely expand to cover the pseudobulbs, leaves and throughout the plant until the orchid dies (Uchida 1994; Cating et al., 2010).

Breeding for disease resistance in orchids through mutation and *in vitro* selection is a powerful and valuable approach which may be more efficient than conventional breeding in improving desirable traits such as agronomic traits and disease resistance under certain circumstances (Hammerschlag et al., 1995; Brar and Jain, 1998; Predieri, 2001). Ethyl methanesulfonate [EMS ($CH_3OSO_2C_2H_5$)], is an alkylating agent which is commonly used as a chemical mutagen for inducing point mutation in DNA. EMS greatly generates cytosine (C)-to-thymine (T) substitutions resulting in a high frequency of C/guanine (G) to T/adenine (A) transitions, low frequency of G/C to C/G or G/C to T/A transversions through 7-ethylguanine hydrolysis or A/T to G/C transitions through 3-ethyladenine pairing errors, and can induce 2 to 10 mutations/Mb of diploid DNA (Krieg, 1963; Greene et al., 2003; Kodym and Afza, 2003; Kim et al., 2006; Till et al., 2007). This chemical has been applied to improve new varieties with high yield or desirable traits, i.e. producing phenotypic variation, disease resistance and salt tolerance in several plants (Rao et al., 1977; Yudhvir, 1995; Muangsorn and Te-chato,

2008; Lethin et al., 2020). Usage of EMS and culture filtrate (CF) that contains phytotoxin/toxin as selective agent for disease resistance has been reported in citrus and banana. While Savita et al. (2017) selected citrus (*Citrus jambihiri*) calli for resistance to *P. parasitica* CF, they found that after 4th selection cycles, calli treated with 100 and 200 mM EMS for 6 hr showed 24 and 52 % tolerance in 100% CF, respectively. Similarly, EMS-derived mutants of the banana (*Musa paradisiaca* L.) cv. Puttable acquired from *in vitro* selection in MS medium supplemented with *F. oxysporum* f.sp. *cubense* (FOC) CFs (5-15%) showed a significant increase in some oxidative enzymes and pathogenesis-related (PR) proteins, reaching higher levels than control. It was also found that FOC symptoms were delayed in EMS-derived mutants, compared to control. (Krishna et al., 2013). For orchids, Dehgahi et al. (2016) selected *Fusarium proliferatum*-tolerant PLBs and assessed the effects of different concentrations of *F. proliferatum* CF (5-20%) *in vitro*. It was found that 2.7-folds increased in peroxidase (POD) activity was observed in CF-treated PLBs as compared to the untreated one. Furthermore, they also found that disease symptoms in all CF-treated leaflets were reduced compared to the control under the assessment of disease development. Besides, Ramírez-Mosqueda et al. (2019) selected the *F. oxysporum* f. sp. *vanillae* (Fov)-resistant *Vanilla planifolia* PLBs on MS medium with various concentrations of Fov CF (30, 40 and 50%, v/v), and obtained 40 Fov-resistant *V. planifolia* plants. Of these, 26.6% had acquired resistance to the pathogen under greenhouse conditions. The EMS-derived resistant mutants should be characterized through several methods such as morphological, cytological and molecular marker analysis, especially in ornamental plants. Visual screening of desirable characteristics, including plant growth and architecture, flowering, maturity, shape and size, disease and pest resistance, and yields has been

used to select for candidate mutants (Østergaard and Yanofsky, 2004; Arisha et al., 2015; Siddique et al., 2020). For orchids, changes in morphological characteristics have been reported in mutants i.e., increased height, larger leaves, thicker roots, higher numbers of nodes, reduced node length, and three types of chimeras (sectorial, mericlinal and periclinal) (Muangsorn and Te-chato, 2008; Samala et al., 2014). These unique characteristics could affect economic values and make these mutants priceless. However, this method may be insufficient because it is influenced by the plant developmental stages and various environmental factors. Meanwhile, cytological evaluation based on flow cytometry has been used to estimate variability in quantitative traits and genome level, as well as changes in nuclear and cytoplasmic organelle DNA (Larkin, 1998). Nuclear DNA content could be increased from 33 to 50% in colchicine-treated orchid mutant when compared with non-mutagenized controls (Choopeng et al., 2019). Similarly, flow cytometry was used to confirm successful induction of polyploidy in *D. secundum* when protocorms and plantlets were treated with colchicine at a concentration of 0.05% (Atichart and Bunnag, 2007). Moreover, Lapjit and Teng (2017) has reported changes in profiles of flow cytometric histograms in several regenerated plants of EMS-treated *Erycina pulsilla* PLBs. Thus, cytological analysis based on flow cytometry should be useful for characterizing *Dendrobium* mutants. However, mutations may not result in an easily identifiable phenotypes or chromosome changes. To avoid this limitation, direct selection based on DNA markers are required (Wannajindaporn et al., 2016). Inter simple sequence repeats (ISSRs) are regions in the genome flanked by microsatellite sequences and has proven to be a powerful, rapid, simple, reproducible and inexpensive markers to assess genetic diversity or to identify closely related cultivars in many plant species (Zietkiewicz et al., 1994; Wang et al.,

2009; Zhang et al. , 2010; Ng and Tan, 2015) . ISSR markers are effective for determining genetic variation and successfully identified the genetic differences in NaN_3- derived black rot resistant *D.* 'Earsakul' mutants, compared with non-mutagenized control (Wannajindaporn et al., 2014; 2016; Hualsawat et al., 2019).

These previous researches obviously showed that *in vitro* mutagenesis and selection is an attractive method for development of disease resistance in orchids. Therefore, the objectives of this study were (i) to breed *D.* 'Earsakul' for black rot resistance via *in vitro* mutagenesis and selection, (ii) to evaluate black rot resistance levels in *D.* 'Earsakul' mutants and controls using detached leaf assay, (iii) to verify genetic changes of putative resistant mutants using ISSR analysis and (iv) to investigate DNA content and genome size using flow cytometry and morphological traits in black rot resistant mutants, compared to controls.

4.3 Materials and methods

4.3.1 Chemical mutagenesis and *in vitro* selection for black rot resistance

D. 'Earsakul' protocorm-like bodies (PLBs) were treated with EMS at 0, 1.00, 1.25, 1.50, 1.75 and 2.00% concentrations for 4 h in 0.01 M phosphate buffer, pH 7.0 for lethal dose 30 (LD_{30}) and LD_{50} determination (Sambrook and Russell, 2001; Muangsorn and Te- chato, 2008) . Treated PLBs were then cultured on VW1 (Tantasawat et al., 2015) medium at 25°C with 12 h photoperiod. Three replications (10 PLBs per replication) per treatment were used, and the percentage of mortality of PLBs was observed daily for 14 days after culturing on the VW1 medium. LD_{30} and LD_{50} were derived from dose-response curve based on the mortality rate of PLBs with various EMS concentrations. After LD_{30} and LD_{50} of EMS were obtained, PLBs were treated

with these EMS concentrations for 4 hr. Treated PLBs were then cultured on VW1 and MS3 for PLB proliferation for 4 months (Tantasawat et al., 2015).

One hundred PLBs per EMS treatment and non-mutagenized control of *D.* 'Earsakul' were cultured in PSB medium together with *P. palmivora* CFs for 3 cycles at final concentrations of 0-60% (0, 30 and 50% (first 2 cycles) and 0, 40 and 60% (3rd cycle)), which were the most suitable medium and concentrations for effective selection of black rot resistance in *D.* 'Earsakul', and were incubated with shaking at 50 rpm for 21 days at 25°C with 12 h photoperiod. After incubation in each cycle, survival PLBs based on necrosis area per PLB <50% were selected and cultured on VW1 medium (Tantasawat et al., 2015) for 2 weeks before proceeding to the next selection cycle. Those that survived through 3 cycles of selection were selected as black rot resistant putative mutants (Khairum et al., 2018).

4.3.2 Evaluation of black rot resistance levels in *D.* 'Earsakul' putative mutants using detached leaf assay in laboratory

A single-spore *P. palmivora* isolate NK-53-9, which was the most virulent isolate from our previous experiment was cultured on CMPA medium to induce sporulation, and 42 *D.* 'Earsakul' mutants which were mutagenized by EMS and survived *in vitro* selection with *P. palmivora* CFs and six non-mutagenized controls were tested for pathogenicity according to Khairum et al. (2016) method. To evaluate for stability of resistance, two additional single-spore isolates of *P. palmivora* (SK-63-1 and SK-63-5 from Songkhla province) were prepared according to Pettongkhao et al. (2020) method. Inoculums of NK-53-9, SK-63-1 and SK-63-5 were adjusted to 10^7 zoospores/mL, and 3 µL drops of each suspension were inoculated on leaves of five black rot resistant *D.* 'Earsakul' putative mutants (SUT13E18301 (HR), SUT13E18303

(R), SUT13E18304 (R), SUT13E18305 (HR) and SUT16E18502 (R)) and a non-mutagenized control (SUT16C014 (S)) to test for pathogenicity according to Nyasse et al. (1995) and Khairum et al. (2016). A scale of disease severity scores from 0 to 5 was as follows; 0 = no symptom; 1 = very small localized lesions; 2 = yellow around lesions; 3 = yellow around lesions, hyphae was revealed; 4 = brown lesions, hyphae was expanded; 5 = brown lesions, hyphae was expanded outside the area covered. Resistance levels were determined from disease severity scores; highly resistant (HR; 0.00-0.50 scores), resistant (R; 0.51-1.50 scores), moderately resistant (MR; 1.51-2.50 scores), moderately susceptible (MS; 2.51-3.50 scores) and susceptible (S; 3.51-5.00 scores).

4.3.3 Characterization of black rot resistant mutants

4.3.3.1 Verification of genetic changes of putative resistant mutants using ISSR analysis

4.3.3.1.1 DNA isolation

From 13 black rot resistant *D*. 'Earsakul' putative mutants, which were evaluated for their black rot resistance levels, fresh young leaves of only ten putative resistant mutants and non-mutagenized control were used, while other putative resistant mutants (SUT17E14303, SUT17E18313 and SUT17E18322) died. These 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).

4.3.3.1.2 ISSR analysis

Twelve 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 4.1). These primers with microsatellite repeats (AG, 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). Each 20 μL of PCR mix contained 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). 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 4.1 Primer sequences and annealing temperature for each ISSR primer used for the analysis of *D.* 'Earsakul' putative mutants and non-mutagenized controls.

Primers	Primer sequences	Annealing temperature (°C)
807	$(AG)_8T$	53.0
811	$(GA)_8C$	53.0
812	$(GA)_8A$	53.0
817	$(CA)_8A$	53.0
818	$(CA)_8G$	53.0
825	$(AC)_8T$	53.0
829	$(TG)_8C$	58.0
835	$(AG)_8YC$	48.0
841	$(GA)_8YC$	54.0
851	$(GT)_8YG$	54.0
868	$(GAA)_6$	48.0
880	$(G(GA)_2)_3$	48.0

Y = T, C

4. 3. 3. 1. 3 Data scoring, cluster and principle coordinate analysis

The clearly amplified bands were coded as 0 or 1 for their absence or presence, respectively. Similarity coefficients between various putative resistant mutants and a non- mutagenized 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 black rot resistant putative mutants and non-mutagenized controls to a specific cluster in the UPGMA cluster analysis was determined by Mantel correlation test (Mantel, 1967) . A principal coordinate analysis (PCoA) was performed by NTSYSpc version 2. 2 to show the multiple dimensions in the scatter-plot of the D. 'Earsakul' line distributions (Keim et al., 1992). The potential of ISSR markers for estimation of genetic variability was evaluated by polymorphism information content (PIC) measurement. PIC values were calculated using the formula $PIC = 1- \sum pi^2$, where pi is the frequency of the ith allele (Smith et al., 1997).

4.3.3.2 Investigation of chromosomal variation

All of those black rot resistant mutants and non- mutagenized controls used in the verification of genetic changes using ISSR markers were used for analysis of DNA content and genome size using flow cytometry. Nuclei for DNA content analysis were extracted from leaves of ten black rot resistant mutants from EMS-mutagenized plants and three non- mutagenized plants. The fluorescence of at least 10,000 DAPI- stained nuclei were measured using flow cytometer (Quantum analysis flow cytometer, (QAFCMQP2), Friesoythe, Germany) at the 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}). The reference standard peak was set at channel 200 of relative fluorescence intensity for instrument calibration. The histograms were computerized by software (CyPAD pantau). The *Dendrobium* 2C DNA contents were calculated based on *D. lindleyi* Steud. DNA using the following formula:

Dendrobium 2C DNA content = $\dfrac{\text{Sample peak mean}}{D.\ lindleyi \text{ peak mean}}$ × 2C DNA of *D. lindleyi* 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).

4.3.3.3 Morphological characterization

Morphological characterization was carried out in all of those black rot resistant mutants and non- mutagenized controls used in the verification of genetic changes using ISSR markers and flow cytometry analysis. In this experiment, a resistant mutant SUT17E18303 was not used in the characterization due to insufficient number of replications. Plant height, number of nodes, node length, leaf length, number of leaves, root length and number of roots were measured by direct morphological observation according to Wannajindaporn et al. (2016).

4.3.4 Statistical analysis

The experimental data were analyzed by analysis of variance (ANOVA) and mean comparison was performed by Duncan's multiple range test (DMRT) using SPSS version 16.0 (Levesque and SPSS Inc., 2006).

4.4 Results

4.4.1 Chemical mutagenesis and *in vitro* selection for black rot resistance

Effects of different EMS concentrations (0 (control), 1.00, 1.25, 1.50, 1.75, 2.00% EMS) on mortality of *Dendrobium* PLBs were evaluated at 7 and 14 days after treatment. The results showed that the percentages of mortality PLB increased as concentrations of EMS increased and the effects of EMS concentrations were highly significant on mortality of PLBs throughout the experiment ($p < 0.01$). After 7 days, the maximum percentage of mortality was obtained at 2.00% EMS (63.33%), which was not significantly different from 1.75% EMS (30.00%). Whereas, control had no percentage of PLB mortality (0%). Similar results were observed until 14 days with a slight increase in mortality over time. The LD_{30} and LD_{50} were obtained with 1.40% and 1.80% EMS, respectively at 14 days (Table 4.2; Figure 4.1). These concentrations were further used for *Dendrobium* PLB mutagenesis.

Mutagenized (1.4 and 1.8% EMS) and non-mutagenized *Dendrobium* PLBs were used for *in vitro* selection for black rot resistance with PSB medium supplemented with 0-60% of *P. palmivora* CFs. When PSB medium supplemented with 0, 30 and 50% (first 2 cycles) and 0, 40 and 60% (3rd cycle) of *P. palmivora* CFs were used for *in vitro* selection of mutagenized and non-mutagenized control *Dendrobium* PLBs, PLB survival declined with the increase in the CF concentrations. All PLBs survived in PSB medium supplemented with 0% of *P. palmivora* CFs (control) (Table 4.3).

At similar cycles and CF concentrations, mutagenized PLBs showed greater survival percentages than control PLBs, and mutagenized PLBs which were induced by 1.8% EMS showed maximum survival percentages. Fifty putative resistant mutants (22 putative mutants from 1.4% EMS and 28 putative mutants from 1.8% EMS) were

obtained after 3 cycles of *in vitro* selection (Table 4.3). After culturing for 4 months, eight of these died, leaving 42 putative mutants (10 putative mutants from 1.4% EMS and 32 putative mutants from 1.8% EMS) for evaluation of resistance levels.

Table 4.2 Percentages of mortality of PLBs treated with different EMS concentrations at 7 and 14 days.

EMS concentrations (%)	The percentages of mortality of PLBs (%)	
	7 days	**14 days**
0 (Control)	0.00 ± 0.00 c [1]	0.00 ± 0.00 c
1.00	0.00 ± 0.00 c	0.00 ± 0.00 c
1.25	8.33 ± 8.33 bc	16.67 ± 12.02 bc
1.50	26.67 ± 13.64 b	31.67 ± 11.67 b
1.75	30.00 ± 10.41 ab	41.67 ± 21.86 ab
2.00	63.33 ± 7.26 a	76.67 ± 14.53 a
F-test	**	**
CV (%)	63.79	62.49

[1] Means $\pm$ SE in the same column with different letters are significantly different ($p < 0.05$) DMRT.
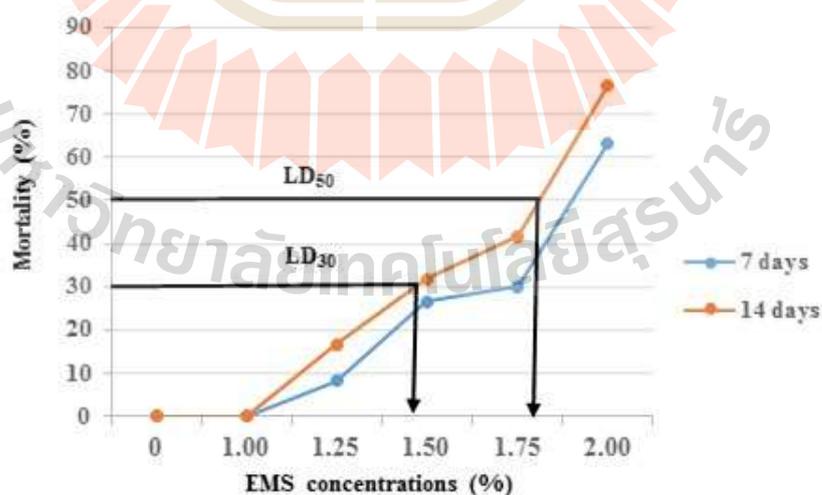

Figure 4.1 Dose-response curve based on the mortality percentage of PLBs treated with different EMS concentrations at 7 and 14 days.

