BIOLOGICAL CONTROL OF ANTHRACNOSE ON GREEN SHALLOT USING ANTAGONISTIC *Bacillus subtilis*

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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GREEN SHALLOT USING ANTAGONISTIC

Bacillus subtilis

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

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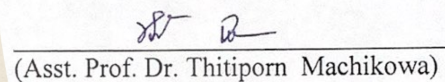

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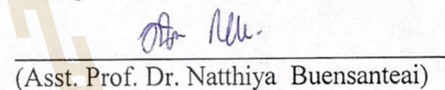

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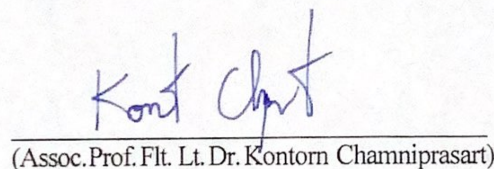

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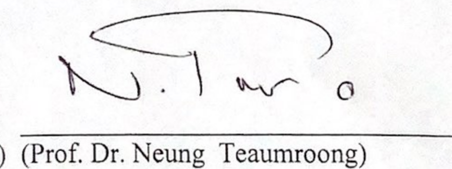

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ณาตยา ทองพรม : การควบคุมโรคแอนแทรคโนสของหอมแบ่งด้วยเชื้อจุลินทรีย์ปฏิปักษ์ *Bacillus subtilis* (BIOLOGICAL CONTROL OF ANTHRACNOSE ON GREEN SHALLOT USING ANTAGONISTIC *Bacillus subtilis*) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.ณัฐธิญา เบือนสันเทียะ, 122 หน้า.

โรคแอนแทรคโนส เกิดจากเชื้อรา *Colletotrichum gloeosporioides* เป็นโรคที่มีความสำคัญของหอมแบ่งในประเทศไทย โดยทำความเสียหายให้กับผลผลิตของหอมแบ่งสูงถึง 80% ในพื้นที่ปลูกหอมแบ่ง การศึกษาครั้งนี้มี วัตถุประสงค์เพื่อประเมินประสิทธิภาพสาร biosurfactants จากเชื้อ *Bacillus subtilis* ต่อการส่งเสริมการเจริญเติบโตและกระตุ้นความต้านทานของหอมแบ่ง โดยทำการทดสอบสาร biosurfactants ที่ผลิตได้จากเชื้อ *B. subtilis* สายพันธุ์ D604 ในการยับยั้งการเจริญของโคโลนีเชื้อราพบว่า สาร biosurfactants จากเชื้อ *B. subtilis* สายพันธุ์ D604 ที่ปริมาณ 150 และ 200 ไมโครลิตร สามารถยับยั้งการเจริญโคโลนีเชื้อรา *C. gloeosporioides* ได้ 70.81 และ 73.48% ตามลำดับ จากนั้นนำสาร biosurfactants จากเชื้อ *B. subtilis* สายพันธุ์ D604 เปรียบเทียบกับสารเคมีโพรคลอราซ และ *B. subtilis* ทางการค้า ต่อการควบคุมและชักนำความต้านทานโรคแอนแทรคโนสในหอมแบ่งที่อายุ 28 วัน ในสภาพโรงเรือนทดลอง และฉีดพ่นทุก 7 วัน จำนวน 4 ครั้ง เมื่อฉีดพ่นครั้งสุดท้าย 7 วัน ทำการปลูกเชื้อรา *C. gloeosporioides* พบว่า กรรมวิธีที่ใช้สารเคมีโพรคลอราซ และ biosurfactants สามารถลดการเกิดโรคได้แตกต่างกันอย่างมีนัยสำคัญที่ 64.70 และ 58.80% ตามลำดับ เมื่อเปรียบเทียบกับกรรมวิธีควบคุม นอกจากนี้ เมื่อฉีดพ่น biosurfactants จากเชื้อ *B. subtilis* สายพันธุ์ D604 พบว่า สารดังกล่าวยังสามารถส่งเสริมการเจริญเติบโตของหอมแบ่งทั้งเพิ่มน้ำหนักสด น้ำหนักแห้ง รวมทั้งความสูงต้นสูงที่สุดที่ 30.34 เซนติเมตร 119.96 และ 32.69 กรัม ตามลำดับ และเพิ่มขึ้นอย่างมีนัยสำคัญ ปริมาณการสังเคราะห์คลอโรฟิลล์ และออกซินสูงที่สุดที่ 25.86 ไมโครลิตรต่อมิลลิลิตร และ 14.76 ไมโครกรัมต่อกรัมน้ำหนักสด จากนั้นทำการทดสอบกลไกการปกป้องตนเอง ได้แก่ ปริมาณรวมของสารฟีนอลิก (PCs) และปริมาณกรดซาลิไซลิก (SA) ปริมาณกิจกรรมเอนไซม์ phenylalanine ammonia-lyase (PAL) และ chitinase (Chi) ที่ 0 24 และ 48 ชั่วโมง ภายหลังการปลูกเชื้อ ผลการทดลองพบว่า ปริมาณรวมของสารฟีนอลิกเพิ่มขึ้นอย่างมีนัยสำคัญภายหลังการปลูกเชื้อ 24 ชั่วโมง ที่ 352.71 316.87 296.87 และ 281.50 ไมโครกรัมต่อกรัมน้ำหนักสด จากการฉีดพ่นด้วย biosurfactants จากเชื้อ *B. subtilis* สายพันธุ์ D604, สารแขวนลอย *B. subtilis* สายพันธุ์ D604, *B. subtilis* ทางการค้า และสารเคมีโพรคลอราซ ตามลำดับ เช่นเดียวกันกับปริมาณ SA ที่มีแนวโน้มเพิ่มขึ้นที่ 24 ชั่วโมง สูงที่สุดที่ 103.58 116.83 90.67 และ 87.04 ไมโครกรัมต่อกรัมน้ำหนักสดตามลำดับ แตกต่างกันอย่างมีนัยสำคัญทางสถิติ เช่นเดียวกับปริมาณกิจกรรมเอนไซม์ PAL มีแนวโน้มสูงที่สุดที่ 15.39 9.93 7.36 และ 5.55 ไมโครโมลต่อมิลลิกรัมโปรตีนตามลำดับ ในขณะเดียวกัน

กิจกรรมเอนไซม์ไคติเนสเพิ่มขึ้นอย่างมีนัยสำคัญ สูงที่สุดที่ 4.11 3.19 2.52 และ 2.46 ไมโครโมลต่อมิลลิกรัมโปรตีน เมื่อฉีดพ่นด้วย biosurfactants จากเชื้อ *B. subtilis* สายพันธุ์ D604, สารเคมีโพรคลอราซ, สารแขวนลอย *B. subtilis* สายพันธุ์ D604 และ *B. subtilis* ทางการค้า จากการทดลองนี้แสดงให้เห็นว่าสาร biosurfactants จากเชื้อ *B. subtilis* สายพันธุ์ D604 มีประสิทธิภาพในการควบคุมโรคแอนแทรคโนสในหอมแบ่งทั้งกลไกทางตรงและทางอ้อม โดยมีความสามารถในการส่งเสริมการเจริญเติบโต มีความสามารถในการยับยั้งการเจริญของเชื้อราสาเหตุโรคโดยตรง และสามารถชักนำให้เกิดกระบวนการปกป้องตนเองต่อการเข้าทำลายเชื้อสาเหตุโรค ดังนั้นการชักนำความต้านทานพืชโดยใช้สาร biosurfactants จึงเป็นทางเลือกในการนำไปใช้ในระบบการผลิตหอมแบ่งปลอดภัย ลดการใช้สารมีในการควบคุมโรค และลดสารเคมีตกค้างซึ่งเป็นอันตรายต่อเกษตรกร ผู้บริโภค และสิ่งแวดล้อม

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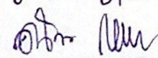
NATTAYA THONGPROM : BIOLOGICAL CONTROL OF ANTHRACNOSE ON GREEN SHALLOT USING ANTAGONISTIC *Bacillus subtilis*. THESIS ADVISOR : ASST. PROF. NATTHIYA BUENSANTEAI, Ph.D., 122 PP.

BIOSURFACTANTS/SECONDARY METABOLITE/LIPOPEPTIDES/INDUCE RESISTANT/*Colletotrichum gloeosporioides*

Anthracnose disease, caused by *Colletotrichum gloeosporioides*, is an important disease of the green shallot in Thailand. It can reduce green shallot products, causing up to 80% yield losses in green shallot growing areas. The objective of this study was to evaluate the efficiency of biosurfactants produced by *Bacillus subtilis* on growth promotion and induced resistance in green shallot. The efficacy of biosurfactant from *B. subtilis* strain D604 on fungal growth colony inhibition was investigated. The results showed that the biosurfactant *B. subtilis* strain D604 at the volumes of 150 and 200 μl can significantly inhibit *C. gloeosporioides* colony growth at 70.81 and 73.48%, respectively. Then biosurfactant from the *B. subtilis* strain D604 was compared with the *B. subtilis* strain D604, Prochloraz, and commercial *B. subtilis* was used to investigate anthracnose disease control and resistant mechanisms induced after 28 days green shallot under greenhouse conditions. When applied biosurfactant at every 7 days for 4 times and then inoculated with *C. gloeosporioides* at 7 days after foliar treatment, the results showed that Prochloraz and biosurfactants could significantly reduce anthracnose disease severity up to 64.70 and 58.80% respectively, when compared with the control. Besides, when applied biosurfactant as a foliar treatment, it was found that biosurfactant from *B. subtilis* strain D604 affects plant growth including fresh weight, dry weight, plant height at 30.34 cm, 119.96 and 32.69 g, respectively, and significantly

increased chlorophyll and auxin content at 25.86 μl mL^{-1} and 14.76 μg g^{-1} fresh weight. Moreover, for the defense mechanism investigation in green shallot leaves such as phenolic compound (PCs) and salicylic acid (SA), the defense enzymes: phenylalanine ammonia-lyase (PAL), and PR-protein: chitinase (Chi) were investigated at 0 24 and 48 hours after inoculation (HAI). The results found that the total of PCs significantly increased at 24 HAI to levels of 352.71 316.87, 296.87, and 281.50 μg g^{-1} fresh weight from foliar treated with biosurfactant *B. subtilis* strain D604, *B. subtilis* strain D604, commercial *B. subtilis*, and Prochloraz, respectively. Likewise, the accumulation of SA significantly increased at 24 HAI to levels of 103.58, 116.83, 90.67, and 87.04 μg g^{-1} fresh weight, respectively. Similarly, PAL activity also significantly increased at 24 HAI with levels of 15.39, 9.93, 7.36, and 5.55 μmol mg^{-1}protein, respectively. On the other hand, Chi activity significantly increased at 24 HAI with levels of 4.11, 3.19, 2.52, and 2.46 μmol mg^{-1}protein from foliar treated with biosurfactant strain D604, Prochloraz, *B. subtilis* strain D604, and commercial *B. subtilis*, respectively. These results indicated that the biosurfactant *B. subtilis* strain D604 has direct and indirect effects that could act as plant growth promotion and as an antimicrobial agent and activate several plant defense mechanisms during host-pathogen interaction. Induced plant resistance using biosurfactants can be an alternative method to control green shallot disease. This is an agricultural safety biosurfactant to reduce chemical fungicide for the farmer, consumer, and the environment.

School of Crop Production Technology Student's Signature Nattaya Thongprom

Academic Year 2019 Advisor's Signature 

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LIST OF ABBREVIATIONS

ANOVA	=	Analysis of variance
ASM	=	acibenzolar-S-methyl
BA	=	benzoic acid
GAP	=	good agricultural practices
GlcNAc	=	N-acetyl glucosamine
CRD	=	Completely randomized design
DAI	=	Days after inoculation
H	=	Hydroxyphenyl
H	=	Hour
HAI	=	Hours after inoculation
HR	=	hypersensitive response
IAA	=	Indole-3-acetic acid
ISR	=	Induced systemic resistance
JA	=	Jasmonic acid
LPS	=	Lipopolysaccharides
MAPK	=	Mitogen activated protein kinases
MAPKK	=	MAPK kinase
MAPKKK	=	MAPKK kinase
mg l^{-1}	=	Milligram per milliliter
µl	=	Microliter (s)

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LIST OF ABBREVIATIONS (Continued)

μm	=	Micrometer (s)
min	=	Minute (s)
ml	=	Milliliter (s)
mM	=	Millimolar
NADP	=	nicotinamide adenine dinucleotide phosphate
NB	=	Nutrient broth
nm	=	Nanometer
NO	=	nitric oxide
O^{2-}	=	Superoxide anion
^{o}C	=	Degree celcius
OH	=	hydroxyl radicals
PAL	=	phenylalanine ammonia-lyase
PAMPs	=	pathogen-associated molecular patterns
PDA	=	potato dextrose agar
PGPR	=	plant growth promoting rhizobacteria
POX	=	peroxidase
ppm	=	part per million
PPO	=	polyphenol peroxidase
PR protein	=	pathogenesis-related protein
RP	=	receptor protein
SA	=	salicylic acid

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CHAPTER I

INTRODUCTION

1.1 Background of the selected topic

Green shallot or welsh onion (*Allium fistulosum*) is a member of the Amaryllidaceae family, perennial herb and crop food that is cultivated worldwide, specifically at tropical countries such as Korea, China, Japan, and Thailand (Nagaki et al., 2012; Liu et al., 2014; Abdelrahman et al., 2017; Abdelrahman et al., 2018). The green shallot is rich in proteins, carbohydrates, vitamins, and contains propylene sulfide, dietary flavonoids, that have anti-inflammatory effects and bactericidal. The green shallot has been used as herbal by preventing colds, activating immunity, anti-cancer properties, having anti-asthmatic activities (Liu et al., 2014; Wu et al., 2016).

cancer properties, having anti-asthmatic activities (Liu et al., 2014; Wu et al., 2016).

Global onion and shallot production were continually increased from around 52 million tons in 2003 to around 85 million tons in 2013. They are the second most grown plant in the world inferior to tomatoes (Brewster, 2008). In 2016, the planting area of green shallot and onion in Thailand was approximately 50,566 hectares, and green shallot yield was approximately 68,649 tons (FAO, 2014; Office of Agricultural Economics, 2014; the Ministry of Agriculture and Cooperative, 2016). However, it is a decreasing trend of green shallot production from 2015-2017, resulting in low supply and high cost in the local market as well as a limited amount of green shallot for exporting (Thailand Foreign Agricultural Trade Statistics, 2016; Department of

International Trade Promotion, 2016). Beyond, green shallot production is affected by various factors such as nutrient deficiency, poor soil quality, pests, and diseases, leading to the reduction of green shallot quality and yield.

Anthracnose-twister disease plays a necessary role in its growth and low productivity. At present, it is regarded to be the most destructive disease of green shallot. The causal pathogen of green shallot anthracnose is identified as *Colletotrichum gloeosporioides* that could reduce yield up to 80% (Brewster, 2008; Yutthasin and Thummabenjapone, 2012; Maneesuwan and Sirithorn, 2013; Sutthisa et al., 2014; Diao et al., 2017; Yutthasin et al., 2018). This fungal pathogen can survive in the soil and plant debris. In recent years, anthracnose disease is the most problems in all planting areas in Thailand. Pathogens can survive for several years until a suitable period. It causes substantial crop losses in most of the major producing regions in the world and has a wide host range This disease generally occurs in the tropical areas of Thailand, Asia, Africa and Latin America (Galván et al., 1997; Sa-ardluan and Sruamsiri, 2005; Vurro and Gressel, 2006; Srisuttee and Nalumpang, 2007; Gajbhiye et al., 2009; Santana et al., 2016; Suwannara et al., 2017). The deaths of nearly 600 people each year are directly a result of the use of insecticides, herbicides, fungicides, and pesticides (Thai-PAN, 2019). Caused the farmers and customers concern about over toxic agrochemical residue in agricultural products due to rapid urban and industrial evolution and increasing reliance on agrochemicals in the past several decades (Vila Nova et al., 2011; Patil, 2013; Sharma and Kulshrestha, 2015). The chemical pesticide residue contamination of agricultural products has become an increasingly severe problem in Thailand and ASEAN countries (Kongtragoul and Nalumpang, 2010; Jaipin and Nalumpang, 2014; Phukang and

Chaisuk, 2016; Suwannarat et al., 2017). The wide use of pesticides carries concerns on the health venture associated with pesticide exposure and gradually affects the health of the consumers (Le Thanh et al., 2015). In Thailand, hygienic crops are in high demand, especially among the growing number of tourists and health-conscious people. Regarding the problems of chemical residue, biological control is a promising alternative crop production system (Brewster, 2008; Panichsukpatana et al., 2011; Sutthisa et al., 2014). The study on the new intact method of disease control, such as cultural practices, biological method, resistant varieties, and induced resistance. Nowadays, induced resistance can be helpful for the management of the pathogen in a susceptible plant. The plant itself has an immunity system, which can be produced by an abiotic or biotic agent against infection by several pathogens (Steiner and Schönbeck, 1995; Heil and Bostock, 2002; Edreva, 2004; Walters et al., 2005; El Hadrami et al., 2010; El Hadrami et al., 2011; Le Thanh et al., 2015). Subsequently, the induced plant can be able to be resistant to the attack of pathogens because of an enhanced potentiality to rapidly express defense mechanisms (Prakongkha, 2011; Phiwthong, 2014; Saengchan, 2014; Wongchalee, 2015; Le Thanh et al., 2015). Plants can be induced to develop enhanced resistance to pathogen infection by treatment with a variety of abiotic and biotic inducers. Abiotic inducers include chemicals that act at various points in the signaling pathways involved in disease resistance, as well as salicylic acid, (jasmonic acid), β-aminobutyric acid (BABA), thiamine (vitamin B1), acibenzolar-S-methyl (ASM), and benzothiadiazole (BTH). Biotic inducers include *Trichoderma* spp. (Hoitink et al., 2006; Palmirei et al., 2012), plant-growth-promoting rhizobacteria (PGPR) such as *Pseudomonas fluorescens* (Vleesschauwer et al., 2008; Verhagen et al., 2010), *Bacillus amyloliquefaciens*, *B. subtilis* (Buensanteai et al.,

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2009; Rudrappa et al., 2010). Plant growth-promoting rhizobacteria (PGPR) belonging to *B. subtilis* are being exploited commercially for plant protection to induce systemic resistance against diseases. Plant-treatment with PGPR causes cell wall structural modifications and biochemical/physiological changes leading to the synthesis of proteins and chemicals involved in plant defense mechanisms. Lipopolysaccharides, siderophores, and salicylic acid are the major determinants of PGPR-mediated induced systemic resistance (ISR). The performance of PGPR has been successful against certain pathogens (Ramamoorthy et al, 2001; Buensanteai et al., 2009; Athinuwat, 2012; Saengchan et al., 2015; Nikaji et al., 2015; Wongchalee and Buensanteai, 2015). Besides, members of the *Bacillus* genus are considered as efficient microbial factories for large scale production of lipopeptides such as iturin, surfacing, and fengycin, inhibiting various fungal pathogens and protecting the crop plants. Biosurfactant is widely used in the research experiment and commercial to control disease in plants and supplement plant growth in the various plants (Harwood 1992; Jin et al., 2014; Singh et al., 2014).