4.4.2 Evaluation of black rot resistance levels in *D.* 'Earsakul' putative mutants using detached leaf assay in laboratory

The severity score of symptoms was evaluated in forty-two *D.* 'Earsakul' putative mutants which were induced by EMS and *in vitro* selected with *P. palmivora* CFs, and non-mutagenized controls by a detached leaf assay. When *D.* 'Earsakul' leaves were inoculated with a 10^7 zoospores/mL suspension of *P. palmivora* single-spore isolate NK-53-9 from Nakhon Ratchasima province, and symptom development was observed at 3 and 5 days after inoculation, a significant difference was observed among *D.* 'Earsakul' lines after inoculation at both 3 and 5 days ($p < 0.01$). From 42 putative mutants that survived *in vitro* selection, four were highly resistant (9.5%), nine were resistant (21.4%), eight were moderately resistant (19.0%), nine were moderately susceptible (21.4%) and twelve were susceptible (28.6%). We obtained 12 black rot highly resistant/resistant *D.* 'Earsakul' putative mutants from 1.8% EMS-mutagenized plants, and only one black rot highly resistant *D.* 'Earsakul' putative mutant from 1.4% EMS-mutagenized plants (a total of 31%). One putative mutant from 1.4% EMS-mutagenesis namely SUT17E14303, and three putative mutants from 1.8% EMS-mutagenesis namely SUT13E18301, SUT13E18305, SUT17E18311, which were selected in PSB medium supplemented with 30-30-40% of *P. palmivora* CFs were highly resistant. Percentages of highly resistant putative mutants obtained after *in vitro* selection were 10 and 9.4% from 1.4 and 1.8% EMS-mutagenesis, respectively.

Six of nine putative mutants from 1.8% EMS-mutagenesis namely SUT13E18303, SUT13E18304, SUT17E18303, SUT17E18313, SUT17E18316, SUT17E18322, which were selected in PSB medium supplemented with 30-30-40% of *P. palmivora* CFs, and three of nine putative mutants from 1.8% EMS-mutagenesis namely

SUT16E18502, SUT16E18503 and SUT16E18505, which were selected in PSB medium supplemented with 50-50-60% of *P. palmivora* CFs were resistant (28.1% from 1.8% EMS-mutagenesis). All together 37.5% of 1.8% EMS-mutagenized plants that survived *in vitro* selection with *P. palmivora* CFs were either highly resistant or resistant to the disease, suggesting the effectiveness of this approach, particularly when using 1.8% EMS. At lower EMS concentration (1.4%), only 10% of survived putative mutants were highly resistant or resistant.

Eight putative mutants from 1.8% EMS-mutagenesis, which were selected in PSB medium supplemented with 30-30-40% of *P. palmivora* CFs, were moderately resistant (25.0% from 1.8% EMS-mutagenesis). Moreover, we also found five putative mutants that were moderately susceptible from 1.4% EMS-mutagenesis, which were selected in PSB medium supplemented with 30-30-40% and 50-50-60% of *P. palmivora* CFs (50.0% from 1.4% EMS-mutagenesis). Similarly, four putative mutants from 1.8% EMS-mutagenesis, which were selected in PSB medium supplemented with 30-30-40% of *P. palmivora* CFs, were moderately susceptible (12.5% from 1.8% EMS-mutagenesis).

Nevertheless, it was found that four putative mutants from 1.4% EMS-mutagenesis, which were selected in PSB medium supplemented with 30-30-40% and 50-50-60% of *P. palmivora* CFs, were susceptible (40.0% from 1.4% EMS-mutagenesis). Likewise, eight putative mutants from 1.8% EMS-mutagenesis, which were selected in PSB medium supplemented with 30-30-40% and 50-50-60% of *P. palmivora* CFs, were found to be susceptible (25.0% from 1.8% EMS-mutagenesis). Conversely, when six non-mutagenized controls which were selected in PSB medium without *P. palmivora* CFs were considered, all of them were susceptible (100%) (Figure 4.2).

To evaluate the stability of resistance against other *P. palmivora* isolates, five highly resistant and resistant black rot *D.* 'Earsakul' putative mutants (SUT13E18301 (HR), SUT13E18303 (R), SUT13E18304 (R), SUT13E18305 (HR) and SUT16E18502 (R) and non-mutagenized control (SUT16C014 (S)) were evaluated for black rot resistance by detached leaf assay with 10^7 zoospores/mL suspension of two additional *P. palmivora* single-spore isolates (SK-63-1 and SK-63-5 from Songkhla province). Necrotic lesions and hyphae were formed differently among *D.* 'Earsakul' lines after inoculation with SK-63-5 ($p < 0.01$). Less disease symptoms were observed on leaves of black rot resistant putative mutants with the disease severity scores of 0.25-0.88, compared to a non-mutagenized control SUT16C014 (3.00). SUT16E18502, SUT13E18305 and SUT13E18301 showed the highest resistance to this isolate (highly resistance) with the scores of 0.25, 0.38 and 0.50, respectively. Moreover, SUT13E18303 and SUT13E18304 also showed resistance response with the scores of 0.83 and 0.85, respectively. Similarly, when these *D.* 'Earsakul' lines were inoculated with NK-53-9, disease severity scores were highly significantly different between *D.* 'Earsakul' putative mutants and a non-mutagenized control ($p < 0.01$). SUT13E18301, SUT13E18305 and SUT13E18303 were classified as highly resistance while SUT16E18502 and SUT13E18304 were resistant to this isolate. By contrast, the non-mutagenized control exhibited susceptible response. On the other hand, disease severity scores were not significantly different among *D.* 'Earsakul' lines after inoculation with SK-63-1 ($p > 0.05$). However, all putative mutants still showed less disease symptoms than non-mutagenized control (Table 4.4).

Table 4.3 *In vitro* selection of *D.* 'Earsakul' PLBs on different concentrations of *P. palmivora* CFs.

Treatments	CFs (%)	No. of PLBs	First selection cycle		Second selection cycle		Third selection cycle		Overall survival rate (%)
			Survival rate (%)	Survival PLBs (n)	Survival rate (%)	Survival PLBs (n)	Survival rate (%)	Survival PLBs (n)	
Control (0% EMS)	0-0-0 [1]	100	100.00	100	100.00	100	100.00	100	100.00
	30-30-40	100	72.00	72	47.22	34	11.76	4	4.00
	50-50-60	100	1.00	1	100.00	1	100.00	1	1.00
1.4% EMS	0-0-0	100	100.00	100	100.00	100	100.00	100	100.00
	30-30-40	100	75.00	75	69.33	52	34.62	18	18.00
	50-50-60	100	56.00	56	28.57	16	25.00	4	4.00
1.8% EMS	0-0-0	100	100.00	100	100.00	100	100.00	100	100.00
	30-30-40	100	76.00	76	64.47	49	46.94	23	23.00
	50-50-60	100	63.00	63	7.94	5	100.00	5	5.00

[1] CF concentrations (%) of first, second and third selection cycles.

Figure 4.2 Evaluation of black rot resistance levels in *D*. 'Earsakul' lines using detached leaf assay in laboratory. A scale of disease severity scores from 0 to 5 was used; 0 = no symptom, 1 = very small localized lesions, 2 = yellow around lesions, 3 = yellow around lesions, hyphae was revealed, 4 = brown lesions, hyphae was expanded and 5 = brown lesions, hyphae was expanded outside the area covered. Disease response based on severity scores was as follows; highly resistant (HR; 0.00-0.50 scores), resistant (R; 0.51-1.50 scores), moderately resistant (MR; 1.51-2.50 scores), moderately susceptible (MS; 2.51-3.50 scores) and susceptible (S; 3.51-5.00 scores).

Table 4.4 Severity scores of symptoms after inoculation with *P. palmivora* single-spore isolates NK-53-9, SK-63-1

and SK-63-5 on 5 black rot resistant putative mutants and non-mutagenized control.

Lines	Severity score of symptoms[1]					
	NK-53-9	Disease response [2]	SK-63-1	Disease response	SK-63-5	Disease response
SUT16C014	4.66 ± 0.67 a [3]	S	3.38 ± 0.24	MS	3.00 ± 0.00 a	MS
SUT13E18301	0.00 ± 0.00 b	HR	1.50 ± 0.87	R	0.50 ± 0.29 b	HR
SUT13E18303	0.50 ± 0.29 b	HR	0.67 ± 0.33	R	0.83 ± 0.44 b	R
SUT13E18304	0.83 ± 0.44 b	R	1.17 ± 0.44	MR	0.88 ± 0.72 b	R
SUT13E18305	0.00 ± 0.00 b	HR	1.50 ± 0.87	MR	0.38 ± 0.24 b	HR
SUT16E18502	0.67 ± 0.33 b	R	1.00 ± 0.58	R	0.25 ± 0.25 b	HR
F-test	**		ns [4]		**	
CV (%)	15.18		25.36		22.94	

[1] A scale of disease severity scores from 0 to 5 was used; 0 = no symptom, 1 = very small localized lesions, 2 = yellow around lesions, 3 = yellow around lesions,

hyphae was revealed, 4 = brown lesions, hyphae was expanded and 5 = brown lesions, hyphae was expanded outside the area covered.

[2] Disease response based on severity scores was as follows; highly resistant (HR; 0.00-0.50 scores), resistant (R; 0.51-1.50 scores), moderately resistant (MR; 1.51-

2.50 scores), moderately susceptible (MS; 2.51-3.50 scores) and susceptible (S; 3.51-5.00 scores).

[3] Means ± SE in the same column with different letters are significantly different ($p < 0.05$) based on DMRT.

[4] ns=not significant

4.3.3 Characterization of black rot resistant mutants

4.3.3.1 Verification of genetic changes and genetic vaiation of black rot resistant mutants

Ten of 13 putative resistant mutants that survived after 6 months were evaluated by 12 ISSR primers for their genetic variability compared with non-mutagenized control. A total of 157 amplified fragments among all *D.* 'Earsakul' lines and 44 polymorphic fragments were produced, giving a polymorphism percentage of 28.02%. Five to 18 reproducible bands were amplified per primer (average 13.08), of these one to seven polymorphic fragments were produced in each primer (average 3.67). The length of amplified ISSR fragments ranged from 170 bp (ISSR 811 and 817) to 1900 bp (ISSR 807). ISSR 851 produced the highest percentage of polymorphism (50.00%), followed by ISSR 829 (46.15%) and 868 (42.89%), respectively. Among different ISSR primers analyzed, ISSR 812 gave maximum PIC value of 0.320, followed by ISSR 811 (0.293) and 880 (0.262), respectively (Table 4.5).

A total of 44 polymorphic bands were used to construct a dendrogram based on cluster analysis using UPGMA. The Mantel test with a cophenetic correlation coefficient value of 0.95 ($p < 0.01$) indicated that data in the similarity matrix were well represented by the dendrogram. All putative resistant mutants were genetically different from non-mutagenized controls and were confirmed as true mutants. Two clusters and eight individuals were differentiated at the genetic similarity of 0.96 (Figure 4.3). Non-mutagenized controls were grouped into cluster I, while SUT16E18503 and SUT16E18505 were grouped into cluster II. Whereas, eight resistant mutants, SUT13E18301, SUT13E18303, SUT13E18304, SUT13E18305, SUT17E18303, SUT17E18311, SUT17E18316 and SUT16E18502 could not be grouped into any clusters.

The Jaccard's genetic similarity coefficients ranged from 0.677 to 0.985 (average 0.908) among the pairwise combinations of all *D.* 'Earsakul' lines. A resistant mutant SUT17E18316 (0.750) was found to be the most dissimilar from non-mutagenized controls On the contrary, the highly resistant mutant SUT17E18311 (0.951) was found to be the most similar with non-mutagenized controls.

The PCoA with three-dimensional plots based on ISSR marker was used to analyze multidimentional relationships. The three coordinates explained 28.77, 20.45, and 18.15% with a total of 67.37% of the total variance. PCoA clearly separated non-mutagenized controls in cluster I from black rot resistant putative mutants, while SUT16E18503 and SUT16E18505 were separated as individuals rather than grouped into cluster II. In addition, SUT17E18316 was obviously separated from the other lines (Figure 4.4).

Table 4.5 Primer sequences, annealing temperature, number of total scorable DNA bands, number of polymorphic DNA band, percentages of polymorphism, and amplified band size for each ISSR primer used for the analysis of *D.* 'Earsakul' putative mutants and non-mutagenized controls.

| Primers	Primer sequences	Annealing temperature (°C)	No. of Total bands	No. of polymorphic bands	Polymorphism (%)	Amplified band size (bp)	PIC
807	$(AG)_8T$	53.0	18	4	22.22	200-1,900	0.161
811	$(GA)_8C$	53.0	13	3	23.08	170-1,000	0.293
812	$(GA)_8A$	53.0	14	3	21.43	185-1,200	0.320
817	$(CA)_8A$	53.0	11	2	18.18	170-1,000	0.235
818	$(CA)_8G$	53.0	5	2	40.00	267-900	0.133
825	$(AC)_8T$	53.0	9	2	22.22	200-800	0.133
829	$(TG)_8C$	58.0	13	6	46.15	175-1,500	0.133
835	$(AG)_8YC$	48.0	16	1	6.67	200-900	0.133
841	$(GA)_8YC$	54.0	14	5	35.71	267-800	0.196
851	$(GT)_8YG$	54.0	14	7	50.00	242-1,000	0.149
868	$(GAA)_6$	48.0	14	6	42.86	200-900	0.133
880	$(G(GA)_2)_3$	48.0	16	3	18.75	236-1,200	0.262
Total			157	44			
Average			13.08	3.67	28.94		0.190
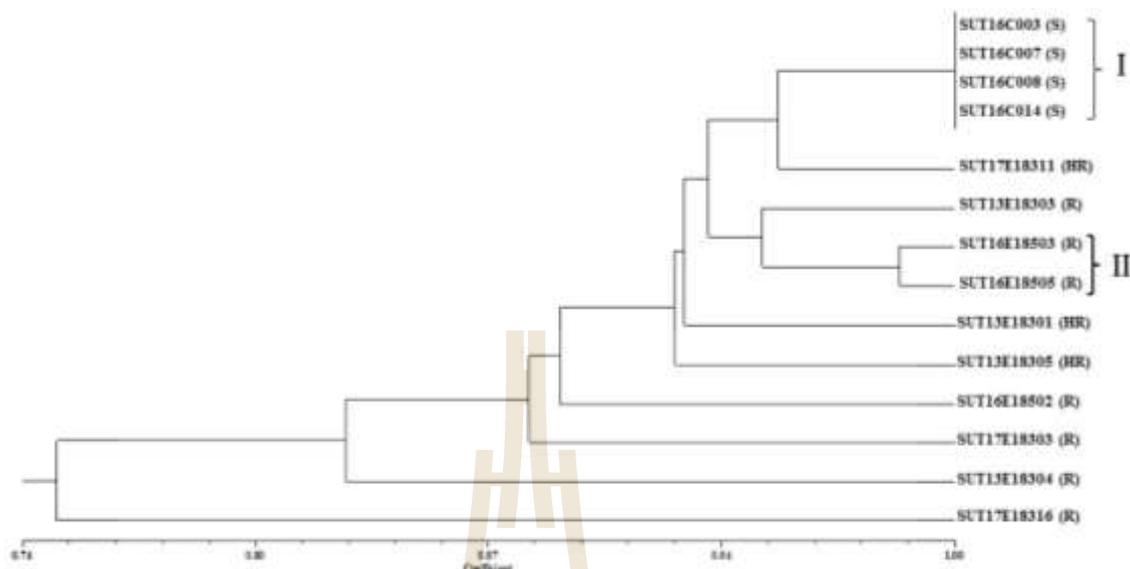
Figure 4.3 ISSR-derived dendrogram of 10 black rot resistant putative mutants and 4 non-mutagenized controls.
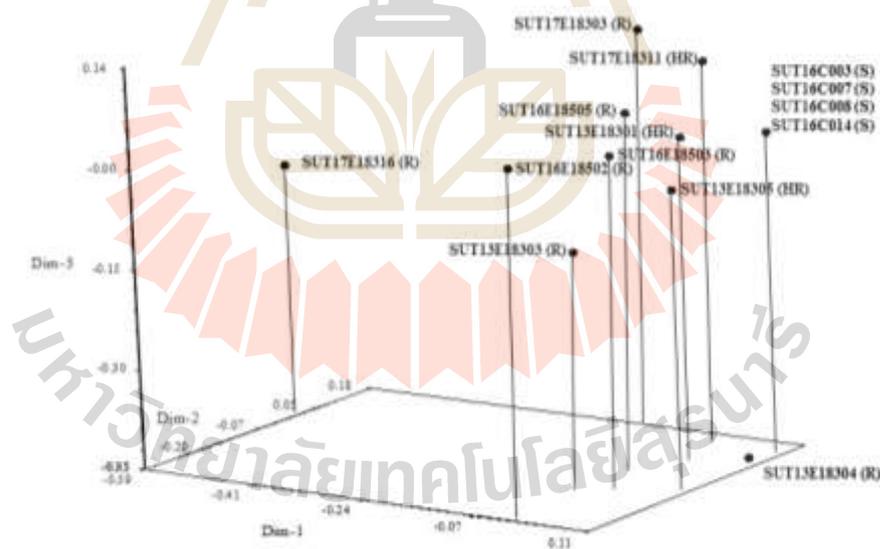
Figure 4.4 ISSR derived three- dimensional plot based on the first three principal coordinates from a principal coordinate analysis of 10 black rot resistant putative mutants and 4 non-mutagenized controls.

4.3.3.2 Investigation of chromosomal variation

Ten *D.* 'Earsakul' resistant mutants and three non-mutagenized controls were further evaluated for nuclear DNA content and genome size using flow cytometry. The results showed that all highly resistant, resistant mutants and three non-mutagenized controls had the same chromosome number of 2n+4n+8n. However, nuclear DNA content and genome size were significantly different in these *D.* 'Earsakul' lines ($p < 0.01$). A mutant SUT17E18316 exhibiting maximum DNA content (4.11 pg. $2C^{-1}$) and genome size (3,965.71 Mbp) was identified, whose DNA content and genome size were significantly higher than those of non-mutagenized controls (3.41-3.50 pg. $2C^{-1}$ and 3,289.31-3,371.94 Mbp) and most of the mutants. However, it was not significantly different from those of SUT17E18303 (3.96 pg. $2C^{-1}$ and 3,818.18 Mbp), SUT16E18505 (3.91 pg. $2C^{-1}$ and 3,770.78 Mbp), SUT16E18503 (3.90 pg. $2C^{-1}$ and 3,765.81 Mbp) and SUT17E18311 (3.90 pg. $2C^{-1}$ and 3,759.98 Mbp) (Table 4.6; Figure 4.5). No relationship was observed between DNA content and genome size and either disease response or concentrations of CFs used for selection (Table 4.6).