1.2 Research objectives of this study

To evaluate the efficiency of biosurfactant produced by *B. subtilis* on growth promotion and induced resistance.

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CHAPTER II

LITERATURE REVIEW

2. 1 Background of the problem

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various factors such as nutrient deficiency, poor soil quality, pests, and diseases, leading to the reduction of green shallot quality and yield. Anthracnose-twister disease plays a necessary role in its growth and low productivity. At present, it is regarded to be the most destructive disease of green shallot. The causal pathogen of green shallot anthracnose is identified as *Colletotrichum gloeosporioides* that could reduce yield up to 80% (Brewster, 2008; Yutthasin and Thummabenjapone, 2012; Maneesuwan and Sirithorn, 2013; RT, 2014; Sutthisa et al., 2014; Diao et al., 2017; Yutthasin et al., 2018). This fungal pathogen can survive in the soil and plant debris. In recent years, anthracnose disease is the most problems in all planting areas in Thailand. Pathogens can survive for several years until a suitable period. It causes substantial crop losses in most of the major producing regions in the world and has a wide host range causing this disease in the tropical areas of Thailand, Asia, Africa and Latin America (Galván et al., 1997; Sa-ardluan and Sruamsiri, 2005; Vurro and Gressel, 2006; Srisuttee and Nalumpang, 2007; Gajbhiye et al., 2009; Santana et al., 2016; Suwannara et al., 2017). Fungicides have been extensively and successfully used to control anthracnose of green shallot. This fungal disease can be controlled by Prochloraz (1H-imidazole-1-carboxamide) at a concentration of 20-30 mL $20L^{-1}$ of water (U.S. National Library of Medicine National Center for Biotechnology Information), but cause environmental pollution in the agricultural soil and on plant products. The deaths of nearly 600 people each year are directly a result of the use of insecticides, herbicides, fungicides, and pesticides (Thai-PAN, 2019). Caused the farmers and customers concern about over toxic agrochemical residue in agricultural products due to rapid urban and industrial evolution and increasing reliance on agrochemicals in the past several decades (Vila Nova et al., 2011; Patil, 2013; Sharma and Kulshrestha, 2015). The

chemical pesticide residue contamination of agricultural products has become an increasingly severe problem in Thailand and ASEAN countries (Kongtragoul and Nalumpang, 2010; Jaipin and Nalumpang, 2014; Phukang and Chaisuk, 2016; Suwannarat et al., 2017). The wide use of pesticides raises concerns about the health of the consumers gradually (Le Thanh et al., 2015).

2.2 The important disease of green shallot

All crops can be infected by a wide range of pathogens. The weather is responsible for what extent pathogens may infect any plant. The disease will be developed rapidly if the required combination of temperature, humidity or moisture, and other climatic parameters are present. The crops can quickly suffer economic damage as a result of an infection by a pathogen. The green shallot is commonly cultivated in Thailand, and is an essential vegetable for cooking. These increased green shallot demand because the rapidly growing population has led to an expansion of green shallot cultivation in many countries (Kim et al., 2008; Buensanteai and Athinuwat, 2012). However, green shallot production is significantly reduced due to attack by diseases as follow.

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2.2.1 Bacterial soft rot

Bacterial soft rot is a significant disease of shallot or onion, and causes severe problems for growers. The affected scales first appear water-damp and yellow color when infected by *Pectobacterium carotovorum*. While several types of bacteria are participatory with decay through secondary infection, but only *P. carotovorum* produces pectolytic enzymes to cause the specific disease known as soft rot. The intrude becomes sticky and soft with the inside of the bulb breaking-down. A soft,

foul-smelling bulky liquid can be a force from the neck of diseased (Walker et al., 2009; Conn et al., 2012). At present, there are non-effective chemical control measures to combat bacterial soft rot (Wright et al., 2005).

2.2.2 Smut disease

Smut disease caused by *Urocystis colchicine* in plants generates black spots that a few bloated can be seen on the cotyledons. Next, this rip opens, and a substantial number of spores are unleashed. Infected plants encounter from retarded growth, and in cases of extreme infection. The symptoms occur during the entire season and will eventually infect the bulb as well. The lesions on the tubers are frequently the point of entry for different secondary pathogens that cause rot. Chemical controls are most effective when combined with sound cultural practices, for example, mancozeb 150-200 g/100 L of water for the control of smut diseases in shallot and onion and the seed treated with captan or thiram at 2.5 g/kg of seed before insemination for controls this disease (Mishra et al., 2014; Schwartz et al., 2016).

2.2.3 Downy mildew

Downy mildew caused by the *Peronospora destructor* arises worldwide when relatively cold moist weather. Downy mildew is the biggest threat and severe illness on onion and shallot crops that can produce essential yield damage in the seed and bulb production. The veritable yield losses in onions of more than 75% recorded (Gilles et al., 2004; Scholten et al., 2007). Seed treatment is the commonly chemical control method. Thiophanate methyl was used at 2.5 g/kg seed, and for a foliar application, spraying the plant with metalaxyl+mancozeb at 250 g/liter of water. Spray at weekly intervals depending on the severity of the disease (Pakistan, 2014).

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2.2.4 Purple leaf blotch (PLB)

Purple leaf blotch (PLB) is a significant disease of *Allium* spp. worldwide and is more supereminent in warm and damp environments (Aveling, 1998). The pathogen is ordinarily participatory with the PLB caused by the *Alternaria porri*. Furthermore, leaf blight is an important disease of *Stemphylium vesicarium* (Wallr.) *Simmons* and PLB is regarded to be a complex disease caused by pathogens (Suheri and Price, 2000). The PLB causes considerable yield losses in *Allium* ssp. production. In onions, it reduced foliar output by 62-92% (Utikar and Padule, 1980; Suheri and Price, 2001; Suheri and Price, 2000). For disease control, healthy seed should be used. Besides, chlorothalonil at 0.2% or mancozeb at 0.25% should be sprayed at fortnightly interval initiation after transplanting (Vinod Kumar, 2012).

2.2.5 Anthracnose disease

Anthracnose also called ‘twister disease’ or ‘seven curls disease’ is an essential cause of yield losses in onion and shallot. The causal agent of the disease is *C. gloeosporioides* (Galván et al., 1997). This airborne disease has an extensively host range and occurs in tropical regions of Africa, Asia, and Latin America (RB, 1990). The possible dispersing of conidia of *C. gloeosporioides* can be completed by wind, rain, and insects (Ebenebe, 1980). This disease symptoms tapering hollow spots on the foliage at the inception of infection trace by the shape of concentric rings of acervuli in hollow necrotic spots. Dieback is observed on heavily infected foliage, which leads to the fall of plants. Curling of foliage hap in plants (Ebenebe, 1980; Rajasab and Chawda, 1994; Sikirou et al., 2011).

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2.2.5.1 General information on anthracnose disease

Anthracnose symptoms are often distinguished on foliage casing of green shallot vegetative. The symptoms appear as jerky brown spots on the foliage casing. Grayish conidial masses are produced possibly on the black spots (Kim et al., 2008). Anthracnose caused by the fungi *C. gloeosporioides* and *C. acutatum* is an important disease having the host species such as inclusive of apple or strawberry (Trkulja, 2003; Živković et al., 2010). Disease outbreaks can happen quickly, and damage can be radical, particularly under long warm, and moist weather conditions. Typical plant symptoms enclose sunken, dark, and circular lesions that produce sticky, orange conidial masses. Under radical disease pressure, the wound can coalesce and the affected plant can drop prematurely, and damage occurs over post-harvest infection in storage (Živković et al., 2010). The foliage necrosis dieback leads to abatement in the active photosynthetic surface area of the plant, and epidemics at the stage before or during tuber formation can result in yield damage more than 85% (Abang et al., 2002).

2.2.5.2 General information of *C. gloeosporioides* causing anthracnose disease

The *C. gloeosporioides* is one major of plant pathogenic genera responsible for anthracnose. It is particularly essential. Owing to the radical economic damage (Chung et al., 2008). The most accepted taxonomic detail of *C. gloeosporioides* is provided hereupon.

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Division: Ascomycota

Class: Sordariomycetes

Order: Phyllachorales

Family: Phyllachoraceae

Genus: *Colletotrichum*

Species: *gloeosporioides*

The *C. gloeosporioides* belongs to the Phyllachoraceae family in division Ascomycota. The fungus includes *C. gloeosporioides* is anamorph defective and arises on a wide of host species by producing acervuli under the host tissues during an asexual phase of their life cycle. The teleomorph state is known for its potentiality to cause radical disease (Gautam, 2014).

2.2.5.3 Disease cycle of onion anthracnose

Relatively cold temperatures assist the infection process. Hence, the disease tends to be more severe during wet, cold springs. After infection, the *C. gloeosporioides* fungus colonies foliage tissues and begins to produce spores, and fruiting structures are able of increasing foliage tissues. Disease evolution may continue throughout the spring into early summer if advisable stay persists. These diseases keep being less of a problem during hot and dry summer weather (K-State Research and Extension, Dept. of Plant Pathology) (Figure 2.1).

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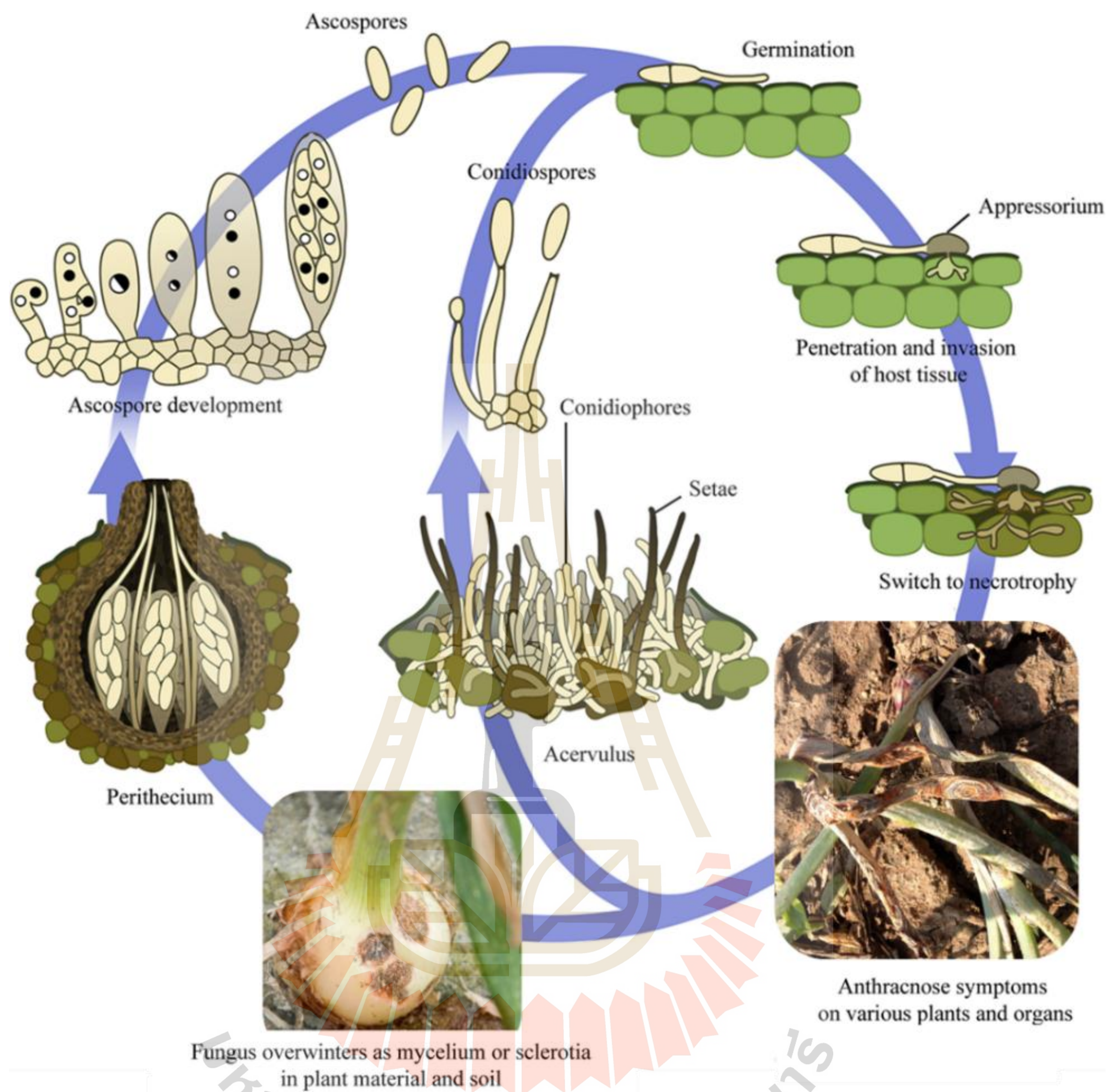

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Figure 2.1 General life cycle of *Colletotrichum* species (Silva et al., 2016).

2.3 Control of anthracnose disease

Anthracnose disease is a global problem and particularly essential owing to the radical economic damage it causes to plants that are grown (Chung et al., 2008). The anthracnose disease management is a method that concentrates on the reduction and after the development of the pathogen on green shallot. These goals can be

accomplished using disease-resistant cultivars, cultural practices, chemicals, biological controls, and induced resistance.

2.3.1 Cultural practices

Cultural practices consist of many methods, and green shallot can be vegetative successfully on which productive. The maximum pH range, irrespective of soil type, is 6.0-6.8, while alkaline soils are also appropriate. The green shallot does not progress in soils under pH 6.0 because of provisionally or manganese toxicity or trace element insufficiency or aluminum. Before cultivation, soils should be furrow and soil mass and disked adequately to rid debris. In commercial area nursery, 0.9-1.0 m wide is formed, and 2-6 rows are seeded or planted on the nursery. They may be two-line rows with plants staggered to obtain suitable spacing and high population intensity (Shanmugasundaram and Kalb, 2001; Kulatunga et al., 2013).

2.3.2 Chemical control

In Thailand, plant pathologists of the Department of Agriculture have been concluded that the cause of the anthracnose disease is *C. gloeosporioides* (Mishra et al., 2012; Wongchalee et al., 2015). Fungicides are generally used in the defense of the disease (e.g., Prochloraz, Carbendazim, Benomyl, and Mancozeb). These chemicals effectively control and suppress a wide variety of anthracnose disease and have been fully used to manage this pathogen (Urkude et al., 2019). Moreover, the capability of these chemicals has degenerated over time because of the development of fungicide-resistant isolates (Kongtragoul et al., 2011). Prochloraz is a fungicide that is popularly used worldwide with non-systemic properties (Kuo, 2001; Vinggaard et al., 2005). This fungicide is known as special inhibition of the ergosterol, fatty acid, the synthesis of which is necessary to the structural formation of preventing

the mycelial growth and fungal cell membranes. Danderson (1986) reported submerging avocados for 5 minutes in a solution comprising 500 ppm of the active ingredient, Prochloraz, and this was found to reasonable control of anthracnose. In the early 1990s (Wicks et al., 1994), applied Prochloraz to preventatively manage anthracnose disease every seven days, under field conditions in head lettuce, and it was found to be more effective than several fungicides. Prochloraz has also proven to be the most effective fungicide for lettuce anthracnose (Broadhurst & Wood, 1996). Moreover, Carbendazim is also suggested for the management of the anthracnose disease of many plants. This fungicide can inhibit microtubule formation during mitosis of cell division and binding to the β-subunit of β-tubulin leading to inhibit microtubule assembly (Davides 1986; Steffens et al. 1996; Prakash, 2004; Ma and Michailides 2005; Duamkhanmanee 2008; Kongtragoul, 2011). A field experiment on disease control showed the most effective function of carbendazim. Carbendazim gave the best result at spraying rates of 10 or 15 g/20 L (Kanlong et al., 1990). In 2016, Patil and Nargund evaluated carbendazim 12%+iprodione 63% (Quintal) and concluded that they inhibited maximum mycelial growth (95.43%).