Table 4.6 Nuclear DNA content and genome size of black rot resistant mutants and non-mutagenized plants.

Lines	DNA content (pg. 2C[-1])	Genome size (Mbp)	CFs (%)	Disease Response [1]
SUT16C003	3.41 ± 0.15 e [2]	3,289.31 ± 144.51 e	0-0-0	S
SUT16C007	3.47 ± 0.05 de	3,343.85 ± 48.25 de	0-0-0	S
SUT16C008	3.50 ± 0.08 de	3,371.94 ± 78.79 de	0-0-0	S
SUT13E18301	3.82 ± 0.02 bc	3,681.15 ± 19.08 bc	30-30-40	HR
SUT13E18303	3.80 ± 0.02 bc	3,670.25 ± 18.75 bc	30-30-40	R
SUT13E18304	3.68 ± 0.02 cd	3,549.15 ± 23.25 cd	30-30-40	R
SUT13E18305	3.58 ± 0.05 de	3,454.92 ± 51.01 de	30-30-40	HR
SUT17E18303	3.96 ± 0.02 ab	3,818.18 ± 19.30 ab	30-30-40	R
SUT17E18311	3.90 ± 0.11 abc	3,759.98 ± 110.07 abc	30-30-40	HR
SUT17E18316	4.11 ± 0.09 a	3,965.71 ± 43.55 a	30-30-40	R
SUT16E18502	3.82 ± 0.04 bc	3,686.52 ± 34.76 bc	50-50-60	R
SUT16E18503	3.90 ± 0.05 abc	3,765.81 ± 48.38 ab	50-50-60	R
SUT16E18505	3.91 ± 0.07 ab	3,770.78 ± 65.64 ab	50-50-60	R
F-test	**	**		
CV (%)	4.04	4.03		

[1] Disease response based on severity scores was as follows; highly resistant (HR; 0.00-0.50 scores), resistant (R; 0.51-1.50 scores), moderately resistant (MR; 1.51-2.50 scores), moderately susceptible (MS; 2.51-3.50 scores) and susceptible (S; 3.51-5.00 scores).

[2] Means ± SE in the same column with different letters are significantly different ($p < 0.05$) based on DMRT.
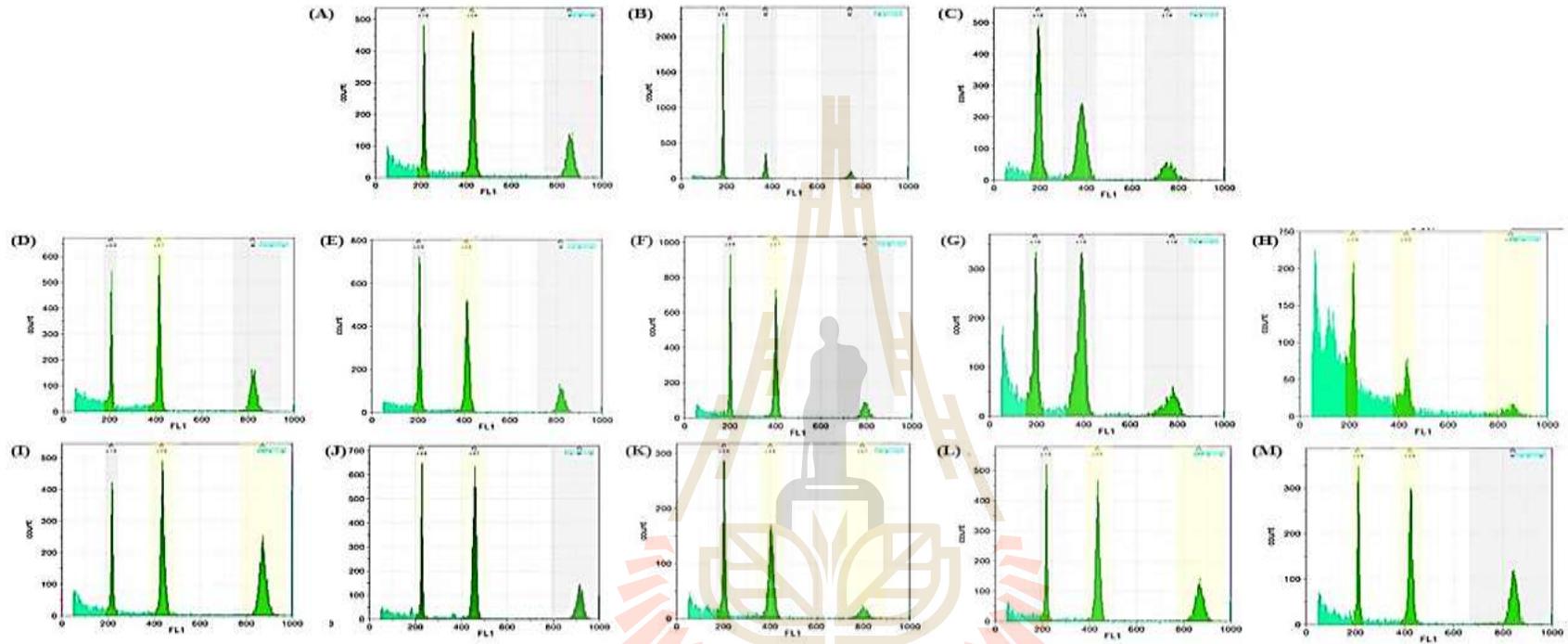
Figure 4.5 Fluorescence intensity histogram of non-mutagenized controls (A-C) and black rot resistant mutants (D-M). SUT16C003 (S)(A), SUT16C007 (S)(B), SUT16C008 (S)(C), SUT13E18301 (HR)(D), SUT13E18303 (R)(E), SUT13E18304 (R)(F), SUT13E18305 (HR)(G), SUT17E18303 (R)(H), SUT17E18311 (R)(I), SUT17E18316 (R)(J), SUT16E18502 (R)(K), SUT16E18503 (R)(L) and SUT16E18505 (R)(M). (FL= fluorescence). Disease response; S (susceptible), R (resistant) and HR (highly resistant).

4.3.3.3 Morphological characterization

The morphological characterization was carried out using 8 traits in nine of six-month-olds black rot resistant mutants, SUT13E18301 (HR), SUT13E18303 (R), SUT13E18304 (R), SUT13E18305 (HR), SUT13E18311 (R), SUT13E18316 (R), SUT16E18502 (R), SUT16E18503 (R) and SUT16E18505 (R) and three non-mutagenized controls SUT16C003 (S), SUT16C007 (S), and SUT16C008 (S). Morphological characterization revealed that most of black rot resistant mutants were morphologically different on some characters from non-mutagenized controls.

The utmost plant height was observed in SUT13E18305 and SUT13E18304 (4.03 and 4.02 cm, respectively), which were significantly higher than non-mutagenized controls (2.60-3.30 cm) and four mutants, SUT17E18311, SUT17E18316, SUT16E18503 and SUT16E18505 (2.34, 2.41, 2.45 and 3.33 cm, respectively), but not significantly different from SUT16E18502, SUT13E18301 and SUT13E18303 (3.87, 3.69 and 3.65 cm, respectively).

Similarly, three black rot resistant mutants, SUT13E18301, SUT13E18304 and SUT16E18502 showed the highest number of nodes (4.80 nodes), which were significantly higher than non-mutagenized controls (1.90-3.30 nodes) and four mutants, SUT17E18311, SUT17E18316, SUT16E18503 and SUT16E18505 (3.60, 3.40, 3.20 and 2.56 nodes, respectively), but not significantly different from SUT13E18305 (4.40 nodes).

Likewise, SUT13E18305 had the maximum node length (0.52 cm), which was not significantly different from those of most mutants (0.44-0.48 cm), but it was significantly higher than non-mutagenized controls (0.31-0.42 cm) and the mutant, SUT17E18311 (0.37 cm).

When the number of leaves was determined, the mutant SUT13E18304 exhibited the highest number of leaves (8.50 leaves), which was not significantly different from SUT13E18305, SUT13E18301, SUT16E18502 (7.80, 7.70 and 7.20 leaves, respectively). While SUT16E18505 showed the lowest number of leaves (3.78 leaves) when compared with other mutants, which was not significantly different from all non-mutagenized controls. Non-mutagenized control SUT16C003, had 2.6-fold lower number of leaves (3.20 leaves) than SUT13E18304.

For leaf length, it was not significantly different among *D*. 'Earsakul' lines. When leaf width was considered, SUT16E18505 showed the maximum leaf width (0.58 cm), which was not significantly different from most of mutants and non-mutagenized controls. However, it was significantly higher than the non-mutagenized control SUT16C003 (0.46 cm) and mutant SUT17E18311 (0.42 cm).

When the number of roots and root length were investigated, SUT13E18303 and SUT13E18305 were identified as having the highest number of roots (8.20 and 7.90 roots, respectively), which was not significantly different from SUT17E18311, SUT17E18316 and SUT16E18503 (7.00, 5.80 and 5.70 roots, respectively). Meanwhile, non-mutagenized control SUT16C003 had the lowest number of roots (2.30 roots), which was significantly lower than SUT13E18303 and SUT13E18305 (3.6 and 3.4-folds, respectively). However, it was not significantly different from other non-mutagenized controls (2.40-3.10 roots) and some mutants (3.44-4.90 roots). Moreover, the mutant SUT13E18301 had the longest root length (2.12 cm), which was significantly higher than all non-mutagenized controls (1.9 to 5.9-folds) and most mutants (1.4 to 2.6-folds), but not significantly different from SUT13E18305 (1.92 cm) and SUT13E18304 (1.56 cm) (Table 4.7; Figure 4.6).

Table 4.7 Morphological characters of 9 black rot resistant mutants and 3 non-mutagenized controls of *D.* 'Earsakul' at 6 months.

Lines	Plant height (cm)	No. of nodes	Node length (cm)	No. of leaves	Leaf length (cm)	Leaf width (cm)	No. of roots	Root length (cm)
SUT16C003	2.60 ± 0.12 d [1]	1.90 ± 0.18 e	0.31 ± 0.03 e	3.20 ± 0.25 d	0.93 ± 0.07	0.46 ± 0.03 bc	2.30 ± 0.58 d	0.36 ± 0.10 f
SUT16C007	3.30 ± 0.22 bc	2.90 ± 0.46 de	0.42 ± 0.05 bcd	3.90 ± 0.46 d	0.94 ± 0.13	0.48 ± 0.05 abc	3.10 ± 0.35 cd	1.13 ± 0.20 cd
SUT16C008	3.01 ± 0.13 cd	3.30 ± 0.65 bcd	0.33 ± 0.04 de	4.00 ± 0.47 d	1.11 ± 0.10	0.47 ± 0.03 abc	2.40 ± 0.73 d	0.41 ± 0.10 ef
SUT13E18301	3.69 ± 0.12 ab	4.80 ± 0.25 a	0.47 ± 0.02 abc	7.70 ± 0.37 ab	1.12 ± 0.09	0.52 ± 0.03 abc	4.30 ± 0.40 cd	2.12 ± 0.36 a
SUT13E18303	3.65 ± 0.24 abc	4.30 ± 0.39 abc	0.48 ± 0.03 ab	6.80 ± 0.68 bc	1.17 ± 0.07	0.56 ± 0.03 ab	8.20 ± 1.32 a	1.35 ± 0.20 bc
SUT13E18304	4.02 ± 0.19 a	4.80 ± 0.36 a	0.48 ± 0.03 ab	8.50 ± 0.62 a	1.26 ± 0.09	0.57 ± 0.03 ab	4.90 ± 0.55 bcd	1.56 ± 0.20 abc
SUT13E18305	4.03 ± 0.28 a	4.40 ± 0.34 ab	0.52 ± 0.02 a	7.80 ± 0.57 ab	1.19 ± 0.15	0.55 ± 0.05 ab	7.90 ± 1.26 a	1.92 ± 0.19 ab
SUT17E18311	2.34 ± 0.15 d	3.60 ± 0.40 bcd	0.37 ± 0.02 cde	6.30 ± 0.56 bc	0.61 ± 0.02	0.42 ± 0.02 c	7.00 ± 0.83 ab	1.02 ± 0.14 cde
SUT17E18316	2.41 ± 0.14 d	3.40 ± 0.27 bcd	0.45 ± 0.03 abc	6.00 ± 0.33 c	0.85 ± 0.07	0.54 ± 0.02 ab	5.80 ± 0.39 abc	0.81 ± 0.13 def
SUT16E18502	3.87 ± 0.27 ab	4.80 ± 0.25 a	0.48 ± 0.03 ab	7.20 ± 0.33 abc	1.04 ± 0.09	0.55 ± 0.05 ab	4.70 ± 1.03 bcd	1.47 ± 0.28 bcd
SUT16E18503	2.45 ± 0.15 d	3.20 ± 0.29 cd	0.44 ± 0.03 abc	5.70 ± 0.56 c	0.80 ± 0.06	0.52 ± 0.03 abc	5.70 ± 1.02 abc	0.83 ± 0.16 def
SUT16E18505	3.33 ± 0.42 bc	2.56 ± 0.38 de	0.48 ± 0.02 ab	3.78 ± 0.64 d	1.26 ± 0.09	0.58 ± 0.03 a	3.44 ± 0.93 cd	0.97 ± 0.13 c-f
F-test	**	**	**	**	ns [2]	*	**	**
CV (%)	21.03	32.22	23.02	26.69	22.75	22.60	31.47	22.75

[1] Means ± SE in the same column with different letters are significantly different (*p* < 0.05) based on DMRT.

[2] ns=not significant

Figure 4.6 Morphological characters of 9 black rot resistant mutants (SUT13E18301, SUT13E18303, SUT13E18304, SUT13E18305, SUT17E18311, SUT17E18316, SUT16E18502, SUT16E18503, SUT16E18505) , and 3 non- mutagenized controls (SUT16C003, SUT16C007, SUT16C008) of *D.* 'Earsakul' at 6 months (Scale bar = 1 cm).

4.5 Discussion

The percentages of mortality of *D*. 'Earsakul' PLBs were influenced by different EMS concentrations. The percentages of mortality of PLBs increased as concentrations of EMS increased. Similarly, the reduced percentage of seed germination has been observed with the increase in EMS concentrations (Talebi et al., 2012; Baghery et al., 2015). Higher concentrations of mutagen would produce higher mutation frequencies (Shah et al., 2015). Conversely, higher survival rate of treated plants was obtained with lower concentrations of EMS but the mutation rate will be lower (Porch et al., 2009). The EMS concentrations commonly used for mutation were between 25-50% of lethality (LD_{25}-LD_{50}) because they could induce high mutation rate, especially at LD_{50}. EMS is widely used at a concentration range of 0.20 to 3.80% EMS (LD_{50}), which has been reported in several plants with the frequencies of phenotypic variants of 0.90 to 5.20% (Zhu et al., 1995; Khatri et al., 2005; Jain, 2010; Emrani et al., 2011; Leitão, 2012; Talebi et al., 2012, Kumar et al., 2013; Shirani et al., 2016; Yadav et al., 2016; Deng et al., 2020). In our study, treated PLBs with 1.8% EMS (LD_{50}) for 4 hr appeared to be most effective for inducing mutation in *D*. 'Earsakul'. By contrast, Muangsorn and Te-chato (2008) and Samala et al. (2014) reported that 0.75 to 1.0% EMS (LD_{50}) with incubation time of 90 min were optimum for inducing mutation in *D. friedericksianum* Rchb.f. and *D*. 'Sonia' PLBs, resulting in the phenotypic variants such as chimera and abnormal morphological characteristics with the frequency of 5 to 11%. Different optimum concentrations and duration of EMS treatment may stem from the effects of genetypes/species or developmental stages of PLBs used for *in vitro* mutagenesis. Moreover, they also found that these mutants showed the highest growth rate with bigger leaves and thicker roots than control, and also gave the highest survival

rate of 91% after acclimatization in greenhouse.

At similar cycles and CF concentrations, mutagenized PLBs showed greater survival percentages than non-mutagenized control PLBs. Fifty putative resistant mutants were obtained after 3 cycles of *in vitro* selection for black rot resistance from 1.4% and 1.8% EMS-mutagenesis, but only five non-mutagenized controls survived. The mortality of PLBs in the PBS medium containing *P. palmivora* CFs was probably due to the presence of toxic metabolites released by *P. palmivora* into the CFs. Rudolph (1976) and Yoder (1983) reported that toxin-induced leakage of electrolytes from plant tissues had plasmalemma as the site of action. The toxin could induce electrolyte leakage in calli and leaves which gradually decreased when the toxin concentration was diluted (Tripathi et al., 2008). At higher CF concentrations, only resistant cells were allowed to grow, while the sensitive cells were eliminated (Savita et al., 2011). Similarly, our results showed greater survival rate of PLBs in PBS medium supplemented with *P. palmivora* CFs at 30-30-40% than at 50-50-60%. Four highly resistant and six resistant putative mutants were obtained from selection with 30-30-40% CFs, while only three resistant putative mutants were obtained from 50-50-60% CFs.

Furthermore, oomycete *P. palmivora* also secretes an extracellular protein with ca. 10.5 kDa molecular weight, known as α-elicitin which acts as elicitor. Elicitins are pathogen-associated molecular patterns (PAMPs). Microbe-associated molecular patterns (MAMPs) or PAMPs were recognized by pattern recognition receptors (PRRs) in plants and lead to the induction of MAMP- or PAMP-triggered immunity (MTI or PTI) that can overcome microbe or pathogen infection by inducing primary defense responses (Capasso et al., 2001; Baillieul et al., 2003; Khairum et al., 2018; Malik et al.,

2020). We found that some EMS-mutagenized PLBs showed lesion mimic resembling hypersensitive response (HR) lesions and had greater survival percentages. By contrast, non-mutagenized controls showed large necrotic lesions throughout portions of PLBs and eventually died.

We obtained 12 black rot resistant *D.* 'Earsakul' mutants from 1.8% EMS-mutagenesis, and only one black rot resistant *D.* 'Earsakul' mutant from 1.4% EMS-mutagenesis after black rot resistance level evaluation by detached leaf assay with *P. palmivora* isolate NK-53-9 from Nakhon Ratchasima province. These results suggest that 1.8% EMS was more effective for generating black rot resistant mutants. Moreover, five of these black rot resistant mutants also showed resistance to two *P. palmivora* isolates from Songkhla province after the stability of resistance evaluation. Similarly, Yudhvir (1995) reported that EMS-mutagenized tomato showed the highest disease resistance to fruit rot disease. Similarly, EMS-mutagegenized abaca plants obtained from *in vitro* selection in shoot induction (SI) medium supplemented with *F. oxysporum* f. sp. cubence (FOC) CF showed higher resistance to fusarium wilt than non-mutagenized control (Purwati et al., 2007). These results suggest that EMS is an effective chemical mutagen that may induce point mutation, or chromosome aberrations that lead to genotypic and phenotypic changes, resulting in lower disease severity scores in black rot resistant *D.* 'Earsakul' mutants than non-mutagenized controls. *In vitro* selection for *Fusarium* spp. resistance has been reported in *Dendrobium* and *Vanilla* orchids. But to the best of our knowledge, we are the first to report *in vitro* mutagenesis using EMS and selection for black rot resistance in *Dendrobium* 'Earsakul'.

EMS is an effective mutagen to enhance genetic variations randomly and mainly generates 70-99% of GC = AT transition mutations (Till et al., 2004; 2007). We were

able to employ ISSR markers to detect genetic variations of all ten putative black rot resistant mutants whose DNA patterns differed from those of all 4 non-mutagenized controls, confirming that they are true mutants and implicating that in vitro EMS mutagenesis can induce genetic alterations in *Dendrobium*. The altered DNA patterns may stem either from the loss/gain of ISSR primer binding sites as a result of point mutations from a single base pair deletion, addition or substitution induced by EMS or changes that alter the size or prevent the amplification of a target DNA i.e., deletion, insertion and inversion (Hofman et al., 2004). Moreover, the randomly induced genetic variability by EMS was evidenced by the genetic dissimilarity ranging from 0.015 to 0.323 among all non-mutagenized controls and ten highly resistant and resistant mutants. Only two resistant mutants were grouped together in cluster II, while eight highly resistant and resistant individuals were not grouped in any cluster, possibly indicating that the mutations in these mutants have occurred in different loci, which may not only affect black rot resistance but also other phenotypes. Previous studies also revealed genetic variability among mutants when assessed by ISSR markers (Wu et al., 2011; Tantasawat et al., 2017).

The results showed that all ten highly resistant and resistant mutants evaluated, and three non-mutagenized controls had the same chromosome number of 2n+4n+8n (mixoploid), consisting of all peaks of 200 (diploid), 400 (tetraploid) and 800 (octaploid) cells. These results substantiate that EMS typically causes a high frequency of gene mutations, while causes low frequency of chromosome aberrations. However, loss of a chromosome segment or deletion was also reported in many plants (van Harten, 1998; Khatri et al., 2005; Kozgar et al., 2011; Jagajanantham et al., 2013; Kashid and More, 2016). The high frequency of gene mutation (transition and transversion) is

naturally occurred during DNA replication. Most of these errors are repaired but some may pass on to the next cell division, particularly the G1 phase. EMS alters the percentages of nuclei distribution within the cell cycle, which resulted in decrease in the percentages of nuclei in G0 + G1 and increase in the G2 + M compartment (Morris et al., 1992). This abnormal phenomenon of the cell cycle may result in higher DNA content and genome size of *D*. 'Earsakul' mutants when compared to non-mutagenized controls.

Morphological characterization revealed that most of black rot resistant mutants were morphologically different on some characters from non-mutagenized controls e.g., plant height and number of roots etc. Likewise, Samala et al. (2014) has reported larger size of guard cells and lower stomatal density in 0.75% EMS-mutagenized *D*. 'Sonia' PLBs and higher growth rate with bigger leaves and thicker roots in 12 months old *D*. 'Sonia' mutants than non-mutagenized control. In addition, 1% EMS induced three types of chimeras, sectorial, mericlinal and periclinal at a frequency of 5, 15 and 11%, respectively in *D. friedericksianum* (Muangsorn and Te-chato, 2008). Interestingly, the black rot resistant mutant SUT13E18305, which was highly resistant to two *P. palmivora* isolates and moderately resistant to one isolate, also possessed outstanding morphological characters such as plant height, node length and number of roots, which may be useful for future commercialization. These black rot resistant mutants will be transferred to the greenhouse for further evaluation of flower and inflorescence characteristics as well as the level of whole plant resistance in the greenhouse.

4.6   Conclusion

EMS is effective for inducing mutation to increase black rot resistance. *In vitro* selection with *P. palmivora* CF offers a potential tool useful for generating black rot resistant mutants in orchids. Fifty putative resistant mutants were obtained after 3 cycles of *in vitro* selection for black rot resistance. Thirteen black rot resistant putative mutants with high black rot resistance levels at the laboratory level were identified. ISSR markers revealed that all black rot putative mutants evaluated were genetically different from non-mutagenized control. In addition, one of these black rot resistant mutants, SUT17E18316 showed maximum DNA content and genome size. Interestingly, SUT13E18305, which was highly resistant to two *P. palmivora* isolates and moderately resistant to one isolate also had distinctive morphological characters. These resistant mutants will be transferred to the greenhouse for further evaluation of flower and inflorescence characteristics, which are important traits for commercialization.

4.7   References

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CHAPTER V

DIFFERENTIAL INDUCTION OF β-1,3-GLUCANASE AND CHITINASE ACTIVITIES IN BLACK ROT RESISTANT MUTANTS OF *Dendrobium* 'Earsakul' IN RESPONSE TO *Phytophthora palmivora* INFECTION

5.1 Abstract

Plants possess several resistance mechanisms to protect themselves from diverse pathogens. Pathogenesis-related (PR) proteins are produced as parts of constitutive and induced chemical defenses. In this current study, we investigated the induction of 2 PR proteins, β-1,3-glucanase and chitinase, and protein profies in response to *Phytophthora palmivora* infection in leaves of non-mutagenized controls and black rot resistant *Dendrobium* 'Earsakul' mutants. The systemic and local induction of both PR-proteins after challenging with the pathogen was also studied using whole plant assay. Accumulation of β-1,3-glucanase and chitinase before the infection was significantly different among resistant mutants and controls in the detached leaf assay. Infection of *P. palmivora* increased the activities of both PR proteins in some mutants and non-mutagenized controls throughout the experimental period of 3 days after inoculation (DAI). Particularly, the highly resistant mutant SUT13E18301 had much higher magnitude of β-1,3-glucanase and chitinase induction as compared to controls. For other highly resistant and resistant mutants, the induction

patterns of both PR proteins were different. Up-regulated protein bands of 15, 16, 39 and 54 kDa were identified in some black rot resistant mutants but not in non-mutagenized controls. These unique protein bands may be related to antifungal activities during the infection of *P. palmivora* and may be used as protein markers for black rot resistance mechanisms in orchids. Using whole plant assay, total protein and both PR proteins were not significantly different among 5 resistant mutants and a non-mutagenized control in non-inoculated treatment. At 3 DAI, the highest total protein was observed in *P. palmivora* infected leaves of SUT16C008, SUT13E18301 and SUT13E18303, compared to the DI water inoculated and non-inoculated treatments. However, no difference was found among the 3 treatments in distal leaves of *D.* 'Earsakul' mutants and non-mutagenized control. Both local and systemic induction of ß-1,3-glucanase were observed in highly resistant mutants SUT13E18301 and SUT13E18305, but only SUT13E18305 exhibited induced chitinase activities in both distal and infected leaves. These biochemical characteristics provide insight into resistance mechanism of *Dendrobium* against *P. palmivora* and may be effectively utilized to characterize other putative black rot resistant *D.* 'Earsakul' mutants in the future. These results can be useful for future breeding programs for black rot resistance in orchids. Moreover, SUT13E18305 may be developed into a new commercial variety or used as new black rot resistance resource for other orchid varieties.

Keywords: Black rot; enzyme activity; local induction; mutants; pathogenesis-related
(PR) proteins; *Phytophthora palmivora*; resistance; systemic induction.

5.2   Introduction

Thailand has more than 1,000 species of orchids and is currently one of the largest exporter of tropical orchids in the world. *Dendrobium* is one of the main species that are exported in international trade (Pizano, 2005; Thammasiri, 2016). Therefore, the production of high- quality orchids is highly important. However, the orchid production nowadays faces with the problem of plant diseases, resulting in low-quality crop yields. Black rot is one of the most serious diseases and is caused by *Phytophthora cactorum* or *Phytophthora palmivora*. *P. palmivora* is a hemibiotrophic oomycete that starts infection like biotrophs and then progresses to the necrotrophic stage (Hine, 1962; Uchida, 1994; Orlikowski and Szkuta, 2006; Cating et al., 2010). More than 170 different species of host plants, including monocots and dicots, were attacked by *P. palmivora* (Erwin and Ribeiro, 1996; Drenth and Guest, 2013; Torres et al., 2016). After infection in the orchid, roots or basal portion of the pseudobulbs initially exhibited small black lesions and onwards the black lesions expanding to completely cover the other parts of the orchid, and eventually, the orchid plant dies (Cating et al., 2010). Applications of systemic fungicides are widely used to control this disease in various crops. However, a broad range of non- target organisms can be highly affected by fungicide toxic, including hazardous effects on human, pollution in soil and water etc. Moreover, high costs, low yields and low price due to poor quality are found in orchid production. Therefore, disease tolerant/resistant orchids are crucial for environmentally friendly and sustainable orchid production by protecting against major diseases that attack orchids.

Plants have evolved constitutive and inducible defense mechanisms against pathogen invasion. Constitutive defenses include cell walls, waxy epidermal cuticles, and bark which are preformed barriers to inhibit the invasion of plants. Subsequently,

the invading pathogens will be detected by plant cells which respond with inducible defenses including production of toxic chemicals and pathogen-degrading enzymes as well as deliberate cell suicide. Because of the high energy costs and nutrient requirements associated with their production and maintenance, plants often wait until pathogens are detected before producing toxic chemicals or defense-related proteins (Freeman and Beattie, 2008; Gupta et al., 2013)

Systemic acquired resistance (SAR) is induced by most pathogens in distal/ uninfected plant tissues following salicylic acid (SA) and pathogenesis-related (PR) protein accumulation (e.g., PR1, chitinase, glucanase). It provides long-lasting and broad-spectrum resistance to subsequent infection. Thus, SAR is a highly desirable type of resistance in plants (Choudhary et al., 2007). PR proteins like ß-1,3-glucanase and chitinase are commonly encoded by multigenic families and may have different isoelectric point (pI) and various functions in plants, including plant growth and development, wounding and defense responses. They are strongly induced when plants respond to infection by several pathogens (Leubner-Metzger et al., 1999; Neuhaus, 1999a; van Loon, 1999; Wu and Bradford, 2003). Induced ß-1,3-glucanase and chitinase activities after fungal infection has been reported in lemon seedlings (Fanta et al., 2003). Similarly, both β-1,3-glucanase and chitinase activities were induced in *Eruca sativa* plants to a higher extent in resistant variety after inoculation with fungal pathogen *Alternaria brassicicola* (Gupta et al. 2013). It was also found that fusarium (*Fusarium oxysporum* f. sp. *melonis*)-infected musk-melon showed higher β-1,3-glucanase activity in resistant plants than susceptible plants (Netzer et al., 1979). In addition, ß-1,3-glucanase and chitinase activities are also parts of the important pre-

formed constitutive defenses.

The protein profiling of germplasm has been widely and effectively used to propose the taxonomic and evolutionary aspects in several crops (Ghafoor et al., 2002; Alghamdi, 2009; Abdel-Razzak et al., 2012; Nagaraja et al., 2016). It was found that ca. 20-44 and 13-43 kDa bands of β-1,3-glucanase and chitinase, respectively which were identified using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were induced in several plants (e.g., grapevine, banana, moth bean and rocket salad) upon pathogen infection (Jayasankar et al., 2000; El-Hadary and Tayel, 2013; Gupta et al., 2013; Krishna et al., 2013; Pareek et al., 2014; Sinha et al., 2014). These protein bands may be used as protein markers for the disease resistance mechanisms in order to characterize putative resistant mutants.

To reduce the use of fungicides for sustainable production of orchids, newly improved disease resistant orchid cultivars should be utilized, and in order to develop durable resistance for black rot in orchid by mutation and molecular breeding, a good understanding of plant defense mechanism is essential. We have previously obtained several black rot resistant *D.* 'Earsakul' mutants from mutagenesis with ethyl methansulfonate (EMS) and *in vitro* selection using culture filtrate (CF) of *P. palmivora*. These mutants have been characterized based on genetic variation, DNA content, genome size and morphological characters. However, their resistance mechanisms have not been investigated. In this research, the induction of two PR proteins i.e., β-1,3-glucanase and chitinase activity levels of some of these black rot resistant *D.* 'Earsakul' mutants and non-mutagenized control were evaluated to explore the resistance mechanisms exhibited by each mutant. In addition, their protein profiles were also investigated before and after *P. palmivora* inoculation to identify protein bands possibly associated with the resistance mechanisms. Moreover, the systemic and

local induction of β-1,3-glucanase and chitinase activities of these black rot resistant *D.* 'Earsakul' mutants and non-mutagenized control was also evaluated after challenging with *P. palmivora* using the whole plant assay. This was the first report of biochemical characterization in these new black rot resistant *D.* 'Earsakul' mutants.

5.3   Materials and methods

5.3.1   Induction of β-1,3-glucanase and chitinase activities following *P. palmivora* inoculation in detached leaf assay

Four month-olds highly resistant (HR) and resistant (R) black rot resistant *D.* 'Earsakul' mutants (SUT13E18301 (HR), SUT13E18305 (HR), SUT17E18303 (R), SUT17E18311 (HR), SUT17E18316 (R) and SUT16E18502 (R)) were obtained from *in vitro* mutagenesis using EMS and selection for black rot resistance on pea sucrose broth (PSB) supplemented with 0-60% *P. palmivora* culture filtrates (CFs) for 3 cycles. These mutants have been shown to be resistant to *P. palmivora* isolates from Nakhon Rachasima and Songkla provinces using detached leaf assay. These resistant mutants and two non-mutagenized controls (SUT16C003 (susceptible; S) and SUT16C008 (S)) were inoculated with the most virulent *P. palmivora* Nakhon Ratchasima isolate NK-53-9 inoculum which was prepared according to the method of Khairum et al. (2016) and then was adjusted to 10^7 zoospores/mL. *D.* 'Earsakul' leaves (nodes 2 and 3 counting from the top) were longitudinally divided into 2 equal halves, and 5 µL of inoculum were inoculated on half of the leaves after pin wounding in a detached leaf assay and incubated at 25°C in the dark for 3 days. The inoculated half-leaves were harvested at different time interval 0-3 days after inoculation. The other half-leaves were not inoculated and were harvested immediately for use as zero-day treatment (before inoculation). After each of half-leaves was weighed, crude protein was

extracted for β-1,3-glucanase and chitinase activity measurement and protein profile evaluation. Four replications per *Dendrobium* line were used for each time point.

5.3.2 Protein extraction

Crude protein extracts were prepared in chilled protein extraction buffer pH 5.0 that contained 0.1 M sodium citrate buffer pH 5.0, 0.1 M KCl, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 0.5% (v/v) Triton X-100, 1 μg/mL leupeptin, 1 mM ethylenediaminetetraacetic acid (EDTA), 3% (w/v) polyvinylpolypyrrolidone (PVPP) (Thipyapong et al., 1995; Gupta et al., 2013) using pre-chilled mortar and pestle. The homogenate was transferred into chilled 1.7-mL eppendorf tubes and subsequently centrifuged at 12,000 rpm and 4 ℃ for 30 min. The supernatant was removed and used for analyzing protein profiles and enzyme activities.

5.3.3 Enzyme activity measurement

5.3.3.1 β-1,3-glucanase activity

The β-1,3-glucanase activity was spectrophotometrically determined with laminarin as a substrate (Sigma-Aldrich L9634, Germany) by dinitrosalicylic acid (DNS) method (Miller, 1959). The modified reaction mixture containing 5 uL of crude protein extract was added to 1% (w/v) laminarin in a 0.05 M sodium acetate buffer pH 5.0 and then incubated at 37 ℃ for 15 min. The reaction was stopped by addition of 50 uL 1% (v/v) DNS reagent and boiling for 8 min. After cooling down at ambient temperature, the colored solution was diluted 1:4 with sterile deionized (DI) water, and enzyme activity was measured at 500 nm absorbance using UV-spectrophotometer. The β-1,3-glucanase activity was defined as nmol of released reducing sugar (D-glucose) per min per milligram leaf fresh weight under the described conditions (Pan et al., 1991; Prakongkha, 2011; Żur et al., 2013)

5.3.3.2 Chitinase activity

The chitinase activity was also spectrophotometrically determined with colloidal chitin as a substrate (Sigma-Aldrich C-9752, Germany) by DNS method (Miller, 1959). The modified reaction mixture containing 20 μL of crude protein extract was added to 1% (w/v) colloidal chitin in a 0.05 M sodium acetate buffer pH 5.0 and then incubated at 37ºC for 60 min. The reaction was stopped by the addition of 60 uL 1% (v/v) DNS reagent and boiling for 20 min. After cooling down, the colored solution was centrifuged at 12,000 rpm for 20 min and supernatant was diluted 1:1 with sterile DI water. The enzyme activity was measured at 520 nm absorbance using UV-spectrophotometer. The chitinase activity was defined as nmol of released N-acetyl-D-glucosamine (GlcNAc)(Carlo Erba reagent, Spain) per min per milligram leaf fresh weight under the described conditions (Prakongkha, 2011; Kuzu et al., 2012; Zhou et al., 2016; Hussin and Majid, 2020).