2.3.3 Biological control

The biological method is present an established sub-discipline in the Science of Plant Pathology (Elad, 1996; Paulitz and Bélanger, 2001). However, absolute control of aforesaid diseases in onion or shallot is yet to be developed. Management strategy for the disease consists of the use of recommended resistant cultivars, disease-free bulbs, and fungicidal sprays. Still, continuous use of fungicides to control the plant diseases results in the maintenance of mischievous chemical residues in the environment (Naguleswaran et al., 2014). Bacterial and fungicidal

biocontrol agents were studied on anthracnose diseases such as *Bacillus* spp., *B. polymyxa*, *B. amyloliquefaciens*, *B. pumilus*, *B. cereus*, *B. subtilis*, and Arthrobacter (Munimbazi and Bullerman, 1998; Havenga et al., 1999; Ashwini and Srividya, 2014; Rungjindamai, 2016). Biological control of diseases in the plant using microbial antagonists and eco-friendly alternatives to replace the use of chemical pesticides and using microbial antagonists as part of integrated disease control have demonstrated to repress a diversity of pathogens (Palaniyandi et al., 2011). Naguleswaran and Pakeerathan (2014) revealed that the effect of the bulb treatment together with foliar application of *T. viride* (biocontrol agents) operate very well for yield (130.7 Mt/ha) with the little disease incidence (1.08 %) and yield (79 Mt/ha). The results achieved in this study helped to control diseases of red onion and are the best promising alternative to sustainable agriculture. According to Freeman et al. (2004), the antagonist capacity of *T. harzianum* strain T-39 from the TRICHODEX (commercial biological control product) was effective in controlling gray mold (*Botrytis cinerea*), and anthracnose disease (*C. acutatum*) in strawberry. Among the biocontrol agents evaluated, *T. harzianum* strain Th-2 was found to be most efficient, giving approximately 77.78-100% inhibition on the mycelial growth. So, *T. harzianum* can apply for the control of anthracnose disease in chili (Begum and Nath, 2015). Galindez et al. (2017) tested the results of *Trichoderma* sp. as a potential biocontrol agent against *C. gloeosporioides* isolated from the onion. The results indicated the percent inhibition of the *Trichoderma* sp. among 14 days of maturing, and *T. longibrachiatum* exhibition the highest percent inhibition of 64.68% compared to *T. harzianum* and *T. asperellum* showing 59.16% and 47.73%, respectively. This was summarized that the

presence of *Trichoderma* sp. could reduce the *C. gloeosporioides* damage and could be used as a biocontrol agent of the pathogen.

2.3.4 Induced resistance

Plants can emerge induced resistance as a result of infectiousness, upon colonization of the roots by the exclusive helpful microorganism (Figure 2.2). The induced condition of resistance features by the actuation of potential defense mechanisms that indicate a challenge from a plant pathogen. Induced resistance is shown not only locally at the predicament of induction but also systemically in plant parts that presume separated from the inducer. Normally, induced resistance provides a supplement level of prevention against a broad spectrum of attackers from the pathogen. Induced resistance is determined by a system of interconnected signaling pathways in that plant hormone having an important regulatory role.

Elicitors have been widely estimated to control plant diseases based on the induced resistance idea (Choudhary et al., 2007; Buensanteai et al., 2009; Buensanteai and Athinuwat, 2012). The Induced Systemic Resistance (ISR) and Systemic Acquired Resistance (SAR) are two kinds of induced resistance wherein plant defenses are pre-conditioned by an early infection that results in resistance against subsequent challenge by a pathogen. One property of SAR is the development of the supplement resistance in uninoculated plant organs and tissues. Another strategy of SAR is its activity against an extensive and dominant spectrum of pathogens (Vallad and Goodman, 2004; Conrath, 2006). The ISR is potentiated by plant growth-promoting rhizobacteria (PGPR). The ISR relies on pathways regulated by ethylene and jasmonate and is not associated with the expression of PR genes (Heil

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and Bostock, 2002; Van Loon and Bakker, 2005; Choudhary et al., 2007; Walters et al., 2008; Okon Levy et al., 2015).

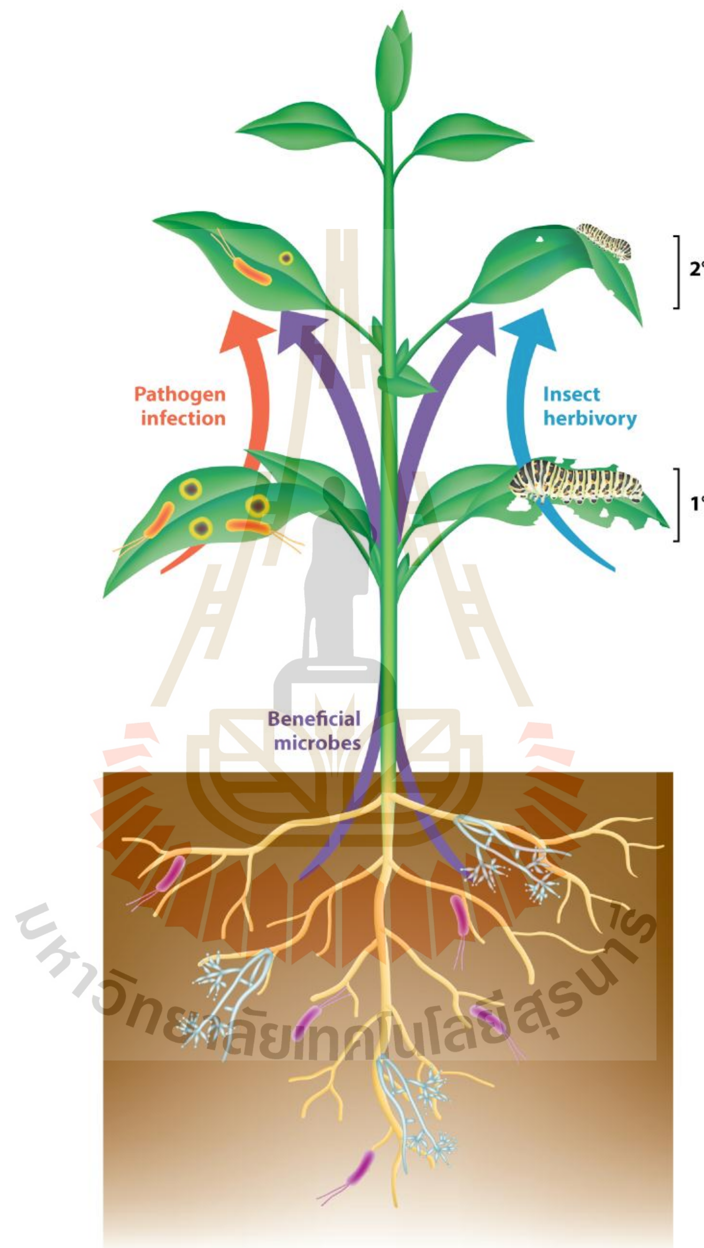

Figure 2.2 The schematic instance of biologically induced resistance stimulus by insect herbivory (blue arrow), pathogen infection (orange arrow) (Pieterse et al., 2014).

2.3.4.1 Mechanisms of defense to pathogens

Plant defenses include both pre-existing barriers as well as defenses induced in the perception of molecules produced from damage as an effect of infectiousness (DAMPs; damage-associated molecular patterns) or pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs). Perception of these molecular patterns and the signaling thus initiated conducts to the deployment of a syndrome of induced defenses, which includes the amplification of the initial signals to neighboring cells and even to else parts of the ISR or SAR (Figure 2.3) (Garcion et al., 2014; Pieterse et al., 2014; Andersen et al., 2018).

2.3.4.2 Structural defense mechanism

The cell wall is considered to be the first obvious barrier to potential pathogens. Progress has been made in elucidating its chemical structure and function (Burton et al., 2010). The cell wall is also a source of molecules (DAMPs) that signal the presence of invading microbes and induce defense reactions (Hückelhoven, 2007; Vorwerk et al., 2004; Wolf et al., 2012). Many observations have shown that plants respond to attempted infections by the formation of cell-wall deposits or papillae targeted at the site of attempted infection. In many cases, this provides an efficient barrier against non-host pathogens. True pathogens might outrun the plant or prevent the deposition of papillae. Several reviews have addressed this topic in the past (Hückelhoven, 2007; McLusky et al., 1999; Nicholson and Hammerschmidt, 1992, Thordal-Christensen, 2003; Thordal-Christensen et al., 1997; Zeyen et al., 2002). The nature of cell wall appositions papillae contains calloses, a β-1,3-glucan polymer with β-1,6-glucan branches. Other components include various phenolics, hydrogen peroxide, or proteins (Stone and Clarke, 1992; Bestwick et al., 1997; Rey et al., 1996;

Smart et al., 1986; von Röpenack et al., 1998; Zeyen et al., 2002). Calloses is a significant component of papillae and is produced after inoculation with pathogens or with chemical potentiators of plant resistance (Hückelhoven et al., 1999; Skalamera and Heath, 1996; Soylu et al., 2004; Kogel et al., 1994; Ton et al., 2005; Zimmerli et al., 2000). During a resistant interaction, papillaes enriched with calloses might offer a mechanical barrier to penetration by non-host pathogens. Still, virulent pathogens are not affected either because they are faster, or they can prevent its formation (Aist, 1976). Some reports have further explored the relevance of calloses as a mechanical barrier for penetration. In Arabidopsis Atgsl5, a gene encoding glucan synthase 5 catalyzes callose biosynthesis. This gene highly expressed in constitutively resistant *Arabidopsis* mapk4 (Ostergaard et al., 2002). Constitutive expression of the NahG gene encoding a bacterial SA hydroxylase in mapk4 abolishes resistance and expression of Atgsl5 (Ostergaard et al., 2002). The content of callose in papillae was reduced in double-stranded RNA interference lines targeted at Atgls5. Still, these plants only showed marginal loss of resistance to a penetration by non-host powdery mildew from barley, however displayed strong resistance against *Peronospora parasitica*, the virulent powdery mildew of *Arabidopsis* (Jacobs et al., 2003).

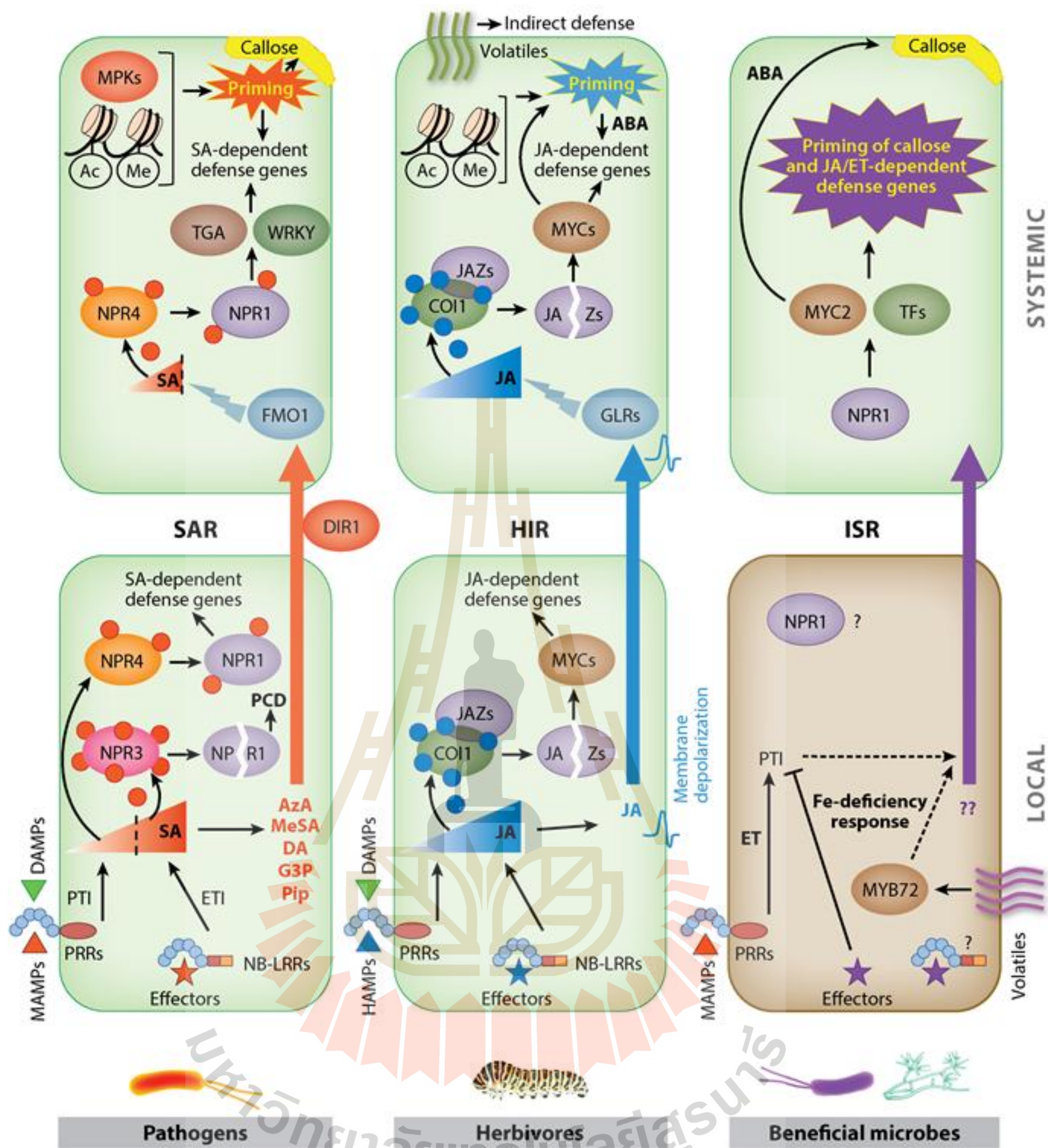

Figure 2.3 The schematic instance of mechanisms involved and molecular components in pathogen-induced systemic acquired resistance (SAR), herbivore-induced resistance (HIR), and induced systemic resistance (ISR) stimulus by the beneficial microbes (Pieterse et al., 2014).

The aromatic polymer lignin's are a major component of secondary walls and has an essential role in plant growth and evolution as well as in the defense against invaders. Lignin makes the wall mechanically rigid and prevents diffusion of water-soluble compounds (enzymes, toxins) released by pathogens (Ride, 1983). Its complex polymeric nature is slowly being deciphered (Boerjan et al., 2003; Humphreys, 2002; Zhao and Dixon, 2011; Zubieta et al., 2002). The lignins deposited in response to the pathogen invasion. Such defense lignins has been observed in cell-wall appositions (Nicholson and Hammerschmidt, 1992; Carver et al., 1992; von Röpenack et al., 1998) but also in entire walls of the infected cells or only at the infection site (Heitefuss, 2001; Moersbacher and Mendgen, 2000; Vance et al., 1980). Besides, defense-related lignin might be of a different composition to developmentally related lignins. As discussed by Hückelhoven (2007), the evidence for the role of lignins in resistance is mostly based on correlative studies (with various inhibitors), and genetic evidence using suppression of gene expression is rare, gave the redundancy of the enzymes involved in lignin biosynthesis. Despite this difficulty, Bhuiyan et al. (2009) recently silenced individually or in combination, several genes involved in monolignol synthesis in wheat using RNAi interference. The transcripts of caffeic acid O-methyltransferase, caffeoyl-CoA O-methyltransferase, ferulic acid hydroxylase, phenylalanine ammonia-lyase (PAL), and cinnamyl alcohol dehydrogenase, were found to accumulate differentially in the epidermis of susceptible or resistant plants after an infection with *Blumeria graminisf.* sp. *tritici* (Bgt). The transient silencing of these genes in the cell layer led to an increased susceptibility to Bgt and reduced resistance to infiltration against the non-host pathogen *Blumeria graminis* f.sp. *hordei*. The autofluorescence of the papillae at the site of contact with the pathogen also

decreased, if evidence for a part of monolignol production in localized defense to pathogens in wheat (Bhuiyan et al., 2009).

2.3.4.3 Biochemical defense mechanism

Plant defense mechanisms against pathogen attacks. Each of these mechanisms is controlled by genes. These mechanisms can be separated into two groups: one elated to nonspecific response to pathogens and another depending on resistance genes; host-pathogen interactions in figure 2.4 (David Guest and John Brown, 1997).

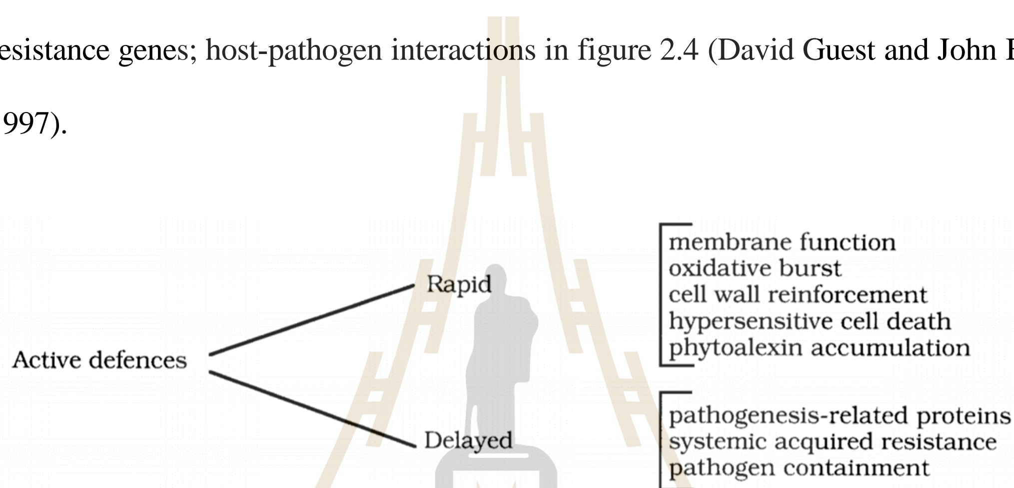

Figure 2.4 Biochemical defense mechanism in plants.

2.3.4.3.1 Rapid active defense

Plant responses to infection are complicated of incidence, almost host-pathogen interaction is special in the particulars of the timing, localization, and activation of each constitutive of the defense responses. As before time stated, resistance is gradually certain, and susceptible depends on the sum of many individual responses or whether a plant ends up being resistant.

2.3.4.3.1.1 The hypersensitive response (HR) was imposed by Stakman (1915), and more recently, by Goodman and Novacky (1994) as the sudden death of plant cells participatory with disease resistance. It happens in resistant plants in response to the pathogens. It is related to biochemical processes that operate these

dead cells, and the nearby living cells, an inhospitable environment for the pathogen (Kombrink and Somssich, 1995; Heath, 1998; Garcion et al., 2014). However, it now seems clear that pathogen-triggered cell death can be dissociated from defense mechanisms and, to some extent, from plant resistance (Clough et al., 2000; Coll et al., 2010; Yu et al., 1998). That is why the term HR is commonly used in the literature for describing the defensive arsenal that is deployed during incompatible interaction, and plant host cell suicide is often referred to as hypersensitive cell death (HCD) in this context. HCD is a form of programmed cell death (PCD), which implies a genetic orchestration of cell suicide. HCD can be induced following plant challenges with viruses, bacteria, or fungi.

2.3.4.3.1.2 The reactive oxygen species (ROS), several plant tissues were reported producing ROS after pathogen infection. ROS are chemically reactive species of oxygen formed by the incessant one-electron abatement of molecular oxygen (O_2) and include the hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot -}$), hydroperoxyl radical ($HO_2^{\cdot}$) or hydroxyl radical ($OH^{\cdot}$). ROS are also produced during plant development and environmental factors (Laloi et al., 2004). After pathogen infection or elicitor treatment, the most abundant ROS produced is H_2O_2, and its production is mainly observed in the apoplastic space and coincides with the induction of cell death during the HR (Grant and Loake, 2000). Adjustment of ROS levels in the plants by lowering ascorbate peroxidase or catalase activity has demonstrated the role of H_2O_2 in limiting pathogen diffuse and involvement in cell death.

2.3.4.3.1.3 Defense-related enzymes including phenylalanine, ammonia-lyase peroxidase, chitinase, and polyphenol oxidase are related to inducing resistance in plants.

Peroxidases are an extra class of pathogenesis-related (PR) proteins. They appertain to PR protein nine subfamily and expressed in limiting the cellular diffusion of disease through the establishment of generation or structural barriers of very toxic environments by enormously producing ROS. Peroxidase activity of peroxidase gene expression in higher plants is induced by bacteria, fungi, viroids, and viruses (Vera et al., 1993; Sasaki et al., 2004; Diaz-Vivancos et al., 2006; Lavania et al., 2006).

Chitinases are an enzyme and one of the critical PR proteins that release chitin; it takes part in plant defenses against chitin containing from pathogens (Jalil et al., 2015). The β-1,3-glucans and chitins the polymer of *N*-acetylglucosamine, are important cell wall constitutive of many fungi because chitinases and β-1,3- glucanase have shown to be efficient in attacking cell walls of fungal pathogens, these enzymes have an offer as direct defense enzymes of plants (Abeles et al., 1970).

Phenylalanine ammonia-lyase (PAL) is an important enzyme that is responsible for conjugate the primary metabolism of aromatic amino acids with secondary metabolic products. PAL catalyzes the non-oxidative deamination of phenylalanine into ammonia and trans-cinnamic acid which is the first step in the biosynthesis of phenolic compounds. PAL is a dependable treatment for the genetic condition phenylketonuria due to the natural potentiality of the enzyme to breakdown L-phenylalanine (MacDonald and Dcunha, 2007).

2.3.4.3.2 Delayed active defense

Phytoalexins are low molecular weight antibiotics from the plants to respond to the pathogen attack. Their intoxication is non-selective for the

chemical togetherness of major phytoalexins for lipids suggests that they were generated in cell membranes. The phytoalexins play a role in disease resistance, and contribute to different inhibitory levels at the further, and infection restricts. Then, over three hundred fifty phytoalexins have been found in more than one hundred plant species from thirty families of monocotyledons and dicotyledons. The structure of a chemical in phytoalexins is multitudinous, they are small organic compounds synthesized from one of three secondary metabolic pathways such as the acetate-malonate, acetate-mevalonate, or shikimic acid pathways.

Pathogenesis-related (PR) proteins are belong to a various group of plant proteins that regard the role of an essential part of plant disease resistance. They are extensively disposed in plants but produced in higher concentrations after the pathogen attack. PR proteins contain in intercellular spaces and also in the plant cells intracellularly, especially in the cell walls of different tissues (Agrios, 2005). The PR proteins have classified agreeable about function, molecular weight, and an amino acid sequence. Nowadays, the PR proteins were divided into Seventeen families, including peroxidases, β-1,3-glucanases, thaumatin-like proteins, oxalate-oxidase-like proteins defensins, chitinases, nonspecific lipid transfer proteins, thionins, ribosome-inactivating proteins, and oxalate oxidase (Table 2.1). The PR proteins are extremely basic either-or extraordinarily acidic, and so are highly reactive, and soluble (Legrand et al., 1987; van Loon and van Strien, 1999). The signal compounds are responsible for the induction of PR proteins consisting of xylanase, ethylene, polypeptide systemin, salicylic acid, and jasmonic acid (Agrios, 2005).

Table 2.1 Accepted families of PR proteins (Prasannath, 2017).

Family	Type member	Properties
PR-1	Tobacco PR-1a	Unknown
PR-2	Tobacco PR-2	ß-1, 3-glucanase
PR-3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII
PR-4	Tobacco 'R'	Chitinase type I, II
PR-5	Tobacco S	Thaumatin-like
PR-6	Tomato Inhibitor I	Proteinase-inhibitor
PR-7	Tomato P69	Endoproteinase
PR-8	Cucumber chitinase	Chitinase type III
PR-9	Tobacco "lignin-forming peroxidase"	Peroxidase
PR-10	Parsley "PR1"	Ribonuclease-like
PR-11	Tobacco "class V" chitinase	Chitinase, type I
PR-12	Radish Rs-AFP3	Defensin
PR-13	Arabidopsis THI2.1	Thionin
PR-14	Barley LTP4	Lipid - transfer protein
PR-15	Barley OxOa (germin)	Oxalate oxidase
PR-16	Barley OxOLP	Oxalate-oxidase-like
PR-17	Tobacco PRp27	Unknown

2.4 Elicitors

The term of elicitor was often used for compounds stimulating to plant defense (Thakur and Sohal, 2013). Ultimately, the induction of plant defense responses maybe leads to an enhanced resistance. This extensive definition of elicitor consists of both

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compounds released from plants by the action of the pathogen and substances of pathogen origin (Angelova et al., 2006). Different kinds of elicitors have been characterized, including lipids, glycopeptides, glycoproteins, and carbohydrate polymers (Table 2.2). Ensuing elicitor insight, the activation of signal transduction pathways ordinarily leads to the production of some of which possess antimicrobial properties, phytoalexin biosynthesis, deposition of calloses, active oxygen species (AOS), the accumulation of PR proteins, and synthesis of defense enzymes (Thakur and Sohal, 2013).

2.4.1 Abiotic elicitors

Various chemicals were discovered that seem to act at various points in these defenses activating networks or parts of the biological activation of resistance. The best-studied resistance activator is acibenzolar-S-methyl (BION) resistance in many crops against a broad spectrum of disease pathogens, including fungi, bacteria, and viruses (Oostendorp et al., 2001). Furthermore, some abiotic inducers from plant extracts studied on induced resistance. In 1995, Cohen and Tosi reported that *β*-Aminobutyric acid, D, L-*β*-aminobutyric acid (BABA), or it is 3-(S)-enantiomer can activate disease resistance, especially against downy mildews in various crops when used at relatively high rates. In 1977, Sticher et al. reported that Probenazole is mainly using on rice against rice blast (*Pyricularia oryzae*) and bacterial blight (*Xanthomonas oryzae*) either as seedling box treatment or a granular treatment of paddy rice and besides, it is registered in several other crops mainly against bacterial pathogens. Le Thanh et al. (2015) studied the efficacy of resistance inducers against bacterial leaf blight (BLB) in rice. Among the inducers applied as seed soak and foliar spray, SA reduced

Table 2.2 List of various types of plant elicitors (Thakur and Sohal, 2013; Wiesel et al., 2014).

No.	Plant	Type of elicitor used	Effects	References
1	*Brassica napus*	Methyl jasmonate	Accumulation of indolyl glucosinolates in the leaves.	Doughty et al. 1995
2	*Oryza sativa*	Benzothiadiazole	BTH protected wheat systemically against powdery mildew infection by affecting multiple steps in the life cycle of the pathogen.	Görlach et al. 1996
3	*Lycopersicon esculentum*	Salicylic acid	Diminished susceptibility to pathogens harm and abiotic stress.	Shirasu et al.. 1997
4	*Brassica oleracea* (var. *Botrytis*)	Benzothiadiazole	BTH induced downy mildew (caused by *P. parasitica*) resistance in both cauliflower seedlings and 30-day old plants.	Godard et al. 1999
5	*Musa acuminata*	Salicylic acid	Delayed ripening of banana fruit.	Srivastava and Dwivedi. 2000
6	*Lycopersicon esculentum*	Salicylic acid	Induced the synthesis of some stress proteins, such as PR proteins, which leads to increased chilling tolerance and resistance to pathogens, thereby decreasing the incidence of decay.	Garcia-Magallon et al. 2002

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Table 2.2 List of various types of plant elicitors (Thakur and Sohal, 2013; Wiesel et al., 2014) (Continue).

No.	Plant	Type of elicitor used	Effects	References
7	*Lilium*	Benzoic acid	Modified the growth, stress tolerance, anatomy, and morphology of eatable and ornamental species.	Ding et al. 2002
8	*Helianthus annuus*	Benzothiadiazole	Prevented infestation by the parasitic weed *Orobanche Cumana*. Root exudates revealed synthesis of the phytoalexin scopoletin, PR-protein chitinase, and H_2O_2.	Sauerborn et al. 2002
9	*Lycopersicum esculentum*	Chitosan	Produced a higher resistance against *Fusarium oxysporum* and *Phytophthora capsici*.	Ortega-Ortíz et al. 2003
10	*Lycopersicon esculentum*	Chitosan and salicylic acid	Increased level of catalase and peroxidase enzyme activity.	Ortega-Ortiz et al. .2007
11	Soybean, rice, and wheat	β-glucans from Phytophthora and Pythium	Produced phytoalexins in soybean and rice plants. Lignification in wheat leaves.	Montesano et al. 2003
12	Arabidopsis, tomato	Oligogalacturonides from bacteria and fungi	Synthesis of protein inhibitors and activation of defense genes.	Montesano et al. 2003
13	Tobacco, tomato	Viral coat protein Harpin from TMV	Activation of hypersensitive response.	Montesano et al. 2003

Table 2.2 List of various types of plant elicitors (Thakur and Sohal, 2013; Wiesel et al., 2014) (Continue).

No.	Plant	Type of elicitor used	Effects	References
14	Tomato	Avr gene products, for example, AVR4 and AVR9 from *Cladosporium fulvum*	Activation of hypersensitive response.	Leach and White. 1996
15	Arabidopsis	Flagellin, flg 15 from gram negative bacteria	Deposition of callose and activation of defense genes in Arabidopsis.	Gómez-Gómez and Boller.2000
16	Oat	Protein or peptide toxin, victorin from *H. victoriae* (rust)	Programmed cell death in oat.	Tada et al. 2001
17	Parsley	Glycoprotein from *Phytophthora sojae*	Synthesis of phytoalexin and activation of defense genes in parsley.	Montesano et al. 2003
18	Soybean	Syringolids from *Pseudomonas syringae*	Activation of hypersensitive response.	Montesano et al. 2003
19	Tobacco	Fatty acid, and amino acid conjugates from Lepidopterans	Synthesis of monoterpenes leading to activation of indirect defense in tobacco.	Montesano et al. 2003
20	Arabidopsis	The bacterial toxin, for example, coronatine from *Pseudomonas syringae*	Activation of defense genes in Arabidopsis.	Kloek et al. 2001

disease severity significantly by about 55.35% compared to that of the non-treated control. Thakur and Sohal (2013) reported that salicylic acid ($C_7H_6O_3$), methyl salicylate ($C_6H_4(OH)(CO_2CH_3)$), benzothiadiazole ($C_6H_4N_2S$), benzoic acid ($C_7H_6O_2$ or C_6H_5COOH) and chitosan can enhance the production of phenolic compounds (containing aromatic rings with attached hydroxyl groups) and activation of various defense-related enzymes in plants. Their introduction to agricultural practice could minimize the scope of chemical control, thus contributing to the development of sustainable agriculture.

2.4.2 Biotic elicitor

The use of biotic elicitors to induce resistance in plants is essential for crop production as the number of available effective synthetic fungicides reduced rapidly because of pathogen resistance to fungicides. Many beneficial microorganisms have studied for controlling plant diseases, such as *Bacillus* spp., *Agrobacterium radiobacter*, *Ampelomyces quisqualis*, *Aspergillus flavus*, *Coniothyrium minitans*, *Gliocladium Catenulatum*, *Pseudomonas* spp., *Trichoderma harzianum*. JM et al. (2016) reported that *B. subtilis* RC 218 could reduce disease severity and the associated mycotoxin (deoxynivalenol) accumulation of *Fusarium* sp., under field conditions. In 2017, Lin et al. found that Fusarium crown and root rot (FCRR) caused by the fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* is a damaging soil-borne disease of tomato. A *Bacillus* sp. strain HN09 isolated from neem tree rhizosphere soil showed on inhibition of the growth, germination, and development of normal morphology of the FCRR pathogen. A substantial level of disease control achieved in the greenhouse. The effect of *T. harzianum* was investigated on plant growth and inhibitory activity against *Pythium ultimum* and *Phytophthora capsici* under

laboratory conditions. The results revealed that mycelial growth of both pathogens was inhibited by *T. harzianum in vitro*. Jangir et al. (2018) conducted characterization of antagonistic *Bacillus* sp., isolated from tomato rhizosphere, and its control mechanisms against *F. oxysporum* f. sp. *lycopersici*. The results showed that *Bacillus* strain B44 had a 36% reduction in disease incidence in tomato plants under greenhouse conditions and could operate as an efficient agent for biocontrol of wilt disease in tomato plants.

2.4.3 *Bacillus* sp.

Bacillus spp. are globally dispersed bacteria producing numerous bioactive compounds with a broad spectrum of activities towards pathogens or inducing host systemic resistance (Wei et al., 2016). The *Bacillus* is a widely biocontrol agent, could be used to control many plant pathogens and also increase plant growth. Furthermore, to directly affecting plant growth and development through produce plant growth regulators, *Bacillus* can colonize roots and trigger plants to produce growth-promoting biomolecules (Saengchan et al., 2015; Wongchalee et al., 2015). The plant growth-promoting rhizobacterium (PGPR), *B. amyloliquefaciens* strain KPS46, can enhance growth in several economic crops such as vegetable soybean, corn, soybean, cassava, rice, cauliflower, and Chinese kale (Buensanteai et al., 2009; Buensanteai et al., 2012; Prathuangwong and Buensanteai, 2007; Saengchan et al., 2015). Plant growth enhancement by KPS46 is mediated in part by the induction of phytohormones, including auxin (indole-3-acetic acid, IAA), extracellular proteins, and lipopeptides (Buensanteai et al., 2009; Saengchan et al., 2015; Wongchalee et al., 2015). Bacterial synthesis of phytohormones that are similar to endogenous plant growth regulators enhances the initial processes of lateral and adventitious root

formation and elongation (Buensanteai et al., 2009; Saengchan et al., 2015; Wongchalee et al., 2015). In 2015, Wongchalee et al. reported that *Bacillus* sp. SPT41.1.3 was applied for controlling anthracnose on chili seeds by standard blotter plate mix with *B. megaterium* SBK5.7 and *Bacillus* sp. SPT41.1.3. These *Bacillus* can reduce the severity of anthracnose on seeds by approximately 41.90%, which were significant as compared with control. Melnick et al. (2008) revealed *B. cereus* isolates BT8 and BP24 was primarily epiphytic, with endophytic populations typically representing 5-15% of total foliar bacteria. In 2015, Meng et al. reported that dry flowable of *B. subtilis* strain T429 formulations at 50 and 75 g/667 m^2 concentrations were as effective as a commercial fungicide to reduce rice blast, control efficiency up to 77.6% and 78.5%, respectively. Besides, disease control efficiency observed between the formulations (12 months of storage at room temperature) and the chemical pesticide tricyclazole (79.5%) was non-significant differences, confirming formulation's long shelf life and high viability. In 2016, Shternshis et al. studied the efficacy of the formulations based on *B. subtilis* and *P. fluorescens* to control the *Didymella applanata*, the causal agent of red raspberry (*Rubus idaeus* L.) spur blight. The results revealed that antagonistic activity towards *D. applanata* depended on the red raspberry cultivar and weather conditions. Efficacy of *B. subtilis* was higher than *P. fluorescens* in bio-control of the raspberry spur blight.

2.4.4 Biosurfactants from beneficial *B. subtilis* for disease control

Biosurfactants are secondary metabolites with surface-active properties and have wide applications in agriculture, therapeutic products, and industrial. The microbial surfactants are a structurally diverse group of surface-active molecules produced by a wide variety of microorganisms, including bacteria and fungi. These

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are amphiphilic molecules with both hydrophilic and hydrophobic moieties that confer the ability to accumulate between fluid phases, thus reducing surface tension at the surface and interface respectively (Mukherjee et al., 2006; Romero et al., 2007; Ongena Jacques, 2008; Kumar et al., 2017). Members of the *Bacillus* genus are considered as efficient microbial factories for large scale production of lipopeptides such as iturin, surfacing, and fengycin, inhibiting various fungal pathogens and protecting the crop plants (Singh et al., 2014; Jin et al., 2014). The *B. subtilis* and *B. licheniformis* are two important bacteria that were used to produce many kinds of metabolites (vitamins, amino acids, and antibiotics) and industrial enzymes (Harwood 1992). Members of the *Bacillus* group (*B. subtilis, B. amyloliquefaciens, B. mojavensis, B. pumilus, B. vallismortis*) are fully sited in the soil, where they help to recycle carbon and nitrogen via the production and secretion of macromolecular hydrolases such as proteases, amylases, and cellulases. *B. subtilis* has been known to produce a range of secondary metabolites including terpenes, polyketides (PKs) and siderophores, as well as ribosomally and non-ribosomally synthesized peptides. The identification of secondary metabolites and antimicrobial peptides (AMPs) were primarily based on their extraction from the culture medium, often because of their inhibitory effect on other bacteria and fungi. *B. subtilis* is widely distributing in the soil, where it helped to recycle nutrients via the production and secretion of macromolecular hydrolases such as amylases, proteases, cellulases and phosphatases. The conditions including pH, osmolarity, radiation, and competition from cohabiting microbes adversely affected to *Bacillus* spp. In brief, the *B. subtilis* group developed a range of strategies at increasing their competitiveness and survival (Hecker and Volker., 2004; Voigt et al., 2014). Secondary metabolites synthesized by the *B. subtilis* such as peptides

synthesized by non-ribosomal peptide synthetases (NRPS) and PKs, synthesized by polyketide synthases (PKS).