5.3.4 SDS-PAGE

Protein profiles were analyzed by SDS-PAGE according to the modified method of Laemmli (1970) and Khairum et al. (2018). Eight uL/well of each sample were loaded in the 1 mm thick polyacrylamide gel (12% separating gel and 4% stacking gel). Electrophoresis was performed at 100 volts for 95 min. Staining was performed by using 0.1% (w/v) silver nitrate (Rockefeller University, 2016). Protein standard (Precision plus protein dual Xtra standards molecular weight (MW) range = 2-250 kDa) (Bio-Rad, USA) was used to determine the size or molecular weight of unknown proteins. Three replications were repeated for consistent results.

5.3.5 Systemic and local induction of *D.* 'Earsakul' lines upon *P. palmivora* infection in whole plant assay

Five hardened six-month olds black rot resistant *D.* 'Earsakul' mutants (SUT13E18301 (HR), SUT13E18303 (R), SUT13E18304 (R), SUT13E18305 (HR)

and SUT16E18502 (R) and a non-mutagenized control (SUT16C008 (S)) with 4 leaves (leaf nodes 1 (top), 2, 3 and 4 (bottom)) were divided into 3 treatments, consisting of 1) non-inoculation; *D.* 'Earsakul' lines were not wounded and inoculated; 2) DI water inoculation; *D.* 'Earsakul' lines were dropped with 10 uL of sterilized DI water after pin wounding and cover with a 2 mm-diameter disc of PDA agar without *P. palmivora* mycelium on leaf nodes 3 and 4, and 3) *P. palmivora* inoculation; *D.* 'Earsakul' lines were dropped with 10 uL of sterilized DI water after pin wounding and cover with a 2 mm-diameter disc of PDA agar containing mycelium of *P. palmivora* isolate NK-53-9 from 5-day-old colonies on leaf nodes 3 and 4. Individual plant of each treatment was covered with a plastic bag and was incubated at 26ºC with 12 hr photoperiod provided by fluorescence light. After 3 days, each leaf node (1, 2, 3 and 4) was separately harvested and extracted in modified protein extraction buffer that contained 0.1 M sodium citrate buffer pH 5.0, 0.1 M KCl, 1 mM PMSF, 0.5% (v/v) Triton X-100, 1 μg/mL leupeptin, 1 mM EDTA and 3% (w/v) PVPP (Thipyapong et al., 1995; Gupta et al., 2013) using pre-chilled mortar and pestle. The homogenate was transferred into chilled 1.7-mL eppendorf tubes and subsequently centrifuged at 12,000 rpm and 4ºC for 30 min. The supernatant was removed and used for analyzing total protein contents and enzyme activities of leaf nodes 3-4 to assess local induction and of leaf nodes 1-2 to evaluate systemic induction.

The total protein contents were determined using spectrophotometry at 590 nm according to Bradford (1976) method using bovine serum albumin (BSA) as standard. The total protein contents were calculated and eventually exhibited as μg per mg of leaf fresh weight. Crude protein extracts were also analyzed for enzyme activities according

to methods as described in 5.3.4. However, the β-1,3-glucanase and chitinase activities were defined as μmol of released reducing sugar and GlcNAc per min per milligram protein under the same conditions, respectively.

5.3.6 Statistical analysis

The experimental data were analyzed by analysis of variance (ANOVA) and mean comparison was performed by Duncan's multiple range test (DMRT) using SPSS version 16.0 (Levesque and SPSS Inc., 2006).

5.4 Results

5.4.1 ß-1,3-glucanase and chitinase activities of *D.* 'Earsakul' lines before and after inoculation with *P. palmivora* in detached leaf assay

The quantitative ß-1,3-glucanase and chitinase activities were evaluated in six black rot resistant *D.* 'Earsakul' mutants (SUT13E18301 (HR), SUT13E18505 (HR), SUT17E18303 (R), SUT17E18311 (HR), SUT17E18316 (R) and SUT16E18502 (R)) and two non-mutagenized controls (SUT16C003 (S) and SUT16C008 (S)) at 0-3 days after *P. palmivora* inoculation. The levels of ß-1,3-glucanase and chitinase activities at the pre-inoculated stage (0 day) were significantly different among *D.* 'Earsakul' lines ($p < 0.01$). The resistant mutant SUT17E18303 showed the highest ß-1,3-glucanase activity level (49.21 nmole.min^{-1}.mg leaf fw^{-1}), which was significantly higher than those of non-mutagenized controls SUT16C008 and SUT16C003 (24.74 and 21.43 nmole.min^{-1}.mg leaf fw^{-1}) and most of black rot resistant mutants (8.58-31.38 nmole.min^{-1}.mg leaf fw^{-1}), but not significantly different from that of the highly resistant mutant SUT13E18305 (35.34 nmole.min^{-1}.mg leaf fw^{-1}). When the chitinase activity was considered, the highest chitinase activity level was observed in black rot

resistant mutant SUT17E18311 and non-mutagenized control SUT16C008 (2.10 and 2.00 nmole.min^{-1}.mg leaf fw^{-1}, respectively), which were significantly higher than two black rot resistant mutants (0.92-1.15 nmole.min^{-1}.mg leaf fw^{-1}), but not significantly different from non-mutagenized control SUT16C003 (1.61 nmole.min^{-1}.mg leaf fw^{-1}) and three other black rot resistant mutants SUT16E18502, SUT13E18305 and SUT17E18316 (1.91, 1.46 and 1.40 nmole.min^{-1}.mg leaf fw^{-1}, respectively). By contrast, the highly resistant mutant SUT13E18301 exhibited minimum activity levels of both enzymes (Table 5.1).

ß-1,3-glucanase activity level slightly increased 1 day after inoculation in some *D.* 'Earsakul' lines (1.2 to 3.2-folds) except SUT16E18502, SUT17E18303 and SUT13E18305. Among these lines, a highly resistant mutant SUT13E18301 demonstrated the highest increasing rate of 3.2-fold when compared with the initial time (0 day). After inoculation for 2 days, ß-1,3-glucanase activity level was continuously increased in most of the resistant mutants (1.8 to 4.2-folds), especially in resistant mutant SUT13E18301 whose ß-1,3-glucanase activity level was highly induced (4.2-fold). Whereas those of non-mutagenized controls were induced with the lesser degree (1.6 to 1.8-folds). ß-1,3-glucanase activity levels in most of the resistant mutants and both non-mutagenized controls tended to decrease at 3 days after inoculation. Interestingly, those of some highly resistant mutants continued to rise at 3 days. Particularly, the highly resistant mutant SUT13E18301 whose ß-1,3-glucanase activity increased 11.1-fold from day 0 to the level 7.4 to 13.9-folds higher than those of non-mutagenized controls at the same time (Figure 5.1).

In summary, the highest induction (1.6 to 1.8-folds) of ß-1,3-glucanase activity was observed at 2 days after inoculation in both non-mutagenized controls (SUT16C003 and SUT16C008). Among the resistant mutants, the responses appeared

to vary in terms of extent and time. Two of them (SUT17E18316 and SUT16E18502) exhibited the highest induction of ß-1,3-glucanase at 2 days after inoculation similar to those found in non-mutagenized controls, but the extent of induction was greater (3.1 to 3.3-folds). Other two mutants (SUT13E18301 and SUT17E18311), however, had the highest ß-1,3-glucanase induction later at 3 days after inoculation (2.4 to 11.1-folds). By contrast, no clear induction was found in the remaining two resistant mutants (SUT13E18305 and SUT17E18303), which possessed higher constitutive ß-1,3-glucanase activity levels before inoculation.

It was also noted that the highest induced ß-1,3-glucanase activity was observed in SUT13E18301 (95.05 nmole.min^{-1}.mg leaf fw^{-1}), which was much higher than those of non-mutagenized controls (33.62 and 44.46 nmole.min^{-1}.mg leaf fw^{-1}). Moreover, maximum induced ß-1,3-glucanase activities were also higher than non-mutagenized controls in three additional resistant mutants (SUT17E18311, SUT17E18316 and SUT16E18502).

When chitinase activity was observed, the chitinase activity levels in most *D.* 'Earsakul' lines were induced within 1 day after inoculation (1.6 to 3.8-folds), while SUT17E18316 showed no increase in activity. The resistant mutant SUT17E18303 exhibited higher increase in chitinase activity level at 1 day than other *D.* 'Earsakul' lines (3.8-fold), followed by SUT16E18502 (2.0-fold), SUT13E18305 (1.9-fold) and SUT17E18311 (1.6-fold), respectively. By contrast, non-mutagenized control SUT16C008 showed the lowest induced chitinase activity level (1.4-fold) when compared with the initial time (0 day). After inoculation for 2 days, the chitinase activity levels were decreased from day 1 in most of *D.* 'Earsakul' lines whereas highly resistant mutant SUT13E18301 and non-mutagenized control SUT16C003 showed only slight increase in activities compared to day 1. When considered at 3 days after

inoculation, the chitinase activity levels continuously increased from day 2 in two black rot resistant mutants (3.0 to 4.4-folds) and a non-mutagenized control (2.2-fold), especially in the highly resistant mutant SUT13E18301 (4.4-fold) (Figure 5.2).

Our results showed that four of resistant mutants (SUT17E18303, SUT16E18502, SUT13E18305 and SUT17E18311) demonstrated the highest induction of chitinase at 1 day after inoculation (1.6 to 3.8-folds) while two others (SUT13E18301 and SUT17E18316) exhibited maximum chitinase induction later at 3 days after inoculation (3.0 to 4.4-folds). On the other hand, non-mutagenized controls had the highest chitinase activity induction at either 1 or 3 days after inoculation (1.4 to 2.2-folds). Moreover, maximum induced chitinase activity was observed in the resistant mutant SUT17E18303 (4.90 nmole.min^{-1}.mg leaf fw^{-1}), followed by SUT17E18316, SUT13E18301 and SUT16E18502 (3.80-4.13 nmole.min^{-1}.mg leaf fw^{-1}), which were higher than those of non-mutagenized controls (2.73-3.54 nmole.min^{-1}.mg leaf fw^{-1}).

5.4.2 Changes in protein profiles of *D.* 'Earsakul' lines following *P. palmivora* inoculation using SDS-PAGE

Different protein profiles were observed among *D.* 'Earsakul' lines when evaluated using SDS-PAGE. Some up-regulated protein bands were observed in most of *D.* 'Earsakul' lines as early as 1 day after inoculation except SUT17E18316 and SUT16C003 whose up-regulated bands were firstly appeared 2 days after inoculation. Eight up- and two down-regulated protein bands were found among *D.* 'Earsakul' lines throughout 3 days after inoculation. Two up-regulated protein bands of 13 and 24 kDa were presented almost in all *D.* 'Earsakul' lines. In addition, a 18 kDa up-regulated protein band was found in SUT16C008 and SUT17E18316 and a 43 kDa up-regulated protein band was expressed in SUT16C003, SUT13E18301 and SUT17E18316. Interestingly, unique up-regulated protein bands, 16 and 39 kDa were only observed in

a highly resistant mutant SUT17E18311 as early as 1 day after inoculation. In addition, a resistant mutant SUT17E18316 also possessed a unique 15 kDa up-regulated protein band at 3 days after inoculation. It is interesting to note that SUT17E18316 and SUT17E18311 did not induce the 13 kDa protein like others, but induced the 15 and 16 kDa proteins instead. A unique 54 kDa up-regulated protein band was also observed in a resistant mutant SUT16E18502 at 2 days after inoculation. Furthermore, we also found two down-regulated protein bands following infection. Two protein bands of 22 and 37 kDa were found in all *D.* 'Earsakul' lines before infection. However, after inoculation, a 37 kDa protein was down-regulated in all *D.* 'Earsakul' lines. Similarly, a protein band of 22 kDa also decreased in all *D.* 'Earsakul' lines after inoculation. Nevertheless, both protein bands appeared to decline later in some black rot resistant mutants, compared to non-mutagenized controls (Figure 5.3).

Table 5.1 β-1,3-glucanase and chitinase activities on 8 black rot resistant mutants and 2 non-mutagenized controls before inoculation (0 d).

Lines	β-1,3-glucanase activity (nmole.min^{-1}.mg leaf fw^{-1})	Chitinase activity (nmole.min^{-1}.mg leaf fw^{-1})
SUT16C003 (S)	21.43 ± 4.73 c [1]	1.61 ± 0.25 ab
SUT16C008 (S)	24.73 ± 3.67 bc	2.00 ± 0.13 a

SUT13E18301 (HR)	8.58 ± 1.62 d	0.92 ± 0.05 c
SUT13E18305 (HR)	35.34 ± 2.64 ab	1.46 ± 0.18 abc
SUT17E18303 (R)	49.21 ± 11.29 a	1.15 ± 0.22 bc
SUT17E18311 (HR)	31.38 ± 5.72 bc	2.10 ± 0.32 a
SUT17E18316 (R)	21.55 ± 3.68 c	1.40 ± 0.15 abc
SUT16E18502 (R)	17.79 ± 1.70 c	1.91 ± 0.45 ab
F-test	**	**
CV (%)	30.63	23.18

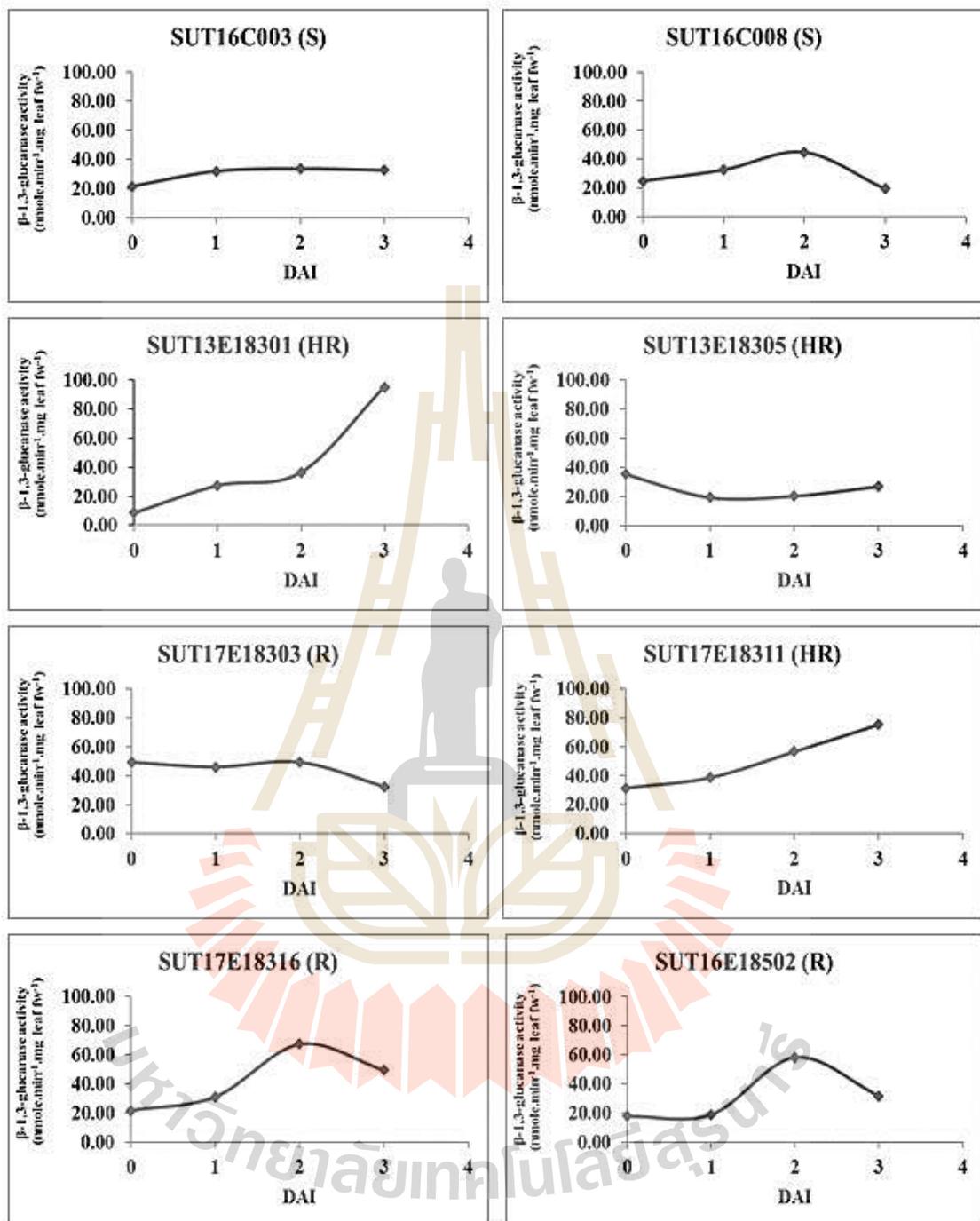[1] Means ± SE in the same column with different letters are significantly different ($p < 0.05$) based on DMRT.
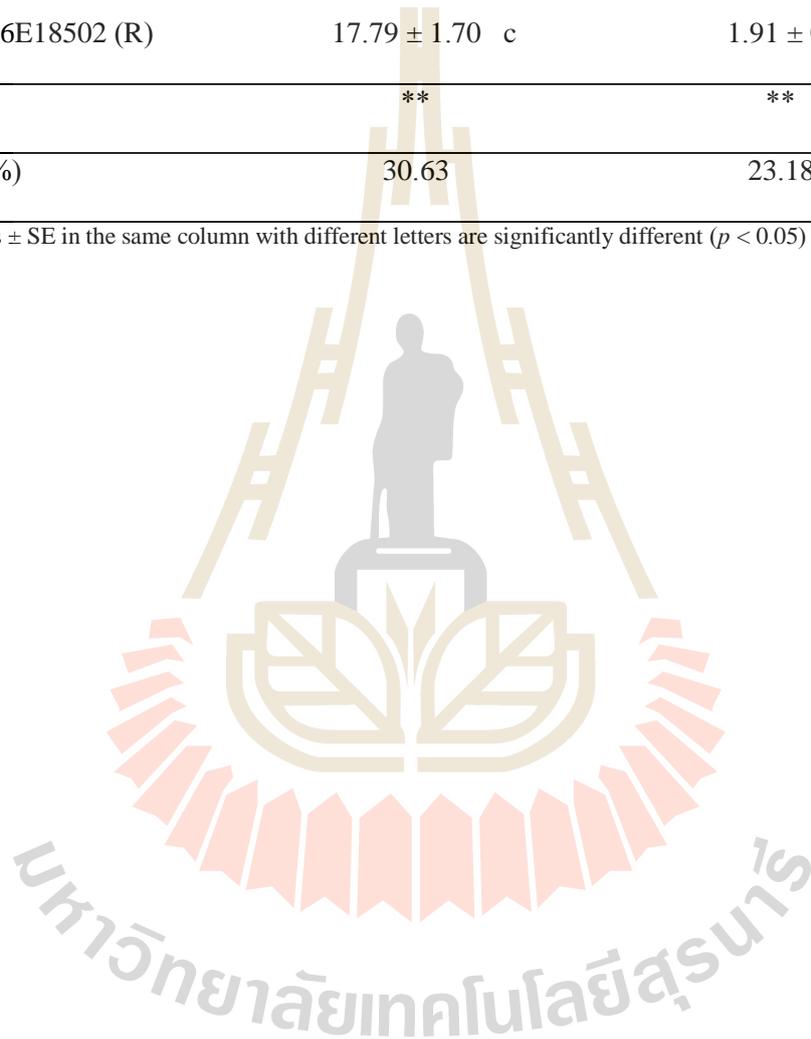
Figure 5.1 Changes in ß-1,3-glucanase activities (nmole.min^{-1}.mg leaf fw^{-1}) of 6

black rot resistant mutants and 2 non-mutagenized controls after *P.*

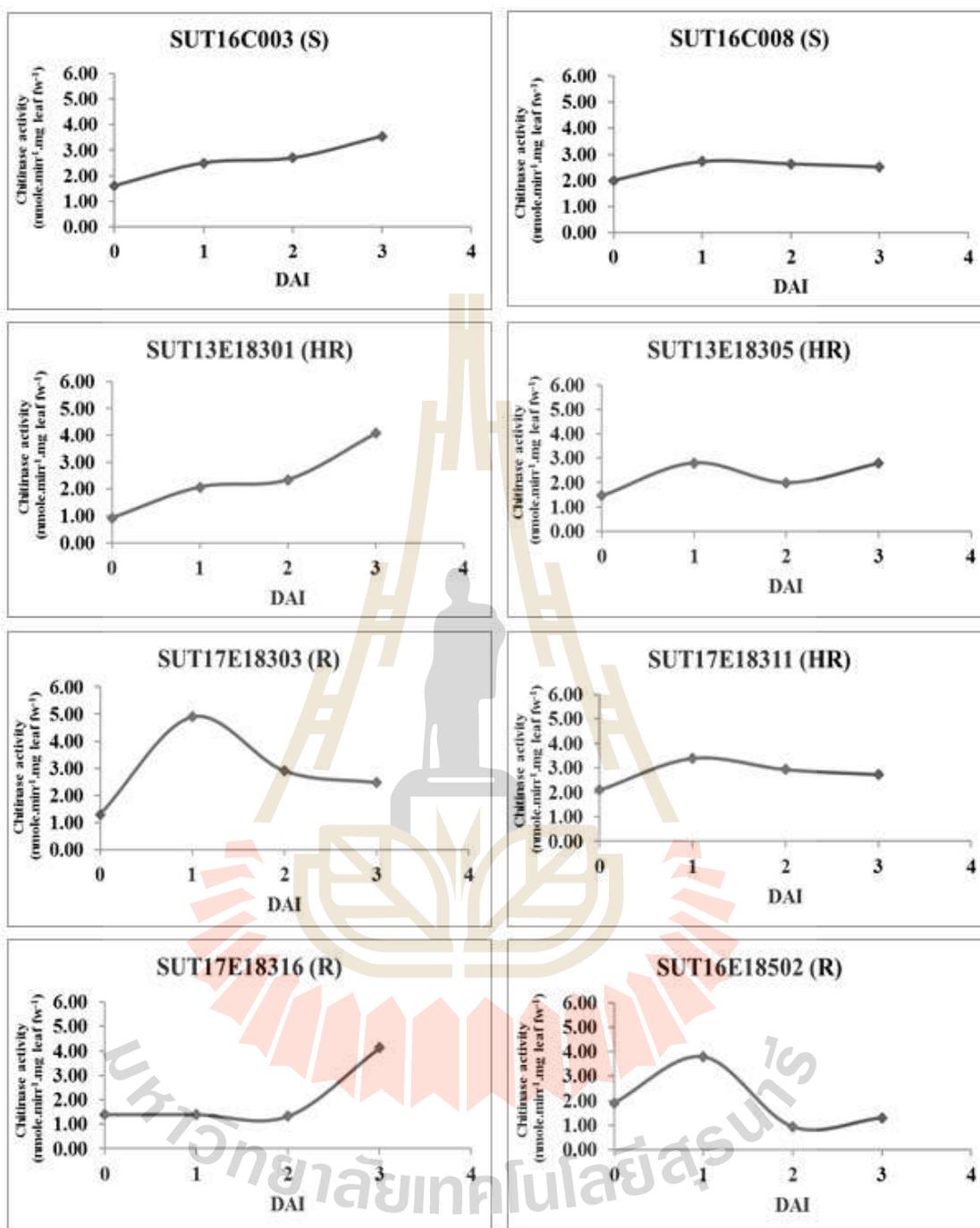*palmivora* inoculation for 0-3 days.

Figure 5.2 Changes in chitinase activities (nmole.min^{-1}.mg leaf fw^{-1}) of 6 black rot resistant mutants and 2 non-mutagenized controls after *P. palmivora* inoculation for 0-3 days.
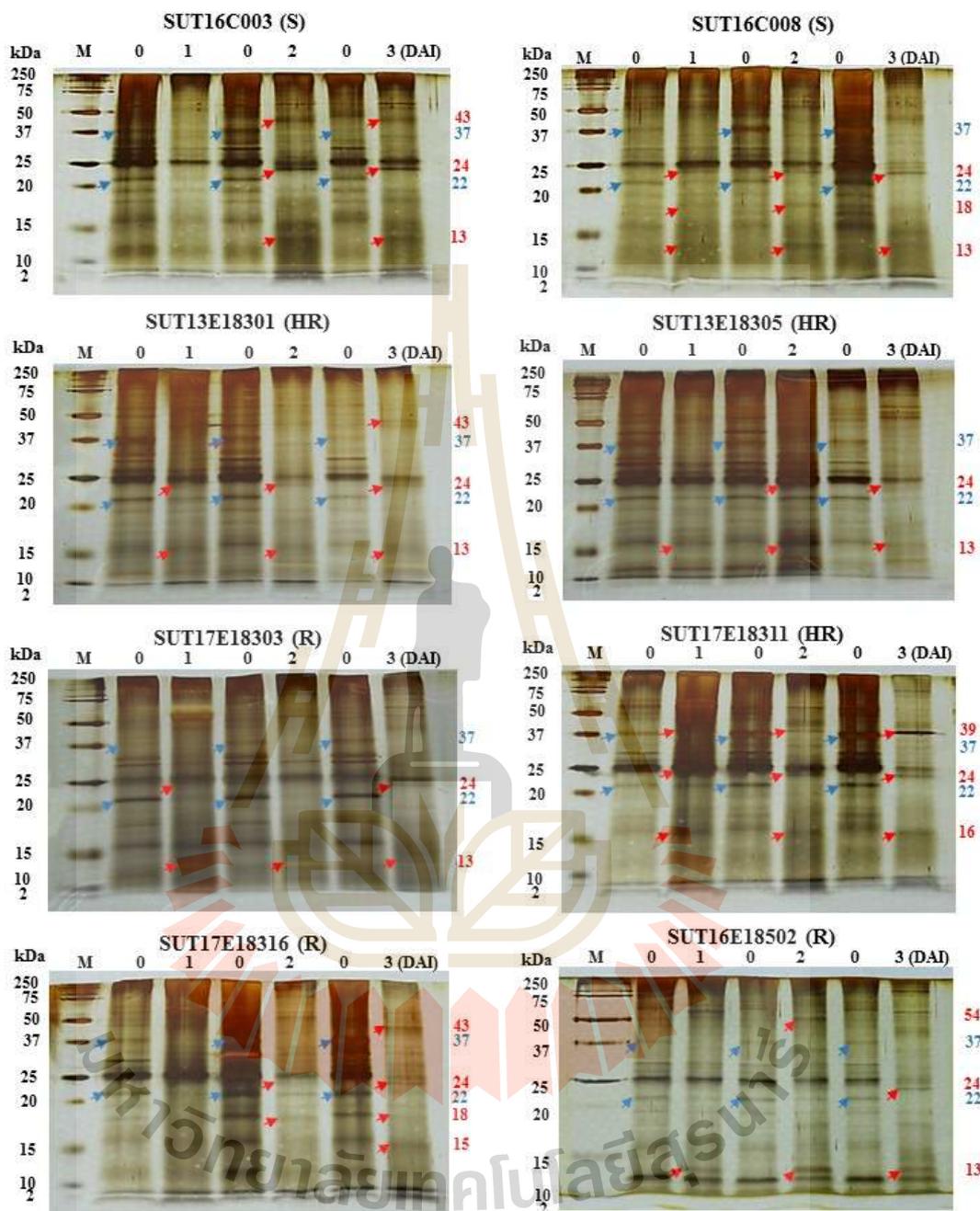
Figure 5.3 Protein profile changes of 6 black rot resistant mutants and 2 non-mutagenized controls at 0-3 days following the *P. palmivora* inoculation using SDS-PAGE. Number and arrow indicated the estimated MW (kDa) of up- (red) and down (blue)-regulated protein bands.

5.4.3 Total protein, β-1,3-glucanase and chitinase activities of black rot resistant mutants and non-mutagenized control upon *P. palmivora* infection in whole plant assay

Total protein, β-1,3-glucanase and chitinase activities were evaluated in 5 highly resistant and resistant black rot *D.* 'Earsakul' mutants (SUT13E18301 (HR), SUT13E18303 (R), SUT13E18304 (R), SUT13E18305 (HR) and SUT16E18502 (R)) and non-mutagenized control (SUT16C008 (S)) at 3 days after the following 3 treatments; 1) non-inoculation, 2) DI water inoculation and 3) *P. palmivora* inoculation. In treatment 2 and 3, DI water and *P. palmivora* were inoculated on leaf nodes 3 and 4, respectively, and total protein, β-1,3-glucanase and chitinase activities were measured to evaluate for both local induction (leaf nodes 3 and 4) and systemic induction (leaf nodes 1 and 2). It was found that total protein, β-1,3-glucanase and chitinase activities were not significantly different among non-inoculated mutants and non-mutagenized control ($p > 0.05$) (Table 5.2).

After 3 days following inoculation, all black rot resistant mutants showed less disease symptoms than non-mutagenized control. Total protein was significantly different among 3 treatments on infected leaves (leaf nodes 3 and 4) in non-mutagenized control SUT16C008 ($p < 0.05$) and two resistant mutants, SUT13E18301 and SUT13E18303 ($p < 0.01$). *P. palmivora* inoculated leaves showed the highest total protein in SUT16C008, SUT13E18301 and SUT13E18303 (3.99, 3.78 and 3.71 µg.mg leaf fw^{-1}, respectively). DI water inoculated leaves of these lines showed significantly lower total protein (2.91, 2.41 and 2.65 µg.mg leaf fw^{-1}, respectively) than *P. palmivora* inoculated leaves, but were not significantly different from those of non-inoculated leaves (2.79, 2.73 and 2.76 µg.mg leaf fw^{-1}, respectively). However, no significant difference among treatments

was found in other resistant mutants ($p > 0.05$). In young leaves distal from the inculation sites, total proteins were not significantly different among the 3 treatments in all resistant mutants and non-mutagenized control ($p > 0.05$) (Figure 5.4).

When β-1,3-glucanase activity assay was performed at 3 days after treatments, the activities were significantly different among the 3 treatments in both infected (leaf nodes 3 and 4) and distal (leaf nodes 1 and 2) leaves of two highly resistant mutants, SUT13E18301 and SUT13E18305 ($p < 0.01$). For infected leaves, the maximum β-1,3-glucanase activities were found in *P. palmivora* inoculated leaves of SUT13E18305 and SUT13E18301 (17.69 and 13.55 µmoles. min^{-1}. mg protein^{-1}, respectively) which were significantly higher than those of DI water inoculated leaves (5.75 and 6.56 µmoles. min^{-1}. mg protein^{-1}, respectively) and non-inoculated leaves (7.31 and 5.55 µmoles. min^{-1}. mg protein^{-1}, respectively). Similar results were observed in distal leaves of these highly resistant mutants. Distal leaves showed the highest β-1,3-glucanase activities after challenged by *P. palmivora* in SUT13E18305 and SUT13E18301 (16.46 and 11.64 µmoles. min^{-1}. mg protein^{-1}, respectively), which were significantly higher than those of DI water inoculated leaves (6.34 and 5.51 µmoles. min^{-1}. mg protein^{-1}, respectively) and non-inoculated leaves (4.21 and 5.88 µmoles. min^{-1}. mg protein^{-1}, respectively. Interestingly, the highly resistant mutants SUT13E18301 and SUT13E18305 exhibited 1.9 to 3.9-folds increase in β-1,3-glucanase activities at distal leaves, and 2.4-fold increase in β-1,3-glucanase activities at infected leaves after inoculation with *P. palmivora* compared to the non-inoculated treatment. These results implicated both systemic and local induction of β-1,3-glucanase in these highly resistant mutants after *P. palmivora* inoculation. By contrast, systemic induction was not evident in other resistant mutants and non-mutagenized control. In these plants, β-1,3-glucanase

activities of the distal leaf nodes 1-2 were not significantly different among 3 treatments ($p > 0.05$). At infected leaves, however, β-1,3-glucanase activities were significantly different in two additional resistant mutants, SUT13E18304 and SUT16E18502 ($p < 0.01$). β-1,3-glucanase activities in *P. palmivora* inoculated leaves of SUT13E18304 and SUT16E18502 (16.63 and 14.11 µmoles.min^{-1}.mg $protein^{-1}$, respectively) were significantly higher than those of DI water inoculated leaves (6.15 and 5.45 µmoles.min^{-1}.mg $protein^{-1}$, respectively), and non-inoculated leaves (3.53 and 3.93 µmoles. min^{-1}. mg $protein^{-1}$, respectively). By contrast, β-1,3-glucanase activities of non-mutagenized control SUT16C008 and resistant mutant SUT13E18303 were not significantly different among all 3 treatments in both infected and distal leaves ($p > 0.05$) although both of them showed a tendency of increased activities in locally infected leaves (Figure 5.5).

When the chitinase activities of *D.* 'Earsakul' lines were considered at 3 days after treatments, the activities were significantly different among 3 treatments in both infected and distal leaves of SUT13E18305 ($p < 0.05$). For infected and distal leaves, *P. palmivora* inoculated leaves had the highest chitinase activities (0.51 and 0.57 µmoles.min^{-1}.mg $protein^{-1}$, respectively), which was significantly higher than DI water inoculated leaves (0.34 and 0.41 µmoles. min^{-1}. mg $protein^{-1}$, respectively) and non-inoculated leaves (0.33 and 0.42 µmoles.min^{-1}.mg $protein^{-1}$, respectively). Moreover, SUT13E18305 demonstrated 1.5 and 1.4-folds increase in chitinase activities at infected and distal leaves after inoculation with *P. palmivora* compared to DI water inoculated and non-inoculated treatments, respectively. This tendency was similar to those of β-1,3-glucanase activities of this highly resistant mutant. Meanwhile, in the other highly resistant mutant, SUT13E18301, it was found that chitinase activities were

significantly different among 3 treatments in only infected leaves ($p < 0.01$) but not in distal leaves. Chitinase activity in *P. palmivora* inoculated leaves (0.56 µmoles.min^{-1}.mg protein^{-1}) was significantly higher than those of DI water inoculated leaves (0.39 µmoles.min^{-1}.mg protein^{-1}) and non-inoculated leaves (0.30 µmoles. min^{-1}. mg protein^{-1}). On the contrary, chitinase activities were not significantly different among 3 treatments in non-mutagenized control SUT16C008 and 3 other resistant mutants, SUT13E18303, SUT13E18304 and SUT16E18502 in both infected and distal leaves ($p > 0.05$)(Figure 5.6).
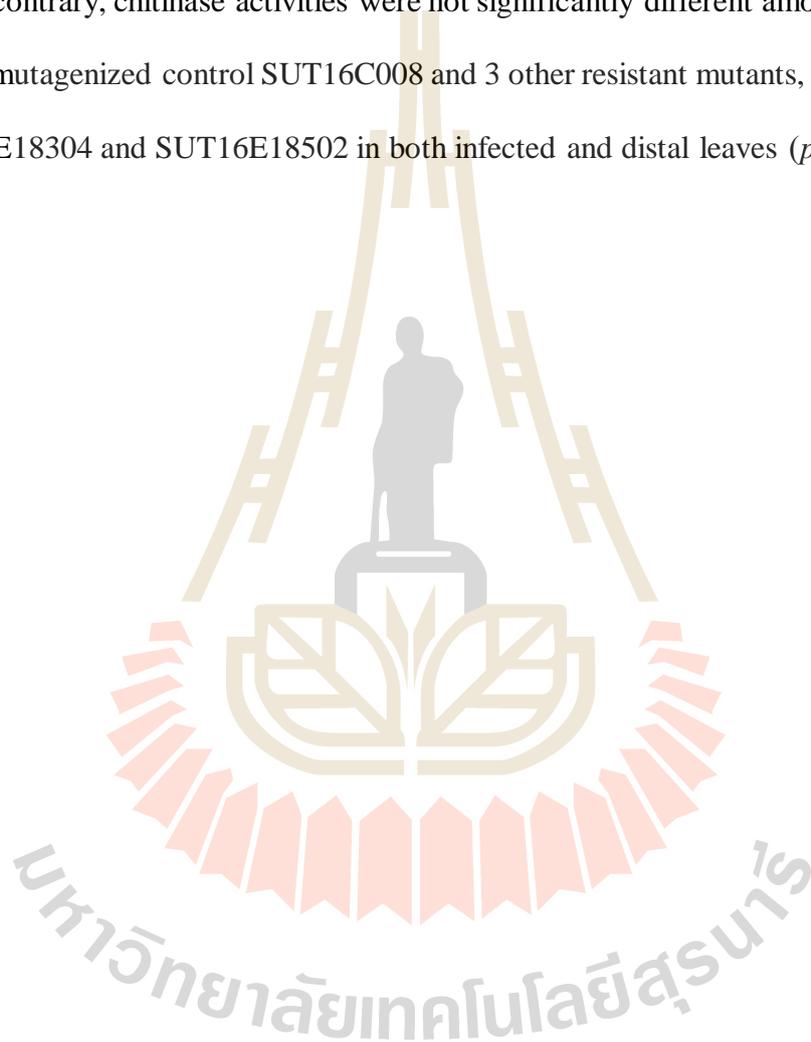
Table 5.2   Total protein, ß-1,3-glucanase and chitinase activities of non-inoculated black rot resistant mutants

and non-mutagenized control at different leaf positions (nodes 1-2 and 3-4).