These peptides have a variety of forms and functions. Iron-chelating siderophores, cytotoxic cyclic lipopeptides, biosurfactants and clinically relevant peptide antibiotics were synthesized by NRPS of *Bacillus* species. Besides, several *Bacillus* strains can produce three families of cyclic lipopeptides being of particular importance, namely surfactins, iturins, and plipastatins or fengycins (Ongena and Jacques 2008). Amino and hydroxy acids are the basic building blocks for NRP, linked by amide or ester bonds, respectively. Each NRPS complex has a loading module, a variable number of elongation modules, and a termination module. PKs are a structurally diverse family of secondary metabolites exhibiting a wide range of biological activities. The PKs discovered so far number into the thousands. They are broadly classified into three structural classes according to the characteristics of the products of the gene clusters responsible for their synthesis in Table 2.3 (Hutchinson 1999).

These surfactants can also play different roles in the development and survival of *Bacillus* strains in their natural habitat, including the genetic makeup of the producer organisms (Das et al. 2008). Studies on molecular genetics and biochemistry of the synthesis of several biosurfactants have revealed the operons, the enzymes, and the metabolic pathways required for their extracellular production. Characteristics of the main PSK and NPRS were characterized by a member of the *B. subtilis* group.

Surfactin, although the synthesis and role of surfactin have been best studied in *B. subtilis*, related species also synthesize very similar NRPs. Surfactin is one of the most potent known biosurfactants; at a concentration of 20 μM, surfactindecreases the surface tension of water from 72 to 27 mN/m. Surfactin structured

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Table 2.3 The list of the secondary metabolites' clusters (non-ribosomal peptides and polyketides) identified in complete genomes.

Species and type	Compound	Prevalence
Bacillus subtilis (n = 68)		
Non-ribosomal peptides	Surfactin	99%
	Plipastatin/Fengycin	97%
	Bacillibactin	99%
	Bacilysin	93%
	Locillomycin	2%
	Xenocoumacin	2%
Polyketides	Bacillaene	77%
	Macrolactin	6%
	Difficidin	6%
	Kalimantacin/Batumin	2%
B. amyloliquefaciens (n = 21)		
Non-ribosomal peptides	Surfactin	100%
	Plipastatin/ Fengycin	95%
	Bacillibactin	100%
	Bacilysin	100%
Polyketides	Bacillaene	100%
	Macrolactin	71%
	Difficidin	67%

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by four isomers (surfactin A–D) that exhibit a wide variety of physiological activities. The chemical structure of surfactin includes a peptide loop of seven amino acids (L-leucine, L-asparagine/L-aspartate, L-leucine, L-valine, L-glutamate, and two D-leucines), attached to a hydrophobic fatty acid chain, the length of which is isoform dependent.

The iturin group is a large family of cyclic heptapeptides with a C14-C17 aliphatic β-amino fatty acid. They have chiral peptide sequences of L- and D- amino acids (LDDLLDL) and are cyclized by the formation of an amide bond between the N-terminal β-amino fatty acid and the C-terminus of the peptide. The group includes iturin (variants A, C, D, and E), bacillomyxin (D, F, L, and Lc) and mycosubtilin, as well as other variants with names that reflect their bacterial source. The β-amino fatty acid linked to the amino acid sequence Asn-Tyr-Asn is a common characteristic of the iturin group (Duitmann et al., 1999; Moyne, Cleveland, and Tuzun 2004). Iturins are synthesized by a ~38 kbp NRPS operon comprising four genes (Figure 5; Duitmann et al., 1999).

Many of these secondary metabolites are of value as antifungal and antibacterial compounds, and, as a result, certain *B. subtilis* group strains have been developed as probiotics and for the production of AMPs and other metabolites for use in humans' animals and agriculture (Cutting 2010).

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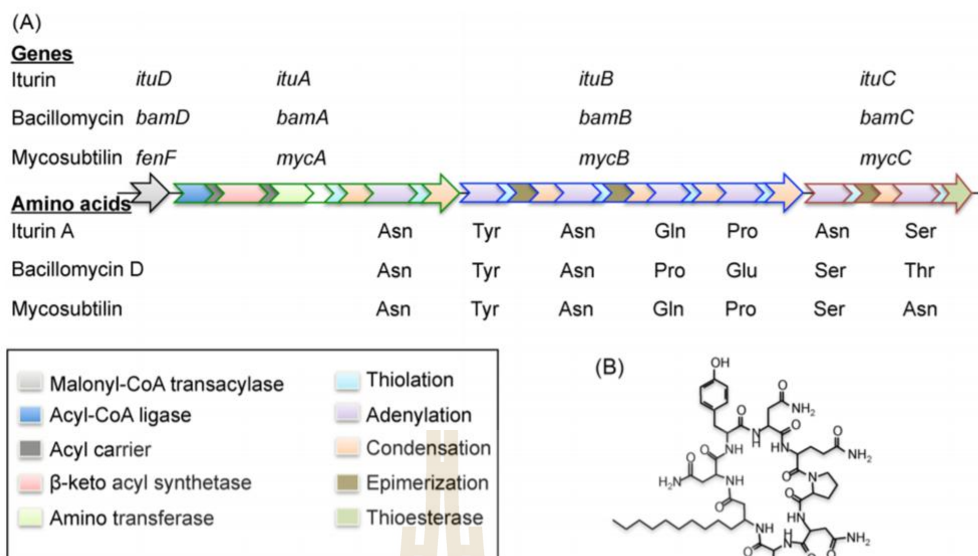

Figure 2.5 Iturin group gene cluster. (A) Representative 4-gene iturin operon showing the gene names and amino acid sequences for bacillomyxin, iturin, and mycosubtilin, color-coded to identify the activities of the various domains. (B) Structure of iturin (modified from Duitmann et al. 1999).

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CHAPTER III

MATERIALS AND METHODS

3.1 Preparation of *C. gloeosporioides* causing anthracnose disease

3.1.1 Isolation of fungal pathogens

Collected infected parts of green shallot were cut into small pieces with a size of 5-10 mm and the tissues were washed with a 10% sodium hypochlorite (NaOCl) solution for 1 minute and sterile distilled water (dH_2O) using flame-sterilized forceps. After that, they were transferred to Petri dishes containing water agar (WA), incubated room temperature. The growth of the fungal pathogens was occasionally observed for 3 days. The mycelium parts were carried to potato dextrose agar (PDA) and incubated 7 days for the absolute growth of fungi (Sompong et al., 2012; Sangpueak et al., 2017; Thilagam et al., 2017; Thumanu et al., 2017).

3.1.2 Pathogenicity test

The healthy green shallot leaves were sterilized with a 10% NaOCl solution for 1 minute. Then NaOCl was removed by washing with sterile dH_2O. The spore suspensions of each fungal isolates were prepared with approximately $1x10^6$ conidia mL^{-1} concentrations and inoculated onto green shallot leaves (Hyun et al., 2001; Sompong et al., 2012). In the negative control treatment, sterile dH_2O was inoculated to compare with others. After that, the green shallot leaves were kept in a box with humidity by adding sterile dH_2O and observed the symptom development for 7 days. The pathogens were re-isolated from the green shallot plants and re-checked

its morphological characteristics by Koch's postulates. Disease reaction on the leaves was evaluated on the 0-5 scale scoring at 7 days after inoculation (Table 3.1). The experiments were performed in a completely randomized design (CRD) with four replications (Schwartz, 2013; Alberto RT, 2014).

Table 3.1 The level of disease incidence of anthracnose disease on green shallot (Bhangale and Joi, 1985).

Scale	Reaction	% Leaf area covered
0	Immune	Non-disease
1	Resistant	Up to 10%
2	Moderately resistant	11-20%
3	Moderately susceptible	21-40%
4	Susceptible	41-60%
5	Highly susceptible	>60%

Disease reduction (%) was calculated by using the formula given by Wongchalee (2015).

$$\text{Disease reduction rate} = ((\text{Negative control} - \text{Treatment})/\text{Negative control}) \times 100$$

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3.2 Efficacy of biosurfactants from *B. subtilis* for inhibition of mycelial growth of *C. gloeosporioides*

3.2.1 Screening of *B. subtilis* as a biological control agent against *C. gloeosporioides*

The experiment was carried out in CRD of *B. subtilis* colony of the 14 isolates (CaSUT007, CaSUT007-1, CaSUT008, CaSUT008-2, CaSUT111, D604, D604-1, D501, D501-1, SUNB1, SUNB2, 37-4, 37-5 and 37-6) were from the stock culture of PPB Laboratory, Suranaree University of Technology. The cock borer with a 5 mm diameter was used to cut *C. gloeosporioides* pure culture of the pathogen was placed at the distance of about 2 cm of a petri dish containing PDA. The *B. subtilis* 14 isolates were inoculated at the opposing corners. In another set, sterile dH_2O was used as a negative control. All Petri-dishes were incubated at room temperature. The inhibition zone of two cultures was measured at 7 days after incubation, and the percentage inhibition in radial growth (PIRG) was estimated by calculating the following formula

$$\text{PIRG}(\%) = ((R_a - R_b)/R_a) \times 100$$

R_a = The radial growth of *C. gloeosporioides* in the control plate

R_b = The radial growth of *C. gloeosporioides* treated with biosurfactants

The most effective 5 strain for inhibition of mycelium fungi would be selected for the next experiments (Sariah, 1994; Sivakumar and Wijesundera, 2004).

3.2.2 Extraction of biosurfactant produced by *B. subtilis*

This experiment was performed in the factorial in CRD with *the B. subtilis* colony of the 5 isolates (CaSUT007, CaSUT007-1, CaSUT008-2, D604, and SUNB2).

The *B. subtilis* was cultured in the nutrient broth (NB) after that incubated at 37°C for 48 h in a shaker 150 rpm. After 2 days of incubation, the surfactant was separated by using a centrifuge 12,000 rpm for 20 minutes, then added 2% potassium sorbate in the supernatant store into the refrigerator (Rajan and Krishnan, 2014).

3.2.3 Screening of potential biosurfactants from *B. subtilis* to control *C. gloeosporioides in vitro*

This experiment was performed in the factorial in CRD with 7 treatments using 4 replications. Plant pathogenic fungus, *C. gloeosporioides* isolate PSKR-SUT10, was grown on PDA media. The cock borer with a 5 mm diameter was used to cut the fungal mycelium colony and placed on the PDA at the center of the Petri dish. The biosurfactants produced by 5 strains of *B. subtilis* were inoculated at the 4 wells with a concentration of 50, 100, 150, and 200 µl, respectively. In another set, sterile dH_2O and 2% potassium sorbate were used as negative control and positive control. All Petri-dishes were incubated at room temperature. The inhibition zone of two cultures was measured at 7 days after incubation, and the percentage inhibition in radial growth (PIRG) was estimated by calculating the following formula (Sariah, 1994, Gamagae et al., 2004; Narendra et al. 2016).

$$PIRG\ (\%) = ((R_a - R_b)/R_a) \times 100$$

R_a = The radial growth of *C. gloeosporioides* in the control plate

R_b = The radial growth of *C. gloeosporioides* treated with biosurfactants

The most effective strain for inhibition of mycelium fungi would be selected for the next experiments.

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3.2.4 Evaluation of potential concentration of biosurfactants from *B. subtilis* strain D604 to control *C. gloeosporioides in vitro*

This experiment was performed in the CRD with 7 treatments using 4 replications. Plant pathogenic fungus, *C. gloeosporioides* isolate PSKR-SUT10, was grown on PDA medium. The cock borer with a 5 mm diameter was used to cut the fungal mycelium colony, which was placed on the PDA medium mixed with the biosurfactants produced by *B. subtilis* strain D604 concentrations 10, 20, 25, 30, 40, and 50% at th e center of the Petri dish. In another set, sterile dH_2O was used as a negative control, and 2% potassium sorbate and Prochloraz was used as a positive control. All Petri-dishes were incubated at room temperature. The inhibition zone was measured at 3, 5, and 7 days after incubate, and the percentage inhibition in radial growth (PIRG) was estimated by calculating follow a formula (Sariah, 1994; Gamagae et al., 2004; Narendra et al. 2016).

$$\text{PIRG (\%)} = ((R_a - R_b)/R_a) \times 100$$

R_a = The radial growth of *C. gloeosporioides* in the control plate

R_b = The radial growth of *C. gloeosporioides* interacting with biosurfactants

The most effective concentration to inhibition of mycelium fungi would be selected from for the next experiments.

3.2.5 Evaluation on the efficacy of biosurfactants from *B. subtilis* for induced resistance against anthracnose disease in green shallot under the greenhouse conditions

This experiment was performed in CRD with 5 treatments using 4 replications. The green shallot was planted in the pot, and foliar spray with 25%

biosurfactant produced from *B. subtilis* strain D604. Distilled water was served as a negative control and Prochloraz fungicide at the concentration of 20 mL/20 L of water was served as a positive control (Table 3.2). Each treatment was applied every 7 days sprayed 4 times, and green shallot plants were inoculated by *C. gloeosporioides* isolate PSKR-SUT10 spore suspension approximately $1x10^6$ conidia mL^{-1} in the suspension. The green shallot plants were incubated with humidity in the greenhouse and observed for symptoms of the disease 7 days after the inoculation of *C. gloeosporioides* isolate PSKR-SUT10. Disease severity was estimated based on the percentage disease intensity and scored using different levels of a 0-5 scoring scale (Table 3.1).

Table 3.2 List of treatments on assessing the biosurfactants from *B. subtilis*.

Treatment	**Foliar spray 7,14, 21 and 28 DAT[1/]**	**Inoculation 35 DAT[1/]**
1	Biosurfactant from *B. subtilis* strain D604	*C. gloeosporioides* inoculation
2	*B. subtilis* strain D604 10^8 CFU mL^{-1}	
3	*B. subtilis* commercial 200 g/ 20 L of water	
4	Prochloraz (positive control) 20 mL 20 L^{-1} of water	
5	Water (negative control)	

[1/]DAT: Days after transplanting

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3.3 Plant growth promotion induced by biosurfactant from *B. subtilis*

3.3.1 Plant growth condition

Plant growth condition was evaluated at 35 days after planting. Parameters included plant height, fresh weight, and dry weight.

3.3.2 Chlorophyll content

The green shallot leaf at a size 4 mm radius, keep in a 1.5 mL tube, add 1 mL of dimethylformamide (DMF), using 5 replicates. The green shallot leaf must be submerged in the DMF solution and incubated at 4°C overnight. The sample solution 150 µl was mixed with 300 µl of DMF in a new microcentrifuge tube, and read the absorbance at 647 nm for chlorophyll a (C_a), and 664.5 nm for chlorophyll b (C_b) by using a spectrophotometer (Harris and Baulcombe, 2015).

C_a content (µg mL^{-1}) = (A664.5 × 12) -(A647 × 2.79)

C_b content (µg mL^{-1}) = (A647 × 20.78) -(A664.5 × 4.88)

Total chlorophyll content (µg mL^{-1}) = $C_a + C_b$

Sample area (mm^2) = $3 \times \pi r^2$

Total chlorophyll content (µg/mm^2) = ($C_a + C_b$)/Sample area

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3.3.3 Indole-3-acetic acid (IAA) analysis in green shallot

The green shallot young leaves with approximately 0.1 g were ground with 1 mL homogenization buffer (0.1 M KCl, 3% (w/v) PVPP, 1 mM PMSF, 1% Triton X-100, 0.1 M Tris-HCl buffer, 1 µg mL^{-1} 1% (v/v) leupeptin, pH 7). The homogenate extract from green shallot 0.5 mL was mixed with 2 mL Salkowski's reagent incubated at room temperature for 20 minutes, and the absorbance at 535 nm wavelength was read by a spectrophotometer. The results were compared to referent

standard 0-50 μg mL^{-1} to obtain the veritable amount of IAA in the sample (Gordon and Weber, 1951; Sarker and Rashid, 2013).

3.4 Characterization of green shallot defense mechanisms after treated by biosurfactants from *B. subtilis* induced resistance against anthracnose disease

3.4.1 Salicylic acid analysis

The modification method followed by Raskin et al. (1990) was used to observe salicylic contents. The green shallot tissue samples 0.5 g were ground with liquid nitrogen and added 90% methanol and centrifuged at 12,000 rpm. They were incubated with 4°C for 5 minutes. The supernatant of 100 µl was mixed with 100 µl of 0.02 M ammonium iron (III) sulfate and was incubated at room temperature for 5 minutes, and then read the absorbance at 530 nm wavelength by using a spectrophotometer. The results were compared to the referent standard to obtain the veritable amount of SA in the sample. The level of SA in the green shallot sample was displayed in μg g^{-1} fresh weight (Prakongkha et al., 2013; Jimkuntod, 2018).

3.4.2 Phenolic compounds analysis

The green shallot leaves 0.5 g ground with liquid nitrogen and adds 1 mL of 90% methanol, after that centrifuged 12,000 rpm, 5 minutes at 4°C. The supernatant of 20 µl was mixed with 2 N Folin-Ciocalteu reagent 100 µl and 7%Sodium bicarbonate 80 µl .The sample was incubated at 30°C for 30 minutes .A spectropho- tometer has used the measure at 765 nm for analysis .Catechol was used as the standard (Kaisoon et al., 2011; He et al., 2015).

3.5 Assay of defense enzymes

3.5.1 Chitinase activity assay

The green shallot leaf 1 g was ground with liquid nitrogen and added homogenized 5 mL (polyvinyl pyrrolidone, 0.1 M sodium phosphate buffer at pH 6.5). The homogenate extraction was centrifuged at conditions including 10,000 rpm and 4^{0}C for 10 minutes. The homogenate extraction was assayed of Chitinase activity analysis. The reaction mixture included a 0.1 mL colloidal chitin, 0.4 mL of enzyme solution, and 10 µL of 0.1 M sodium acetate buffer (pH 4.0). After that, the mixture was incubated at 37^{0}C for 2 hours, then absorbed at 585 nm. One unit of chitinase activity was expressed as one mole of GlcNAc per minute under the assay (Boller and Mauch, 1988; Saravanakumar et al., 2009; Le and Yang, 2018).

3.3.2 Phenylalanine ammonia-lyase (PAL) activity assay

The green shallot leaf 1 g was ground with liquid nitrogen after that added homogenized 5 mL (0.1 M sodium borate buffer, pH 8.8 containing PVP), and then centrifuged at 10,000 rpm, for 15 minutes at 4^{0}C. The reaction mixture 0.2 mL homogenate extract, 0.1 mL of 12 mM L-phenyl alanine, and 3 mL of 0.1 M sodium borate with buffer pH 8.8, and then incubated at 40^{0}C for 30 minutes and stopped reaction by adding 0.2 mL of 3 N HCL. The homogenates were assay of PAL activity with a spectrophotometer at 290 nm by examining the rate of exchanging of L-phenylalanine to trans-cinnamic acid. The result was compared to the referent standard to obtain the veritable amount of PAL in the sample. The activity of PAL was expressed as µmol mg^{-1}protein (Dickerson et al., 1984; Nair and Umamaheswaran, 2016).

3.6 Data analysis

The experiments were analyzed using an examination of variance (ANOVA) to evaluate the differences, and variation among treatments was determined according to Duncan's Multiple Range Test (DMRT) at P = 0.05 by SPSS version 20.

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CHAPTER IV

RESULTS

4.1 Preparation of *C. gloeosporioides* causing anthracnose disease

4.1.1 Isolation of pathogen from anthracnose lesions of green shallot

The fungus was isolated and characterized from the green shallot anthracnose disease sample that was collected from the fields at Muang districts, Nakhon Ratchasima province, Thailand. The fungal mycelium appeared in the PDA medium within three days after incubation. Six different *C. gloeosporioides* strains were isolated from green shallot samples. The color of *C. gloeosporioides* varied from white to grey. The growth pattern was either circular, with the mycelia showing a uniform growth pattern or radial in a ring-like pattern. Subsequently, one representative most virulence isolate was selected for further study (Figure 4.1).

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Table 4.1 List of isolates of *C. gloeosporioides* obtained from green shallot anthracnose disease.

No.	Fungal isolate	Infected tissue
1	PCNK01	leaf
2	PCNK02	leaf
3	PCNK03	leaf
4	PSKR-SUT02	leaf
5	PSKR-SUT10	leaf
6	PSKR-SUT11	leaf

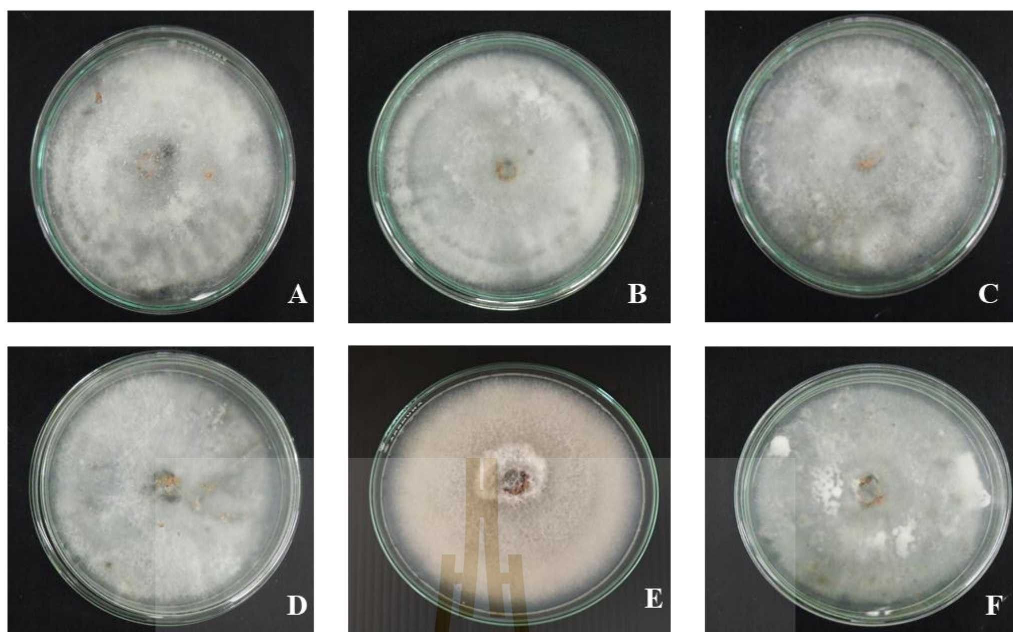

Figure 4.1 Colonies of *C. gloeosporioides* isolated from diseased shallot plants, cultured on PDA medium for 14 days. (A) isolate PCNK01, (B) isolate PCNK02, (C) isolate PCNK03, (D) isolate PSKR-SUT02, (E) isolate PSKR-SUT10, (F) isolate PSKR-SUT11.

4.1.2 Pathogenicity test

Five days after inoculation, the inoculated green shallot leaves began to show lesions (Figure 4.2). The 6 isolates tested on the green shallot, all isolate was pathogenic, but each isolate showed varying degrees of virulence (Table 4.2). The result found that *C. gloeosporioides* isolate PSKR-SUT10 showed high severity scores of 4.66 (most virulent). Additionally, *C. gloeosporioides* isolate PSKR-SUT11 showed severity scores of 4 (highly virulent). Moreover, *C. gloeosporioides* isolate PCNK01 and PCNK02 showed severity scores of 3 (moderately virulent); only two isolates were assessed slightly virulent with a score of 2 is PCNK03 and PSKR-SUT02 (least virulent) respectively, significantly when compared with the control.

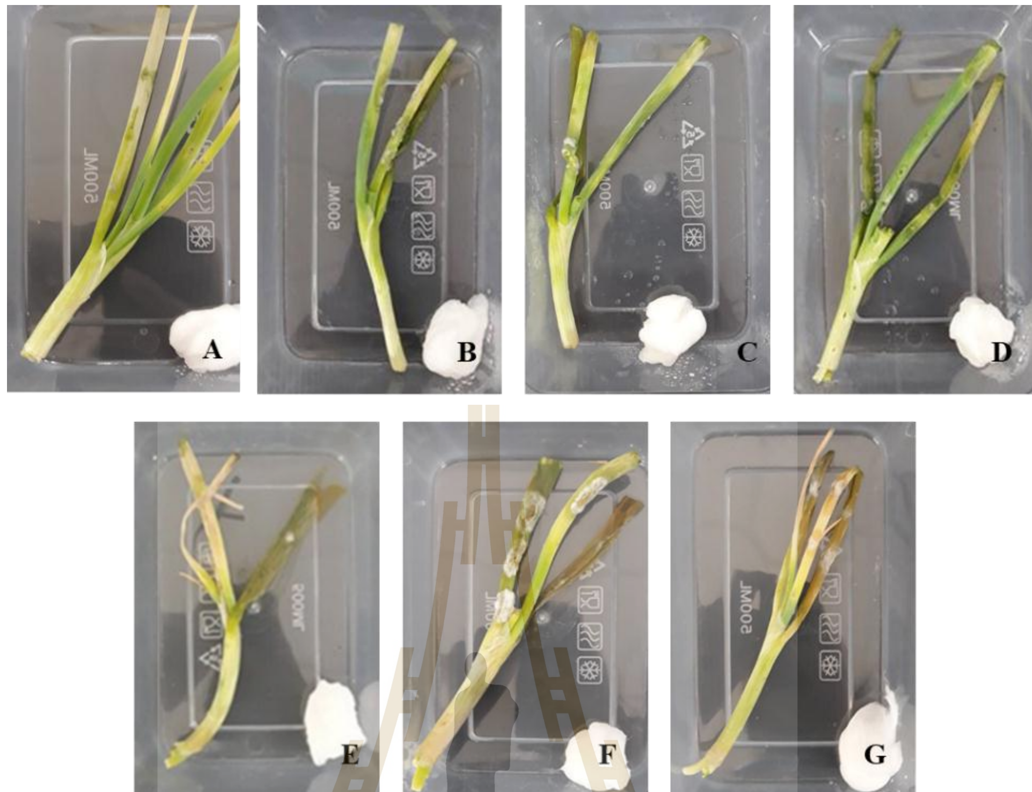

Figure 4.2 Symptoms developed on detached leaves of green shallot at 5 days after inoculation with spore suspension at 1×10^6 conidia mL^{-1} (A) uninoculated control, (B) isolate PCNK01, (C) isolate PCNK02, (D) isolate PCNK03, (E) isolate PSKR-SUT02, (F) isolate PSKR-SUT10, (G) isolate PSKR-SUT11.

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Table 4.2 Severity scores of representative *C. gloeosporioides* 6 isolates obtained from green shallots samples with anthracnose disease.

Isolates	Severity scores [1/]	Virulence level
PCNK01	3.00^{c}[2/]	Moderately virulent
PCNK02	3.00^{c}	Moderately virulent
PCNK03	2.00^{d}	Least virulent
PSKR-SUT02	2.33^{d}	Least virulent
PSKR-SUT10	4.66^{a}	Most virulent
PSKR-SUT11	4.00^{b}	Highly virulent
Water (control)	0.00^{e}	Non-pathogenic
F-Test	**	
CV (%)	11.35	

[1/] Severity scores: 0 = no symptoms, 1 = <1%, 2 = 1-10%, 3 = 11-25%, 4 = 26-50% and 5 = >50%., [2/] Mean in the column followed by the same letter are not significantly different according to the DUNCAN test (α= 0.05).

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4.2 Efficacy of *B. subtilis* as a biological control agent against *C. gloeosporioides*

The *B. subtilis* 14 strains including CaSUT007, CaSUT007-1, CaSUT008, CaSUT008-2, CaSUT111, D604, D604-1, D501, D501-1, SUNB1, SUNB2, 37-4, 37-5 and 38-4 were assessed for their abilities to control pathogenic fungi on green shallot. Their antifungal activities were evaluated in vitro by dual culture with the fungal pathogen, *C. gloeosporioides.* The screening was conducted using *B. subtilis* 5 strains including

D604, CaSUT008-2, SUNB2, CaSUT007-1 and CaSUT007 with strong antagonistic activity to *C. gloeosporioides* was observed. The highest percentage of inhibition of *C. gloeosporioides* was 49.55, 46.00, 45.66, 44.50, and 43.78% respectively, Subsequently, the *B. subtilis* strains CaSUT008, D501, D604-1, D501-1, SUNB1, 38-4, 37-5, 37-4, and CaSUT111 had the percentage of inhibition at 41.72, 40.61, 39.33, 38.61, 36.33, 34.83, 33.11, 31.77, and 27.77% respectively, significantly when compared with the control (Figure 4.3 and 4.4).

4.3 Efficacy of biosurfactants from *B. subtilis* for inhibition of mycelial growth of *C. gloeosporioides*

The biosurfactants from 5 *B. subtilis* strains including CaSUT 007, CaSUT 007-1, CaSUT 008-2, SUNB2, and D604 were assessed for their abilities to control pathogenic fungi on green shallot. Their antifungal activities were evaluated in vitro by dual culture with the fungal pathogen, *C. gloeosporioides.* The screening was conducted using biosurfactants from *B. subtilis* 5 strains and one strain namely strains D604 with strong antagonistic activity to *C. gloeosporioides* was observed. The highest percentage of inhibition of *C. gloeosporioides* was around 73.48% at the volume of 200 µl. Moreover, the biosurfactant *B. subtilis* strain D604 at the volume 150 µl inhibited mycelial growth at 70.81%. Subsequently, the biosurfactant *B. subtilis* strains CaSUT007, CaSUT007-1, CaSUT008-2, SUNB2, and 2% potassium sorbate at volume 200 µl had the percentage of inhibition around 62.74, 63.93, 62.15, 61.04, and 44.44 respectively, significantly lower than the control. This result indicated that biosurfactants *B. subtilis* strain D604 at volume 150 to 200 µl had at approximately 70.81-73.48% of effective inhibition on mycelial growth (Figure 4.5 and 4.6).

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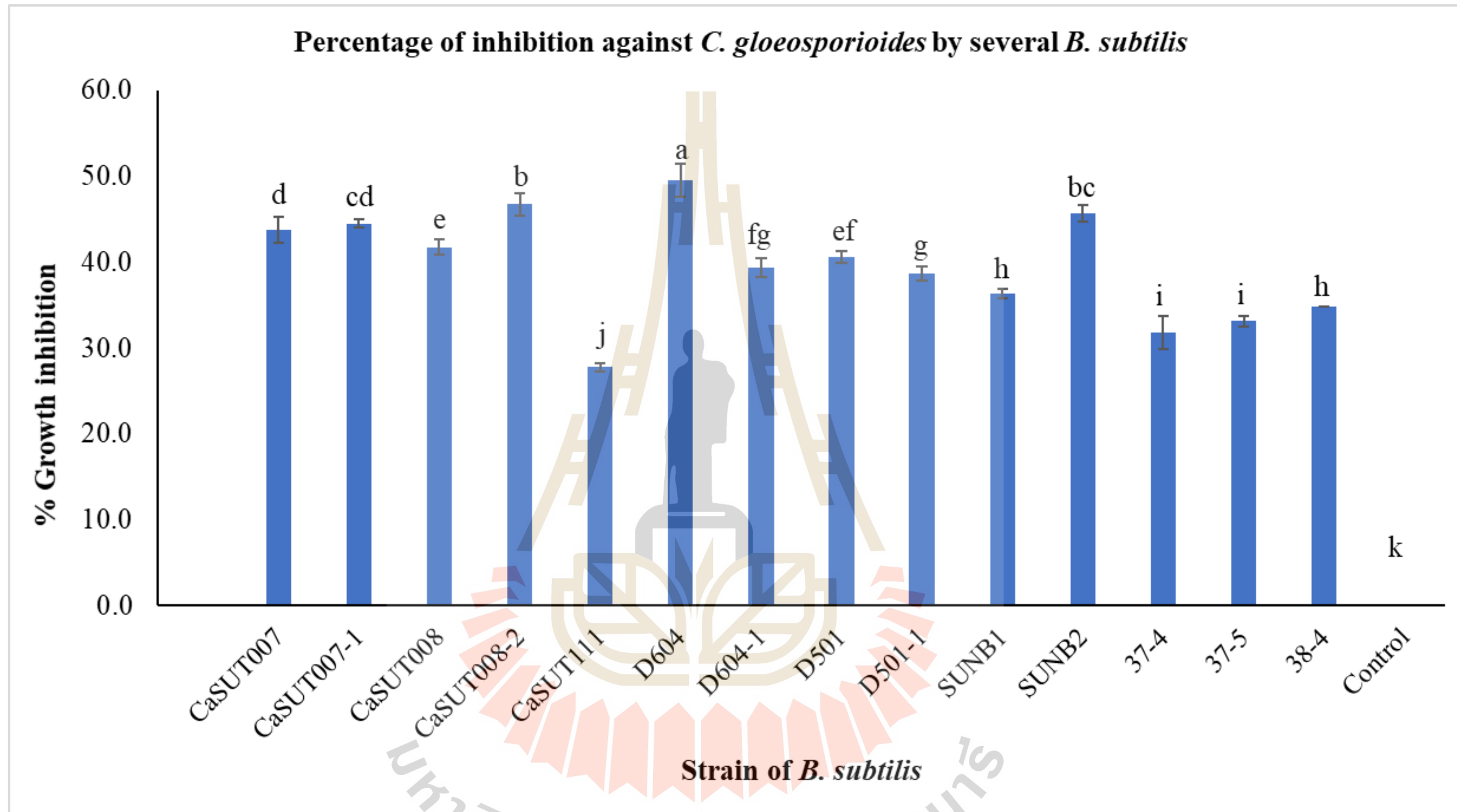

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Figure 4.3 Percentage of inhibition of *B. subtilis* 14 strains against *C. gloeosporioides* at 7 days by PDA medium.