| Lines	Total protein (µg. mg leaf fw^{-1})		ß-1,3-glucanase activity (µmoles. min^{-1}. mg protein^{-1})		Chitinase activity (µmoles. min^{-1}. mg protein^{-1})	
	1-2	3-4	1-2	3-4	1-2	3-4
SUT16C008 (S)	3.07 ± 0.27	2.79 ± 0.22	5.84 ± 2.97	5.92 ± 1.28	0.31 ± 0.05	0.35 ± 0.04
SUT13E18301 (HR)	2.69 ± 0.22	2.73 ± 0.20	5.88 ± 1.09	5.55 ± 0.93	0.38 ± 0.03	0.30 ± 0.03
SUT13E18303 (R)	3.04 ± 0.30	2.65 ± 0.20	6.34 ± 1.43	6.42 ± 1.41	0.38 ± 0.04	0.31 ± 0.08
SUT13E18304 (R)	3.17 ± 0.20	3.15 ± 0.28	4.74 ± 2.58	3.53 ± 1.34	0.43 ± 0.06	0.43 ± 0.06
SUT13E18305 (HR)	3.04 ± 0.30	2.65 ± 0.20	4.21 ± 0.76	7.31 ± 0.99	0.42 ± 0.06	0.33 ± 0.03
SUT16E18502 (R)	3.23 ± 0.26	3.23 ± 0.39	3.72 ± 1.06	3.93 ± 0.39	0.33 ± 0.03	0.33 ± 0.04
F-test	ns [1]	ns	ns	ns	ns	ns

[1] ns=not significant
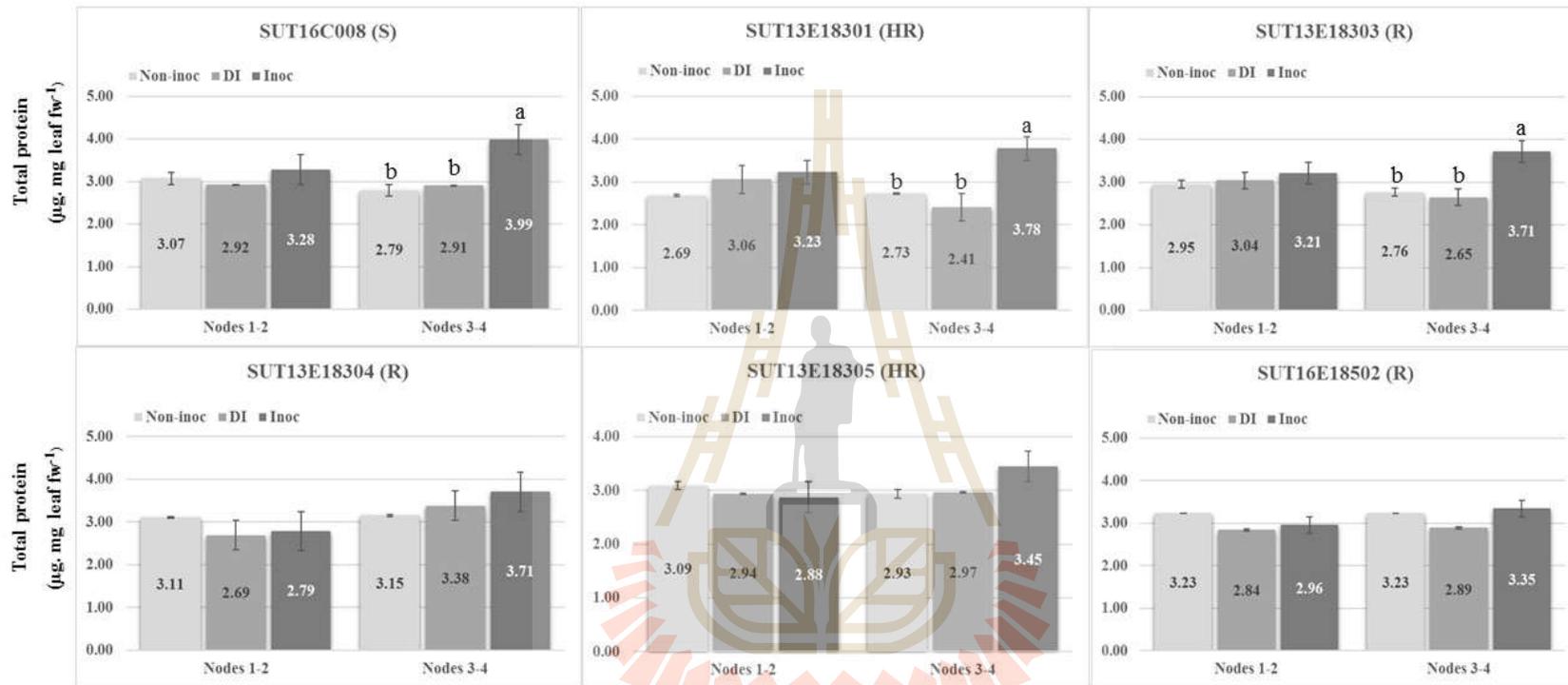
Figure 5.4 Changes in total protein in infected (leaf nodes 3 and 4) and distal (leaf nodes 1 and 2) leaves of 5 highly resistant and resistant black rot *D.* 'Earsakul' mutants (SUT13E18301 (HR), SUT13E18303 (R), SUT13E18304 (R), SUT13E18305 (HR) and SUT16E18502 (R)) and non-mutagenized control (SUT16C008 (S)) at 3 days after 3 treatments; 1) non-inoculation (Non-inoc), 2) DI water inoculation (DI) and 3) *P. palmivora* inoculation (Inoc). Different letters are significantly different ($p < 0.05$) based on DMRT.

Figure 5.5   Changes in ß-1,3-glucanase activities in infected (leaf nodes 3 and 4) and distal (leaf nodes 1 and 2) leaves of 5 highly resistant and

resistant black rot *D.* 'Earsakul' mutants (SUT13E18301 (HR), SUT13E18303 (R), SUT13E18304 (R), SUT13E18305 (HR) and

SUT16E18502 (R)) and non-mutagenized control (SUT16C008 (S)) at 3 days after 3 treatments; 1) non- inoculation (Non-inoc), 2) DI

water inoculation (DI) and 3) *P. palmivora* inoculation (Inoc). Different letters are significantly different ($p < 0.05$) based on DMRT.
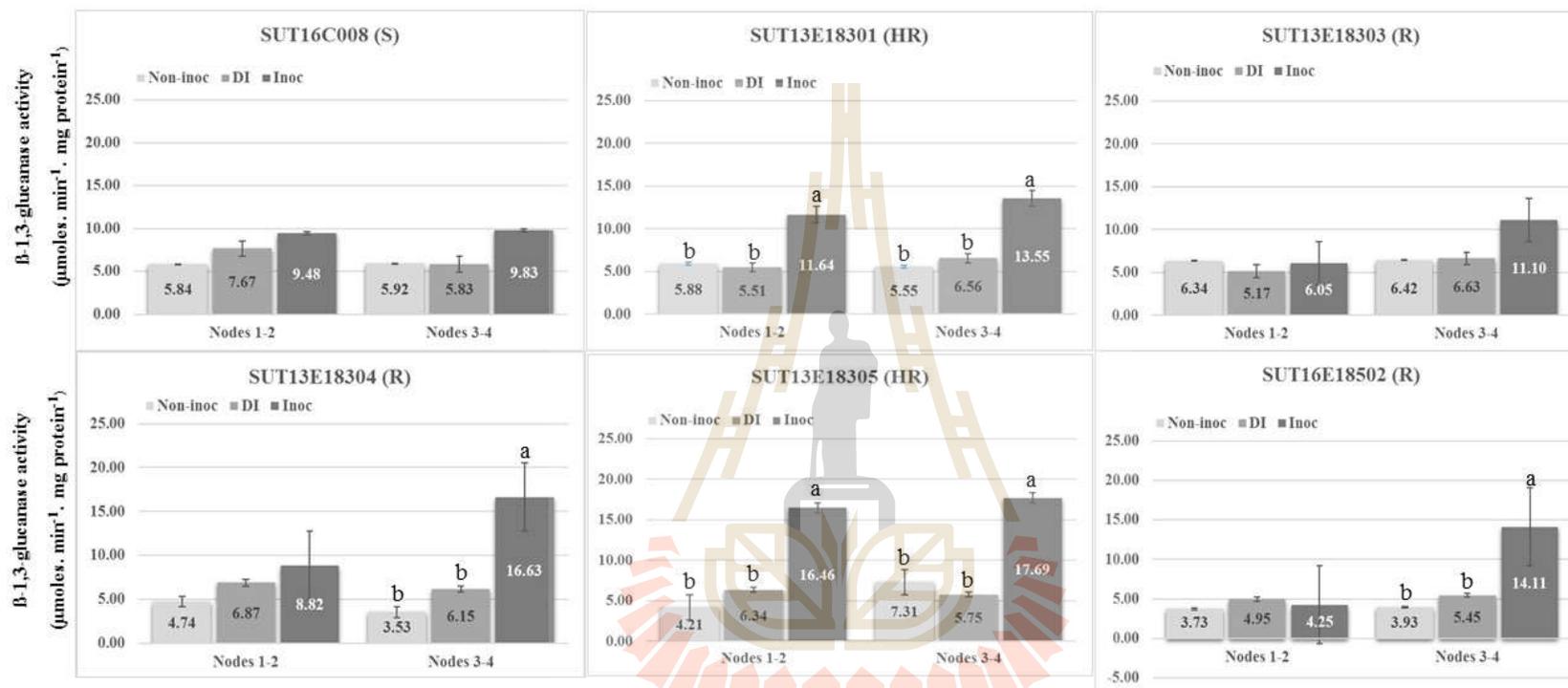
Figure 5.6 Changes in chitinase activities in local (leaf nodes 3 and 4) and systemic (leaf nodes 1 and 2) induction of 5 highly resistant and resistant black rot *D*. 'Earsakul' mutants (SUT13E18301 (HR), SUT13E18303 (R), SUT13E18304 (R), SUT13E18305 (HR) and SUT16E18502 (R)) and non-mutagenized control (SUT16C008 (S)) at 3 days after 3 treatments; 1) non- inoculation (Non-inoc), 2) DI water inoculation (DI) and 3) *P. palmivora* inoculation (Inoc). Different letters are significantly different ($p < 0.05$) based on DMRT.
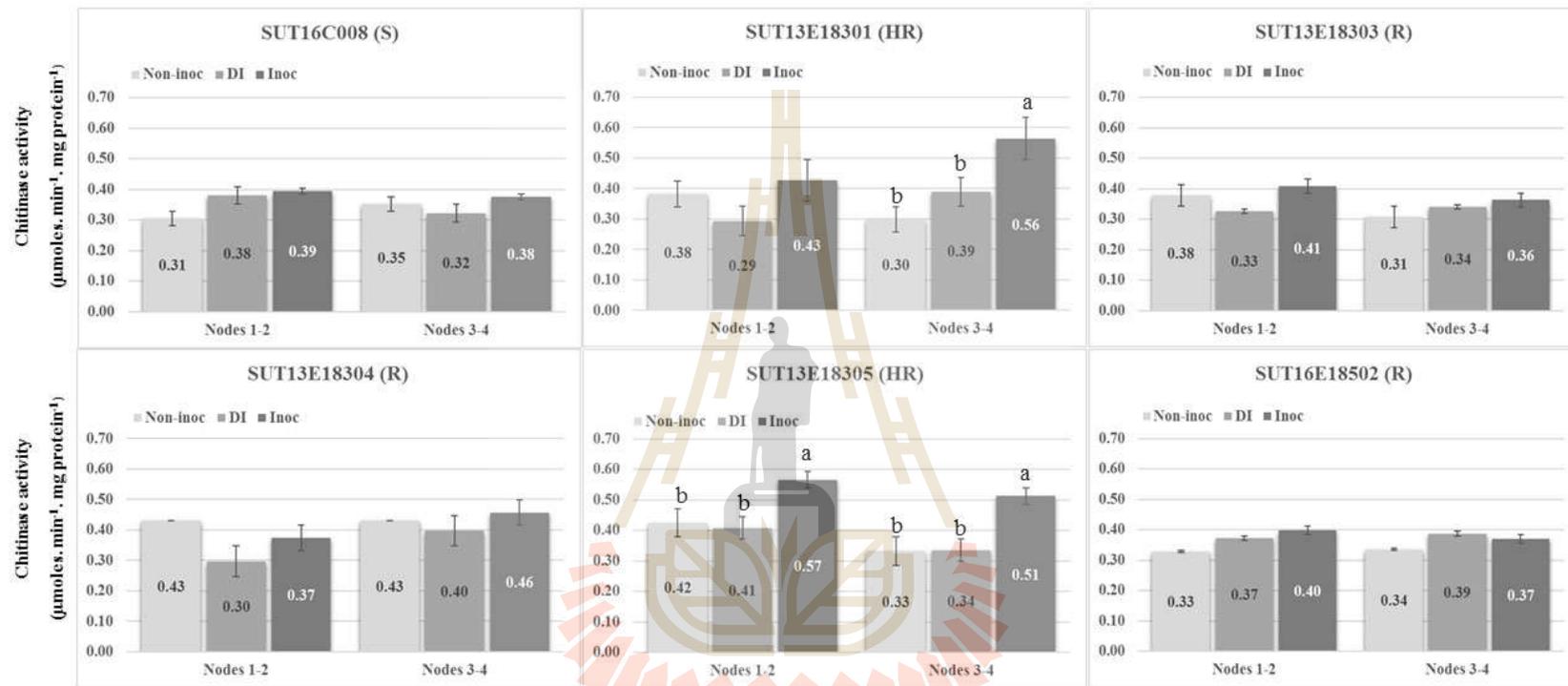
5.5 Discussion

ß-1,3-glucanase and chitinase activity levels varied significantly among black rot resistant mutants and non-mutagegized controls before inoculation. The highest ß-1,3-glucanase activity was found in a resistant mutant SUT17E18303, which was significantly higher than both non-mutagenized controls and most black rot resistant mutants. Likewise, Solanki et al. (2015) reported that the average ß-1,3-glucanase activity was highest in fusarium wilt resistant tomato varieties, followed by moderately resistant and susceptible varieties, respectively at the pre-infection stage. While, Ebrahim et al. (2011) reported that resistant mango cultivars had the highest β-1,3-glucanase (80.54-82.06 units) and chitinase activities (1.98-2.01 units) in resistant mango cultivars, which were higher than those of susceptible mango cultivar (25.21 and 1.01 units, respectively). However, a highly resistant mutant SUT13E18301 exhibited minimum activity levels of both enzymes. It is possible that some mutants may have low levels of some constitutively expressed PR proteins but they will be rapidly turned on in response to pathogen attack to save energy costs and nutrients (Freeman and Beattie, 2008; Gupta et al., 2013). Moreover, the resistance of some mutants may be mediated by other mechanisms not related to PR proteins.

We also found that ß-1,3-glucanase and chitinase activities were induced to higher levels in black rot resistant mutants than non-mutagegized controls during the initial stage of infection. Particularly, the highly resistant mutant SUT13E18301 showed 3.2-fold induced ß-1,3-glucanase activity level at 1 day after inoculation with *P. palmivora*, which was 2.1 to 2.7-folds higher than the induction in non-mutagenized controls (1.3 to 1.5-folds). Similarly, the resistant mutant SUT17E18303 showed the

highest induced chitinase activity levels (3.8-fold), which was 2.4 to 2.7-folds higher than the induction in non-mutagegized controls (1.4 to 1.6-folds). In summary, four and four of these black rot resistant mutants exhibited higher induced ß-1,3-glucanase and chitinase activities, respectively than those of non-mutagenized controls. Generally, such changes are a consequence of up- and down- regulation of a broad variety of plant genes and mainly results in the production of PR-proteins. And these changes appeared to be induced to a higher extent in resistant interactions than susceptible ones (Sudisha et al., 2012). Similarly, a maximum ß-1,3-glucanase and chitinase activities in arugula resistant cultivar (RTM-2002) were induced to a higher level than susceptible cultivar (T-27) after inoculation with a fungal pathogen *A. brassicicola* for 2-3 days (Gupta et al., 2013). The differences of ß-1,3-glucanase and chitinase induction patterns in various black rot resistant mutants may depend on genetic changes induced by mutation. These mutants were obtained from EMS-mutagenesis, which causes high frequency of gene mutations, resulting in amino acid changes, which may change or abolish functions of many proteins including PR proteins.