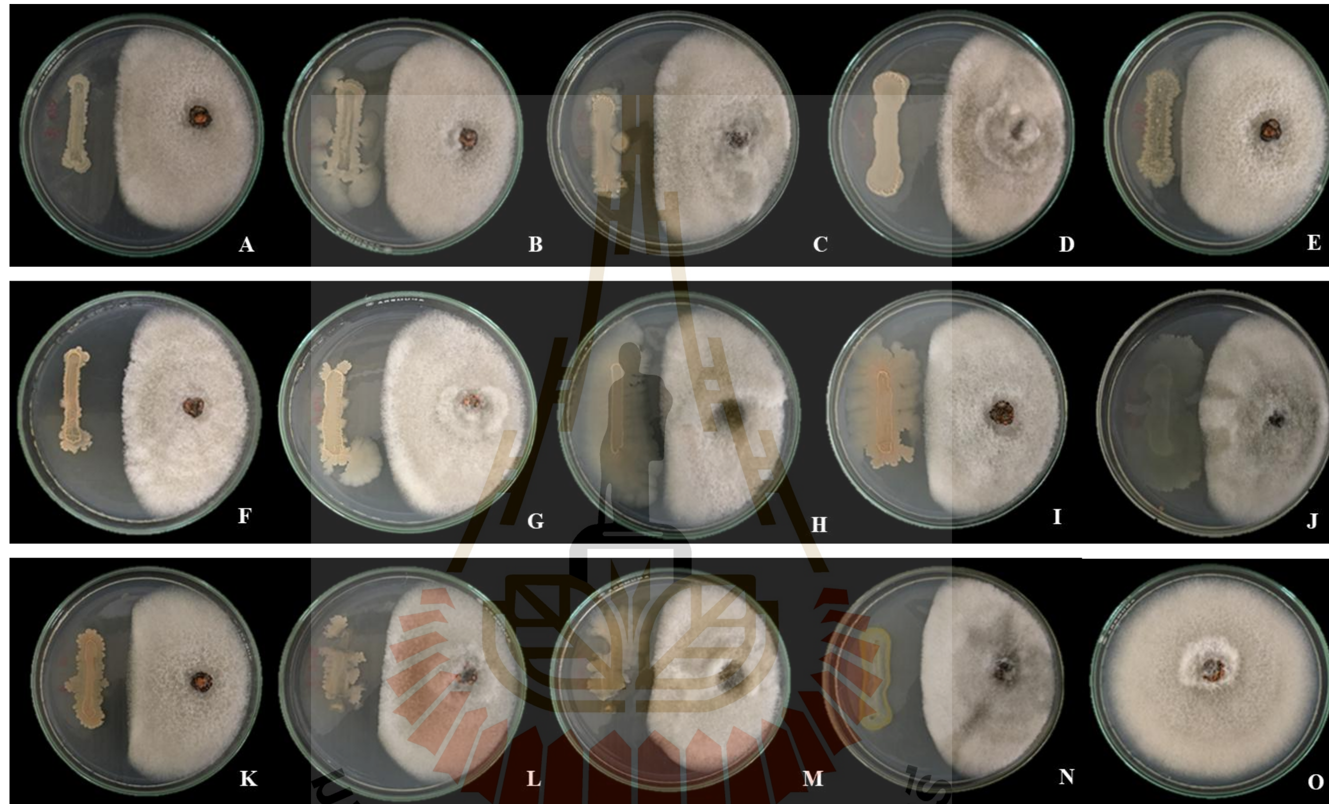

Figure 4.4 A dual culture test of the antagonistic *B. subtilis* activity. (A) CaSUT007 (B) CaSUT007-1 (C) CaSUT008 (D) CaSUT008-2 (E) CaSUT111 (F) D604 (G) D604-1 (H) D501 (I) D501-1 (J) SUNB1 (K) SUNB2 (L) 37-4 (M) 37-5 (N) 38-4 and (G) control.

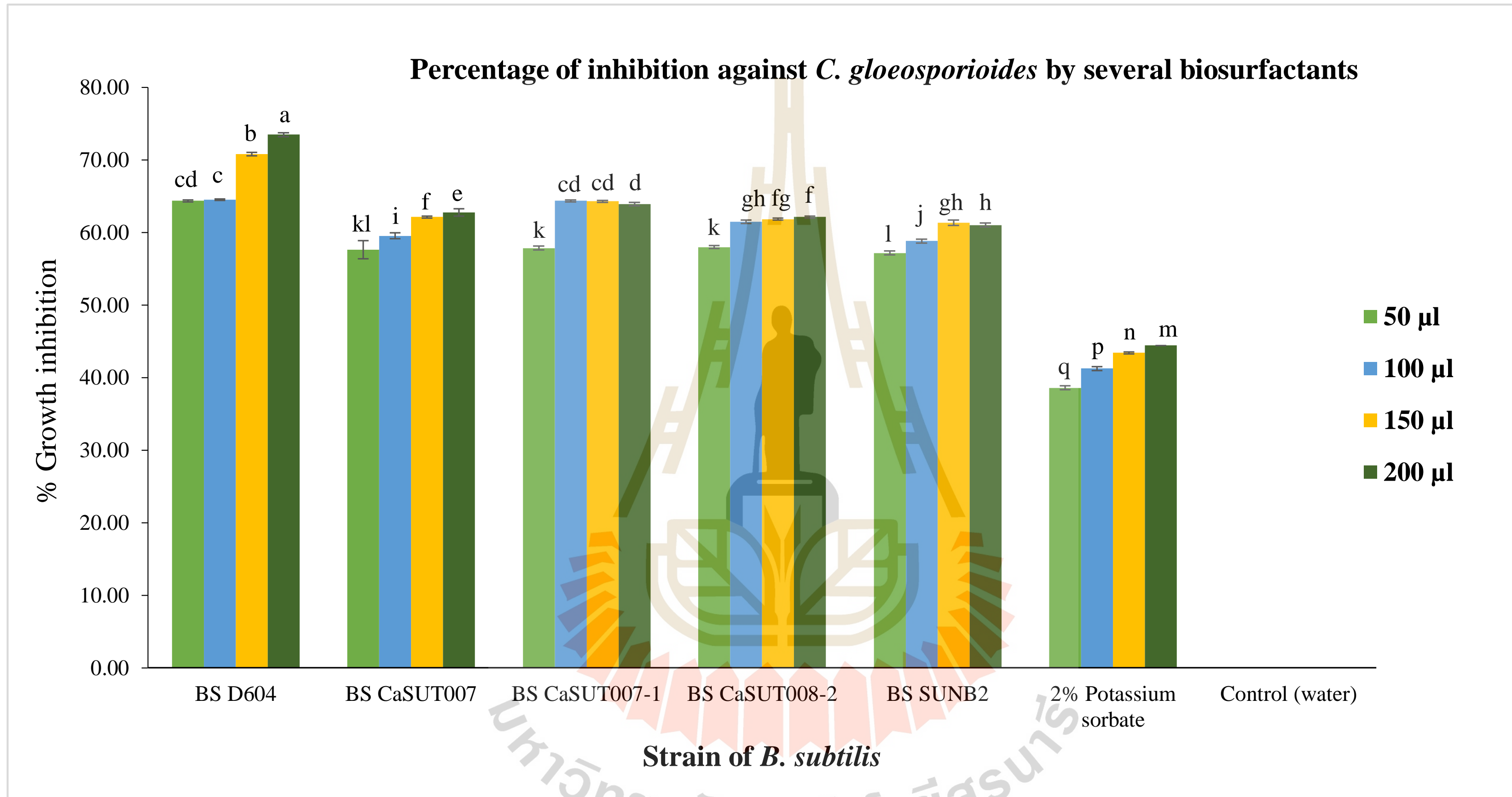

Figure 4.5 Percentage of inhibition of biosurfactants produce 5 strains (D604, CaSUT 007, CaSUT 007-1, CaSUT 008-2, and SUNB2) of *B. subtilis* against *C. gloeosporioides* at 7 days by PDA medium at the concentration 50, 100, 150, and 200 µl.

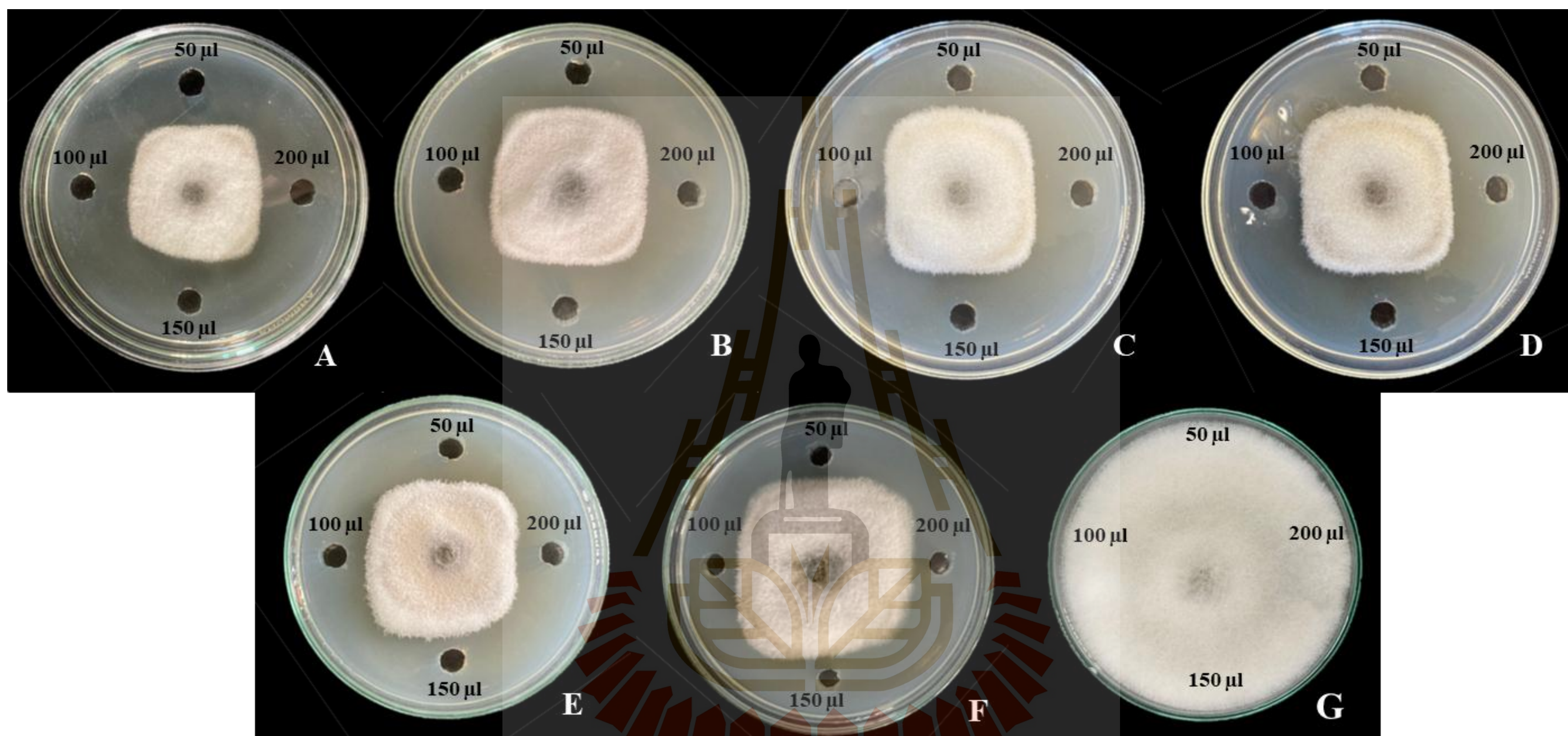

Figure 4.6 A dual culture test by agar well method of the biosurfactant antagonistic activity. (A) biosurfactant strain D604 (B) biosurfactant strain CaSUT007 (C) biosurfactant strain CaSUT007-1 (D) biosurfactant strain CaSUT008-2 (E) biosurfactant strain SUNB2 (F) 2% potassium sorbate and (G) control.

4.4 Efficacy of concentrations of biosurfactants from *B. subtilis* strain D604 in controlling *C. gloeosporioides in vitro*

The biosurfactants from *B. subtilis* strain D604 was assessed for their abilities to control pathogenic fungi on green shallot. Their antifungal activities were assayed *in vitro* by culturing the fungal pathogen in the PDA mixed with the biosurfactants at the concentrations of 10, 20, 25, 30, 40, and 50%. In another set, sterile dH_2O is negative control and Prochloraz, and 2% potassium sorbate is a positive control. The colony diameter was measured at 3, 5, and 7 days, and the percentage inhibition in radial growth (PIRG) was estimated (Sariah, 1994, Gamagae et al., 2004; Narendra et al. 2016). The result revealed that all treatments were significantly different when compared with the control. At 3 to 7 days, PDA medium mixed with biosurfactant at the concentration of 25, 30, 40 50%, and Prochloraz fungicide had the highest percentage inhibition at 100%. While the biosurfactants at the concentrations of 10, 20%, and 2% potassium sorbate had percentage inhibition at 47.03, 77.08, and 37.64%, respectively while comparing with the control (Figure 4.7-4.10).

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4.5 Efficacy of biosurfactants from *B. subtilis* for induced resistance against anthracnose disease under the greenhouse conditions

The green shallot at 28 days old was treated with biosurfactants from *B. subtilis* strain D604 every 7 days. After that, inoculation was conducted with *C. gloeosporioides* isolate PSKR-SUT10 at concentrations of 10×10^6 conidia mL^{-1}. Disease severity was estimated at 7 days after inoculation. The result shows that biosurfactant from *B. subtilis* strain D604 could reduce anthracnose disease severity at

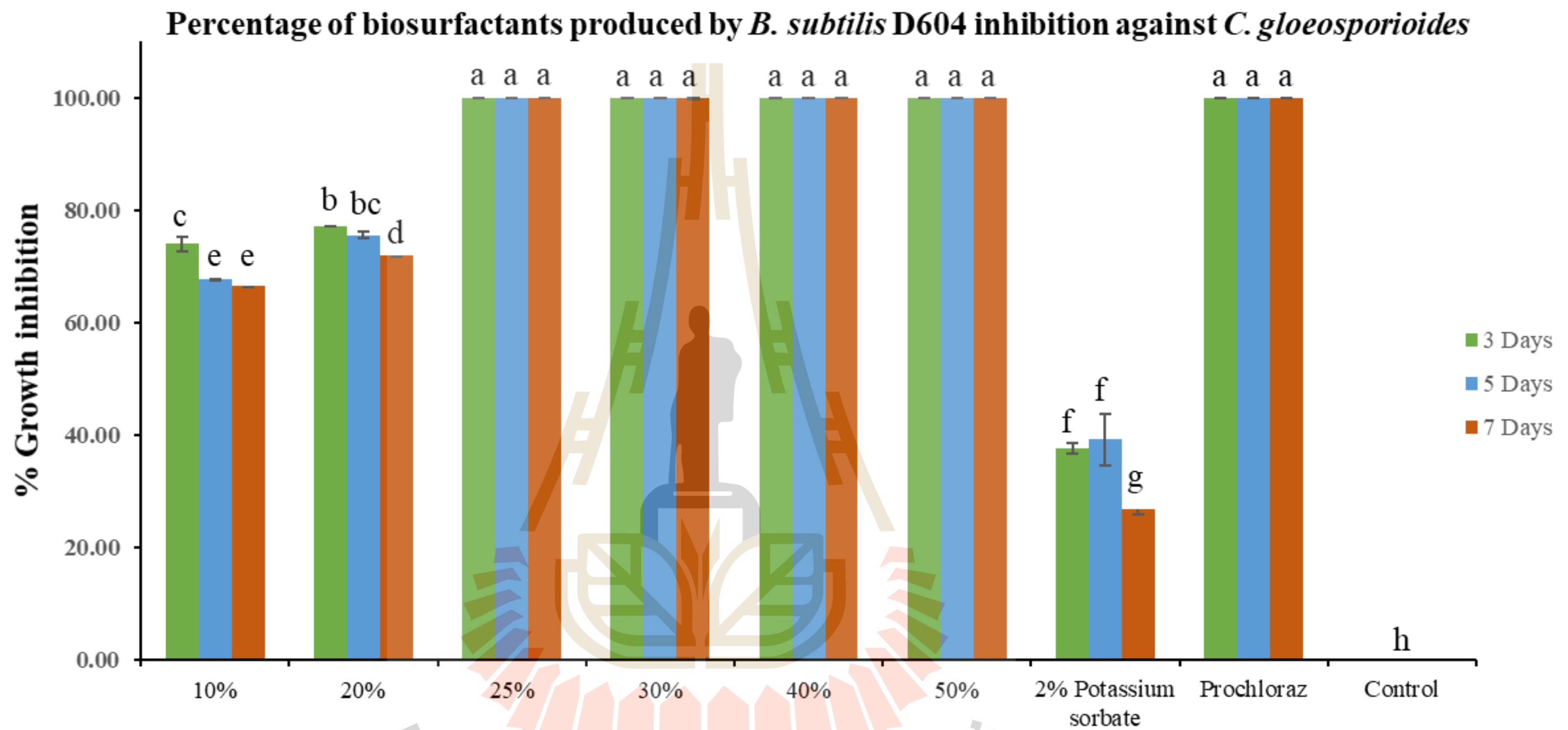

Figure 4.7 Efficacy of biosurfactant produced by *B. subtilis* strain D604 against *C. gloeosporioides* at 3, 5, and 7 days on PDA medium at the concentrations of 10, 20, 25, 30, 40, and 50%.