SDS-PAGE analysis revealed the presence of up-regulated protein bands with estimated MW of 13-54 kDa in various *D.* 'Earsakul' lines (non-mutagenized susceptible control and black rot highly resistant and resistant mutants). The up-regulated proteins with molecular size of 13 and 24 kDa were found in most highly resistant and resistant mutants as well as both black rot susceptible *D.* 'Earsakul' lines, while that with molecular size of 18 kDa appeared only in SUT16C008 (susceptible) and SUT17E18316 (resistant mutant). SUT17E18316 also had a 43 kDa up-regulated protein which was found in SUT13E18301 (highly resistant mutant) and SUT16C003 (susceptible). Moreover, up-regulated proteins with the molecular size of 15 and 54 kDa

were uniquely presented only in two resistant mutants (SUT17E18316 and SUT16E18502) . While those with the molecular size of 16 and 39 kDa appeared only in a highly resistant mutant SUT17E18311. Interestingly, up-regulated proteins with the molecular sizes of 39, 43 and 54 kDa in some black rot resistant mutants (SUT13E18301, SUT17E18311, SUT17E18316 and SUT16E18502 and non-mutagenized control (SUT16C003) were found to be associated with the induction of ß-1,3-glucanase after inoculation. Meanwhile, three up-regulated proteins with the molecular size of 13 kDa found in most *D.* 'Earsakul' lines, 15 kDa found in SUT17E18316 and 16 kDa found in SUT17E18311 appeared to be associated with the induction of chitinase activities after inoculation. These up-regulated protein bands may be related to ß-1,3-glucanase and chitinase induced as parts of defense mechanism against *P. palmivora* infection. By contrast, up-regulated proteins with the molecular sizes of 18 and 24 kDa were not associated with both ß-1,3-glucanase and chitinase activity induction and may be related to other antifungal proteins. Previous studies reported the molecular size of ß-1,3-glucanase (PR-2) varying from 33 to 41 kDa in tobacco and *Nicotiana* spp. (Leubner-Metzger et al., 1999). Likewise, Kim and Hwang (1997) found the induction of a basic 34 kDa ß-1,3-glucanase in pepper stem in response to *P. capcisi* infection. Moreover, they found an expression of a protein with molecular size of 39 kDa, coinducing with the 4-fold induction of ß-1,3-glucanase activities in potato tuber following *P. infestans* infection (Tonón et al., 2002). Moreover, Kabir et al. (2016) also reported the antifungal activity of 39 kDa chitinase from *Trichosanthes dioica* seeds against *Aspergillus niger* and *Trichoderma* sp. Whereas, the protein with low molecular weight of 13 to 19 kDa were advocated to chitinase (PR-4) (reviewed in El-Hadary and Tayel, 2013). β-1,3-glucanases and chitinase are generally encoded by

multigenic families that are widely distributed in the plant kingdom and have diverse roles (plant growth and development, including microsporogenesis, embryogenesis, germination, flowering, and abscission, as well as in wounding and defense responses). They were classified by sequence similarity into four and six families, respectively (reviewed in Meins et al., 1992; Leubner-Metzger et al., 1999; Neuhaus, 1999b; Gomez et al., 2002; Leubner-Metzger, 2003). The different MW protein bands induced in some resistant mutants may be encoded by different members of β-1,3-glucanase and chitinase gene family, possessing diverse amino acid sequences and/or functions. These proteins may be related to antifungal activities during the process of infection by *P. palmivora*. Interestingly, some unique up-regulated protein bands (e.g., 15 and 54 kDa in resistant mutants, and 16 kDa in highly resistant mutant) induced during *P. palmivora* infection may be used as protein markers for resistance mechanisms in orchids. Similarly, Mahmoud and Abd El-Fatah (2020) revealed that SDS-PAGE protein patterns are specific and can differentiate resistant faba bean genotypes from susceptible genotypes. The 26.2 kDa protein band was induced in all faba bean genotypes infected with fusarium wilt (FW; *F. oxysporum* Schlecht.), while the down-regulated protein band with molecular weight of 36.1 kDa appeared only in infected highly susceptible faba bean genotype. In addition, the protein band of molecular weight 29.7 kDa was only induced in all infected resistant genotypes as compared with non-inoculated plants. In this study, some apparent down-regulation of 22 and 37 kDa protein bands was also observed in all *D.* 'Earsakul' lines. However, the down-regulation of both protein bands in some black rot resistant mutants was observed later than those of non-mutagenized controls. Similarly, several identified protein bands of 21.75, 22.7, 36.5, 36.8, 37.25, and 37.75 were down-regulated during papaya ringspot

virus and *F. oxysporum* f. sp. *cucumerinum* (FOC) infection in papaya and cucumber, respectively. These proteins involve in photosynthesis, ribosomal proteins, and carbohydrate and energy metabolism (Siriwan et al., 2013; Jada et al., 2014; Du et al., 2016; Ahmed et al., 2018; Spechenkova et al., 2021). Pineda et al. (2010) reported that after pathogen infection in plants, symptoms (chlorosis and yellowing of the leaves) and toxin attributed to the irregularity of chloroplasts, resulting in reduced photosynthesis process.

Total protein was significantly different among 3 treatments on infected leaves (leaf nodes 3 and 4) in non-mutagenized control SUT16C008 and two black rot resistant mutants, SUT13E18301 and SUT13E18303. It has been reported that total soluble proteins are produced in much greater concentration following pathogen attack, mainly in incompatible interaction which may help impede the pathogen progress. These proteins display high degree of pathogen specificity and are coordinated at the level of transcription. The transcriptional induction of a large suite of defence-related genes, caused accumulation of antimicrobial secondary metabolites and PR proteins. These PR proteins may accumulate up to 10% of the total protein content. Similarly, Meng et al. (2021) reported expression of 38 defense-related genes in resistant tobacco cultivar (BH) including those encoded PR proteins whereas only 9 defense-related genes were expressed in susceptible tobacco cultivar (XHJ) in response to infection by *P. nicotianae* under comparative transcriptome analysis. It is possible that some black rot resistant mutants might have expression of more R genes than non-mutagenized control in response to *P. palmivora* infection. In addition, PR proteins are induced in association with necrotic lesions in plants. Therefore, higher total protein found in infected leaves of non-mutagenized control SUT16C008 may result from more severe

disease symptoms than those in black rot resistant mutants (Agrios, 2005; Golshani et al., 2015).

Both local and systemic induction of ß-1,3-glucanase were observed in highly resistant mutants SUT13E18301 and SUT13E18305. But only SUT13E18305 exhibited induced chitinase activities in both distal (nodes 1-2) and infected leaves (nodes 3-4). The other highly resistant mutant SUT13E18301 only showed local chitinase induction. While no induction of both enzymes was found in non-mutagenized control. Normally, high amounts of PR protein mRNAs were localized in outermost cell layer (epidermal cells, guard cells of the stomata, glandular trichomes, crystal idioblasts) and cells of the vascular system. After infection, they were strongly accumulated at infected sites and also observed in neighbouring or uninfected leaves after *P. infestans* infection in potato (Hoegen et al., 2002). β-1,3-glucanase and chitinases have also been reported to be localized in the cap structure of glandular trichomes of potato leaves and epidermal cells of tobacco and bean leaves after infection (Keefe et al., 1990; Mauch et al., 1992; Garcia-Garcia et al., 1994). Therefore, the higher systemic and local induction of ß-1,3-glucanase and chitinase activities found in some black rot resistant mutants may result from higher accumulation of PR protein mRNAs in specific cells/ tissues due to changes in expression of gene (s) related to resistance. Similarly, Campbell et al. (2012) has reported higher levels of the transcript abundance of four *PR* genes (e.g., *PR1* (antifungal), *PR2* (ß-1,3-glucanase), *PR3* (chitinase), and *PR9* (peroxidase)) in resistant wheat mutant (MNR220; EMS mutagenesis-derived) than in susceptible wheat (mnr220 NIL) which enhanced resistance to three rusts and powdery mildew. Consequently, the resistant wheat mutant had delayed disease development at the seedling stage and completed resistance at the adult plant stage. The differences in

resistance responses of highly resistant mutants found in our study may be associated with altered defense response regulation, leading to increased expression of PR genes.

Both PR proteins have been shown to have potentially strong antifungal activities when worked syringistically (Lorito et al., 1998) and may be responsible for the black rot resistance in some resistant mutants. ß-1,3-glucanase activity which were induced in most black rot resistant mutants may play a major role in defense mechanisms against *P. palmivora*. While the chitinase activity which showed lower activity levels in all *P. palmivora* infected *D.* 'Earsakul' lines, and only significantly induced in highly resistant mutants may play a supportive role. These results are consistent with the fact that *P. palmivora'* s cell walls compose of β-glucan mainly 1,3-, 1,6- and 1,4-links (ca. 90% dry weight) and small amount of chitin (<1%), which were substrates for various ß-glucanase and chitnase degradation (Tokunaga and Bartnicki-Garcia, 1971; Hamid et al., 2013; Mélida et al., 2013).In addition, the lower chitinase activity induction may reflect lower amount of chitin in *P. palmivora*' s cell walls. The hydrolyzed cell wall fragments could also act as elicitors for PAMPs or MAMPs's recognition, inducing hypersensitive response (HR), biochemical changes associated with defense responses such as phytoalexin accumulation and eventually SAR. Then SAR induces secondary resistance response after HR and leads to expression of PR-proteins including ß-1,3-glucanase and chitinase which play a direct defensive role against broad spectrum of pathogenic agents (Capasso et al., 2001; Baillieul et al., 2003; Malik et al., 2020). We found that all black rot resistant mutants had less disease symptoms than non-mutagenized control at 3 DAI in whole plant assay, although some black rot resistant *D.* 'Earsakul' mutants showed no induction of ß-1,3-glucanase and chitinase activities. These results implicate involvement of different

resistance mechanisms in these mutants. For example, a 24 kDa osmotin and thaumatin-like protein (PR-5) was abundantly expressed in grapevine fruits in response to *Erysiphe necator* infection, and also enhanced lytic activity when tested in combination with β-1,3-glucanases and/or chitinases (Lorito et al., 1996; Monteiro et al., 2003). From these reports, it was possible that the induced 24 kDa protein found in all *D.* 'Earsakul' lines may be osmotin. Nevertheless, the protein bands with similar in molecular weight may confer different functions. Therefore, further study is needed to elucidate these proteins by proteomics. A top-down proteomics technique or two-dimensional (2-DE) gel electrophoresis which based on the individual protein separation contained in a proteome by pI (first dimension) and then by molecular weight (second dimension), and subsequent mass spectrometry-based identification is best suited for revealing the complexity of proteins in these black rot resistant mutants during pathogen infection, particularly, the highly resistant mutant SUT13E18305 compared with non-mutagenized control (susceptible). The different expression patterns of proteins can indicate various plant defense mechanisms. These results expand our current knowledge towards a better understanding of the orchid under pathological conditions. Our findings represent an early step toward uncovering mechanisms underlying black rot resistance in orchid, which is essential for the development of disease resistant varieties in the future.

5.6 Conclusion

These results suggest that the constitutive expression of β-1,3-glucanase and chitinase activities were significantly different among *D.* 'Earsakul' lines. The responses to *P. palmivora* infection appeared to vary in terms of extent and time. The newly up-

regulated protein bands of 15, 16, 39 and 54 kDa which were identified in some black rot resistant mutants may be used as protein markers to predict black rot resistance mechanisms in orchids. In addition, a highly resistant mutant SUT13E18305 demonstrated higher magnitude of local and systemic β-1,3-glucanase and chitinase induction than other black rot resistant mutants and non-mutagenized controls in the whole plant assay, suggesting that these enzymes may contribute significantly to resistance in this mutant. These results are useful for future breeding programs for black rot resistance in orchids. Moreover, SUT13E18305 may be developed into a new commercial variety. New black rot resistance resource in this mutant can also be utilized in other orchid varieties.

5.7   References

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CHAPTER VI

CONCLUSION

6.1 The evaluation of different culture media for *P. palmivora* growth and sporulation revealed that the best culture media for surface mycelial growth of *P. palmivora* were PSA and V8A, while CMPA induced sporulation earlier than other media. The preliminary evaluation of resistance levels of the potentially resistant lines in *D.* 'Earsakul' found that NK-53-9 is the most virulent single-spore *P. palmivora* isolate for future screening of black rot resistance. A *Dendrobium* mutant, SUT13E18301 was resistant to all single-spore *P. palmivora* isolates.

6.2 *In vitro* mutagenesis of *D.* 'Earsakul' PLBs for black rot resistance showed that the optimum EMS concentrations for EMS-mutagenesis in *D.* 'Earsakul' PLBs were 1.4% (LD_{30}) and 1.8% (LD_{50}). In addition, fifty putative resistant mutants (22 mutants from 1.4% EMS and 28 mutants from 1.8% EMS) were obtained after 3 cycles of *in vitro* selection for black rot resistance with PSB medium supplemented with 0-60% of *P. palmivora* CFs.

6.3 Thirteen black rot resistant mutants with high black rot resistance levels at the laboratory level, including four highly resistant mutants and nine resistant mutants were identified using detached leaf assay with NK-53-9 isolate from Nakhon Ratchasima province. Moreover, less disease symptoms were also exhibited on leaves of black rot resistant mutants when compared to a non-

mutagenized control after evaluating for the stability of resistance against other two additional *P. palmivora* isolates from Songkhla province.

6.4   ISSR analysis revealed that all black rot putative mutants evaluated were genetically different from non-mutagenized control and were confirmed as true mutants.

6.5   From the investigation of DNA content and genome size using flow cytometry, it was found that all black rot resistant mutants and three non-mutagenized controls had the same chromosome number of 2n+4n+8n. Moreover, a mutant SUT17E18316 was found to exhibit maximum DNA content and genome size among *D.* 'Earsakul' lines. Meanwhile, morphological characterization revealed that most of the black rot resistant mutants were morphologically different on some characters from non-mutagenized controls including plant height and number of roots etc. A mutant SUT13E18305 appeared to possess outstanding characters and may be useful for future commercialization.

6.6   The characterization of the protein changes before and following *P. palmivora* infection in black rot resistant mutants and non-mutagenized controls revealed that the constitutive expression of β-1,3-glucanase and chitinase activities were significantly different among *D.* 'Earsakul' lines and the responses to *P. palmivora* infection appeared to vary in terms of extent and time. Infection of *P. palmivora* increased the activities of both PR proteins in some mutants and non-mutagenized controls throughout the experimental period of 3 DAI. Particularly, the highly resistant mutant SUT13E18301 had much higher magnitude of induction as compared to controls. The newly up-regulated protein bands of 15, 16, 39 and 54 kDa which were identified by SDS-PAGE analysis in some black rot

resistant mutants but not in non-mutagenized controls may be used as protein markers to predict black rot resistance mechanisms in orchids. In addition, a highly resistant mutant SUT13E18305 demonstrated higher magnitude of local and systemic β-1,3-glucanase and chitinase induction than other black rot resistant mutants and non-mutagenized control in the whole plant assay, suggesting that these enzymes may contribute significantly to resistance in this mutant. These results can be useful for future breeding programs for black rot resistance in orchids. Moreover, SUT13E18305 may be used as new black rot resistance resource for other orchid varieties.
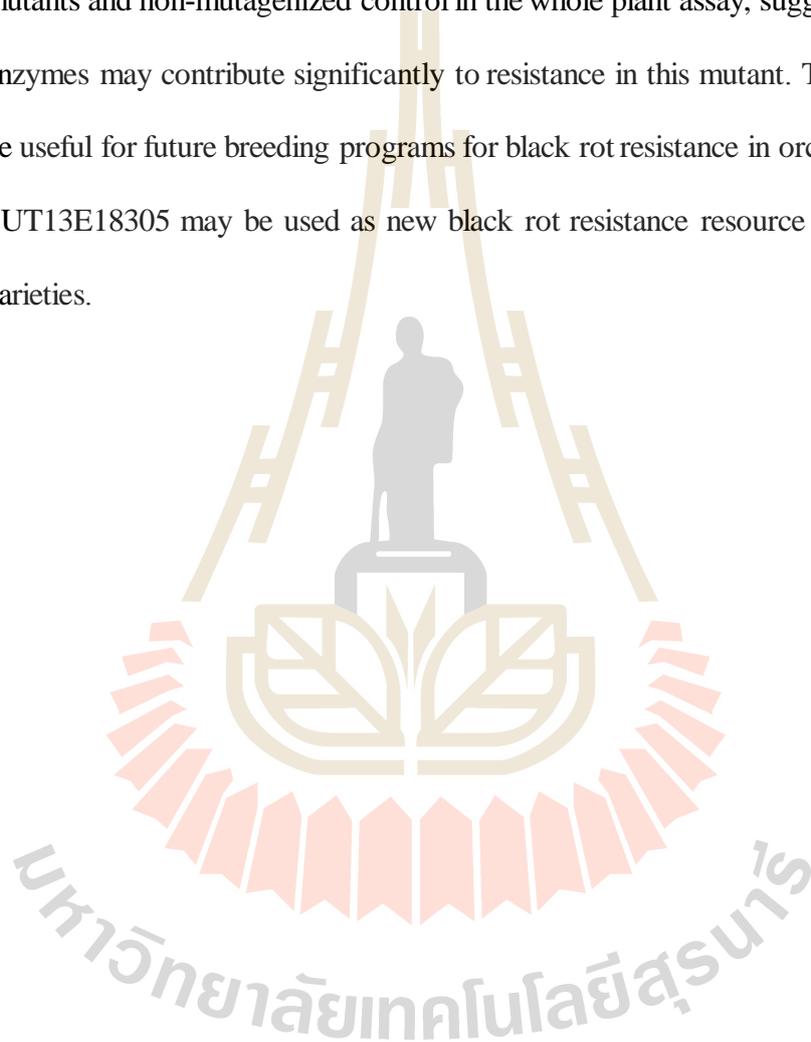
BIOGRAPHY

Ms. Apinya Khairum was born on April 26, 1987 at Buriram, Thailand. She recieved Bachelor of Science Degree Program in Crop Production Technology, Suranaree University of Technology in 2010. Then, in the same year, she had worked as a research assistant in Plant Breeding Laboratory for 4 years at Suranaree University of Technology, Nakhon Ratchasima, Thailand. After that, she decided to further study for a Ph.D. program 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 SUT to support her tuition and fee. Her research topic was breeding for black rot resistance in *D.* 'Earsakul' via *in vitro* mutagenesis and selection under supervision of Professor Dr. Piyada Alisha Tantasawat. The results from some parts of this study have been presented in the International Conference on Agricultural and Biological Sciences (AAFBHS-16), August 22-24, 2016, Kuala Lumpur, Malaysia (Poster presentation in "Cultural characteristics and pathogenicity analysis of *Phytophthora palmivora*, causual pathogen of black rot in orchids").