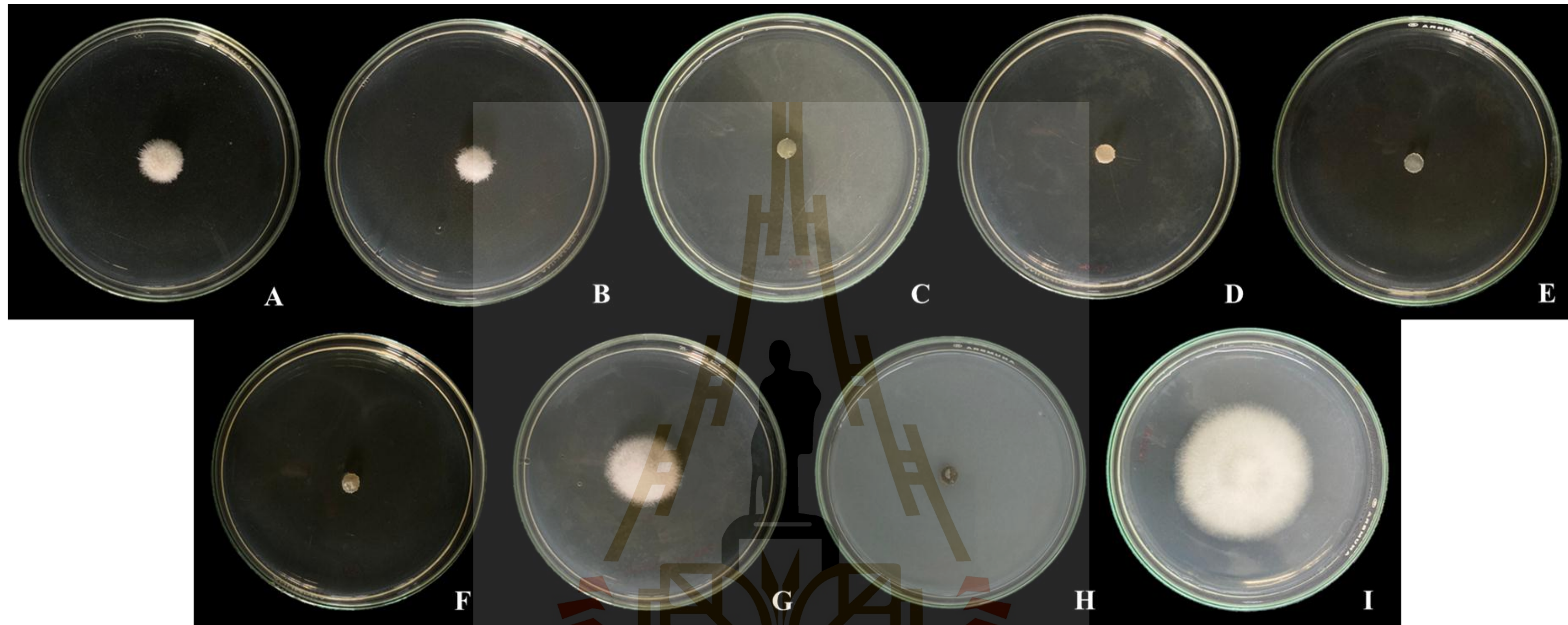

Figure 4.8 Efficacy of biosurfactant produced by *B. subtilis* strain D604 against *C. gloeosporioides* at 3 days by PDA medium at the concentrations of 10% (A), 20% (B), 25% (C), 30% (D), 40% (E), 50% (F), 2% Potassium sorbate (G), Prochloraz fungicide (H), and control (I).

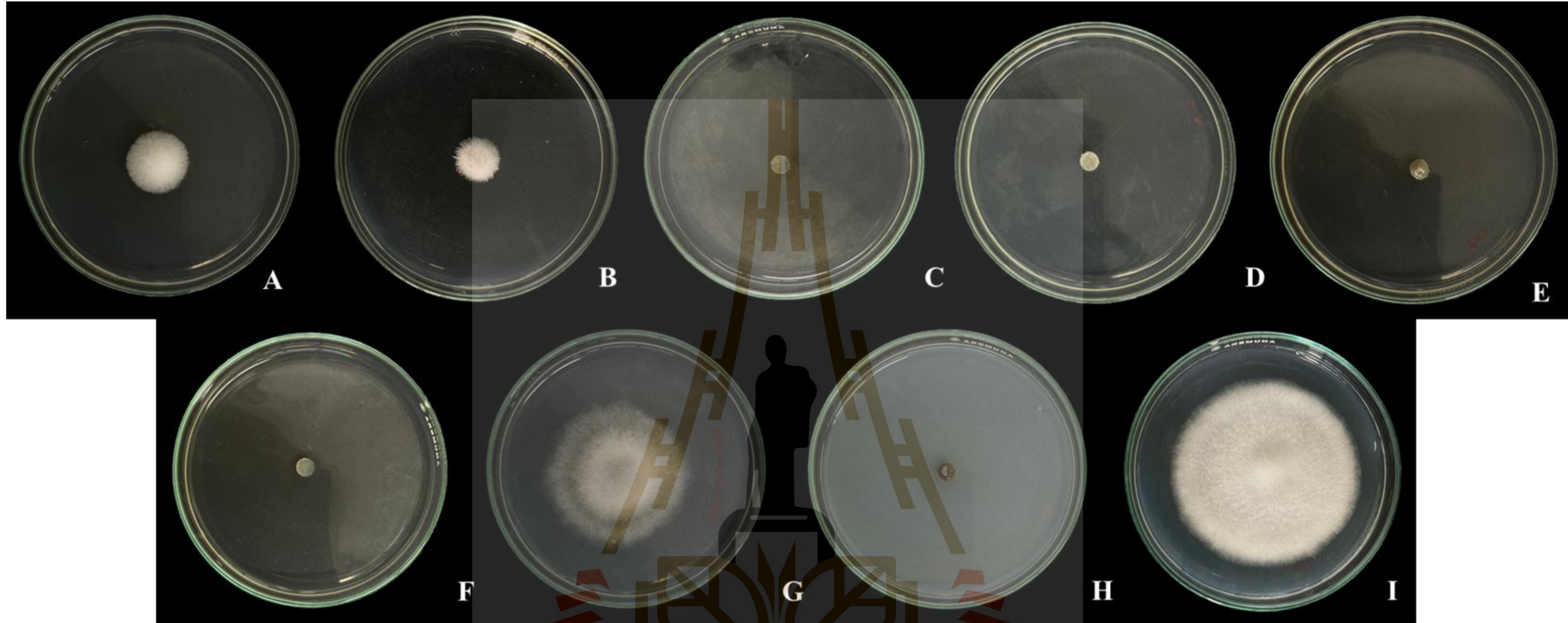

Figure 4.9 Efficacy of biosurfactant produced by *B. subtilis* strain D604 against *C. gloeosporioides* at 5 days by PDA medium at the concentrations of 10% (A), 20% (B), 25% (C), 30% (D), 40% (E), 50% (F), 2% Potassium sorbate (G), Prochloraz (H), and control (I).

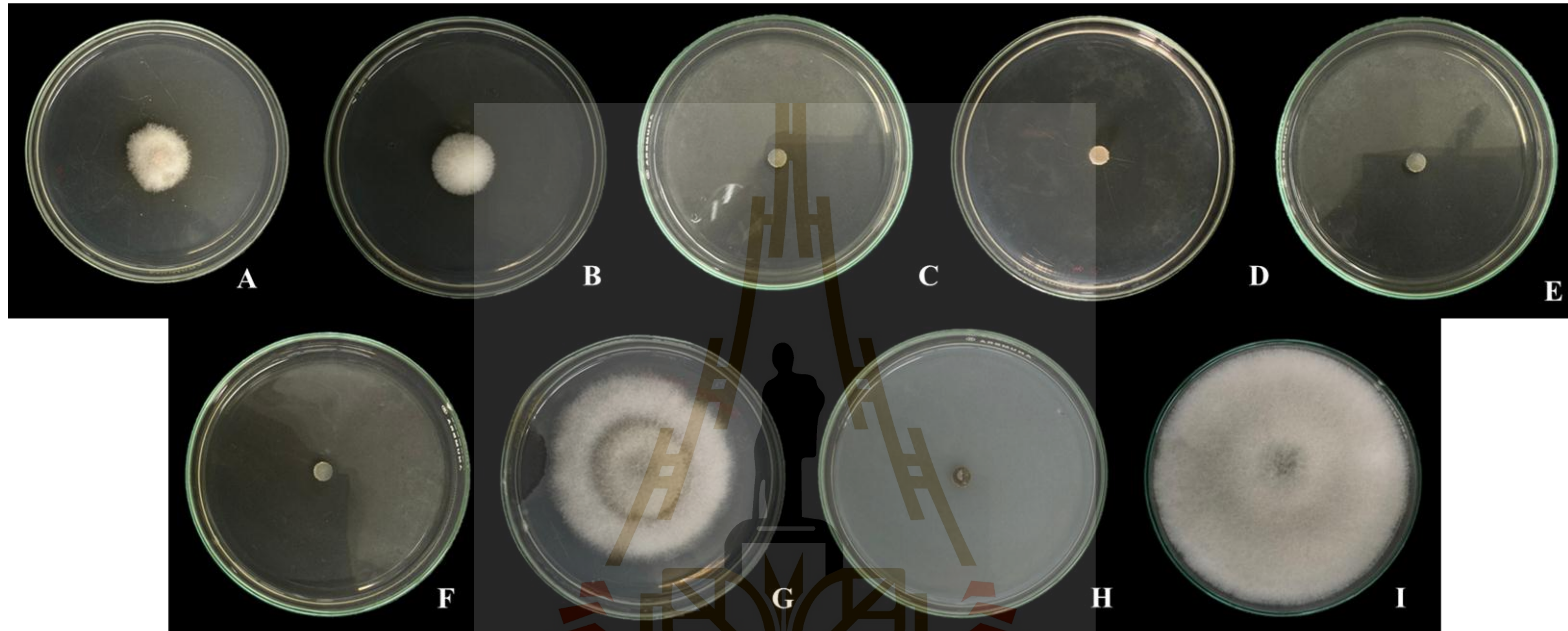

Figure 4.10 Efficacy of biosurfactant produced by *B. subtilis* strain D604 against *C. gloeosporioides* at 7 days by PDA medium at the concentrations of 10% (A), 20% (B), 25% (C), 30% (D), 40% (E), 50% (F), 2% Potassium sorbate (G), Prochloraz (H), and control (I).

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58.80% on the leaves of green shallot, indicating that the induction of systemic resistance may occur. Besides, the results showed that treatment with the chemical fungicide (Prochloraz) reduced the severity of anthracnose disease in green shallot by 64.70%. Both Prochloraz and biosurfactant from *B. subtilis* strain D604 resulted in disease reduction when compared with the control. Other than, green shallot affected by leaves treatment of the cell suspension of *B. subtilis* strain D604 and *B. subtilis* commercial had disease severity of 52.90 and 41.20% on the leaves of green shallot, respectively. Each treatment was significantly different compared with the control (Table 4.3).

Table 4.3 Effect of biosurfactants produced by *B. subtilis* strain D604 on disease reduction of anthracnose disease in green shallot in greenhouse condition.

Treatment	Disease reduction (%)	Reaction
Biosurfactant from *B. subtilis* strain D604	58.80	Resistant
B. subtilis strain D604	52.90	Moderately resistant
B. subtilis commercial	41.20	Moderately susceptible
Prochloraz (positive control)	64.70	Resistant
dH_2O (negative control)	0.00	Susceptible

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4.6 Efficacy of biosurfactant produced by *B. subtilis* strain D604 for growth promoting

4.6.1 Plant growth parameter

Morphological changes of green shallot were observed at 35 days after inoculation. Each treatment affected plant growth and development and significantly increased the plant height, the weight of the shoot, and the dry weight of shoot green shallot plants. Biosurfactant from *B. subtilis* strain D604 potentially increased plant

height with 30.34 cm, the weight of shoot with 119.96 g, and the dry weight of shoot with 32.69 g, respectively. Furthermore, the inoculated green shallot plants with *B. subtilis* strain D604, *B. subtilis* commercial and Prochloraz had increased plant height at 31.91, 28.67, and 25.80 cm, the weight of shoot at 94.97, 89.77, and 76.53 g, and the dry weight of shoot at 27.01, 18.04 and 16.04 g, respectively, compare with control plants which had plant height with 26.10 cm, the weight of shoot with 84.53 g, and the dry weight of shoot with 18.90 g (Table 4.4). This finding suggests that using biosurfactant and different *B. subtilis*, especially biosurfactant could be to promote green shallot growth under greenhouse condition.

Table 4.4 Effect of the biosurfactants from *B. subtilis* on plant growth-promoting in green shallot under greenhouse condition.

Treatment	Plant growth of green shallot		
	Plant height (cm/plant)	**Fresh weight (g/plant)**	**Dry weight (g/plant)**
Biosurfactants from *B. subtilis* strain D604	30.34ab[1/]	119.96a	32.69a
B. subtilis strain D604	31.91a	94.97b	27.01b
B. subtilis commercial	28.67b	89.77bc	18.04cd
Prochloraz (positive control)	25.80c	76.53c	16.04d
dH_2O (negative control)	26.10c	84.53bc	18.90c
F-test	**	**	**
CV (%)	0.28	0.50	0.40

[1/]Mean in the column followed by the same letter is not significantly different according to the DUNCAN test (α = 0.05).

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4.6.2. Analysis of chlorophyll content, Indole-3-acetic acid (IAA) in green shallot

The chlorophyll content in green shallot leaf was significantly different among all treatment. The highest contents of chlorophyll a, b, and total chlorophyll after treated green shallot leaf with biosurfactant from *B. subtilis* strain D604 were high at 19.78, 6.07, and 25.86 µg mL^{-1} respectively, while the leaf treated with *B. subtilis* strain D604 had the contents of chlorophyll a, b, and total chlorophyll at 17.22, 5.26, and 22.49 µg mL^{-1} respectively, The treatment with *B. subtilis* commercial gave the contents of chlorophyll a, b, and total chlorophyll at 13.41, 5.19, and 18.61 µg mL^{-1}. While the lowest values of 11.29, 3.46, and 14.75 µg mL^{-1} were obtained from Prochloraz compare with the control (Table 4.5).

Table 4.5 Chlorophyll and Indole-3-acetic acid content in green shallot.

Treatment	**Chlorophyll (µg mL^{-1})**			**IAA (µg g^{-1} fresh weight)**
	Chla	**Chlb**	**Total Chl**	**IAA**
Biosurfactants from *B. subtilis* strain D604	19.78a[1/]	6.07a	25.86a	14.76a
B. subtilis strain D604	17.22b	5.26ab	22.49b	14.41a
B. subtilis commercial	13.41c	5.19b	18.61c	8.78b
Prochloraz (positive control)	11.29d	3.46c	14.75d	6.98b
dH_2O (negative control)	10.62d	2.25d	12.87e	6.20b
F-test	**	*	**	**
CV (%)	3.64	10.37	0.478	22.18

[1/]Mean in the column followed by the same letter is not significantly different according to the DUNCAN test (α = 0.05).

The indole-3-acetic acid content in green shallot leaf after treated with biosurfactants from *B. subtilis* strain D604 and *B. subtilis* showed the highest amount of 14.76 and 14.41 µg g^{-1} fresh weight respectively, followed by *B. subtilis* commercial (8.78 µg g^{-1} fresh weight). However, the IAA value of the green shallot after treated with Prochloraz (6.39 µg g^{-1} fresh weight) and dH_2O (6.20 µg g^{-1} fresh weight) were not significantly different (Table 4.5).

4.7 Green shallot defense mechanisms after induction of biosurfactants from *B. subtilis* against anthracnose disease

4.7.1 The accumulation of salicylic acid content

Salicylic acid (SA) accumulation in green shallot leaves after treatment inoculation of *C. gloeosporioides* at 0, 24, and 48 hours (HAI) was analyzed in a spectrophotometer at 530 nm wavelength (Raskin et al., 1990). At 0 h after challenge inoculation. The *B. subtilis* commercial reaching the maximum contents of 7.42 µg g^{-1} /fresh weight. Secondly, the biosurfactants from *B. subtilis* strain D604, distilled water (dH_2O), and *B. subtilis* strain D604 were followed by 5.02, 4.05, and 3.67 µg g^{-1} fresh weight respectively, and treatment of Prochloraz was lower at 3.14 µg g^{-1} fresh weight. Moreover, the levels of the SA-associated significantly increased at 24 h after challenge inoculation among all treatment, The *B. subtilis* strain D604 helped plants accumulated the highest SA of 116.83 µg g^{-1} fresh weight. Secondly, *B. subtilis* commercial' SA of 90.67 µg g^{-1} fresh weight was significant treatment compared to the Prochloraz and distilled water (dH_2O) at 87.04 and 85.31 µg g^{-1} fresh weight, respectively. These results indicated that green shallot within 0-24 hours after inoculation as a hypersensitive response (HR) through the SA signaling pathway

while SA was the signal transduction molecule. However, at 48 h after challenge inoculation, each treatment showed a liable decrease of the levels of SA (Table 4.6).

Table 4.6 Accumulation of salicylic acid in leaves of green shallot with the foliar application and after inoculation with *C. gloeosporioides*.

Treatment[1/]	Salicylic acid ($\mu g\ g^{-1}$ fresh weight)		
	0 HAI[2/]	**24 HAI**	**48 HAI**
Biosurfactants from *B. subtilis* strain D604	3.67c[3/]	103.58b	81.01bc
B. subtilis strain D604	5.02b	116.83a	66.83d
B. subtilis commercial	7.42a	90.67c	86.54a
Prochloraz (positive control)	3.41c	87.04c	85.02ab
dH_2O (negative control)	4.05bc	85.31c	79.53c
F-test	**	**	**
CV (%)	14.01	4.44	3.13

[1/] Salicylic acid were evaluated at after 7 days foliar treatments, Prochloraz fungicide or distilled water (control) and at 0, 24 and 48 hours after challenging with *C. gloeosporioides*.; [2/]HAI: hours after inoculated; [3/]each value represents a mean of three replicate plants with two leaves per plant. Mean in the column followed by the same letter are not significantly different according to the DUNCAN test (α= 0.05).

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4.7.2 Total phenolic compound

Phenolic compound (PC) accumulation in each treatment after inoculation with *Colletotrichum* sp. at 0, 24, and 48 hours (HAI) was analyzed using a spectrophotometer at 765 nm wavelength. The results showed that the levels of the PC at 0 h after challenge inoculation among all treatment significantly different. The leaves treated with *B. subtilis* strain D604, biosurfactants from *B. subtilis* strain D604, and fungicides (Prochloraz) showed the maximum content of 255.29, 243.24, and

241.87 μg g^{-1} fresh weight, respectively. On the next positions, the contents observed from the treatment of the *B. subtilis* commercial were followed by 231.50 μg g^{-1} fresh weight significantly when compared with the control. Besides, the number of PC varied widely in green shallot. Among treatment of Prochloraz, *B. subtilis* commercial, *B. subtilis* strain D604, and dH_2O, low levels were found in green shallot (316.87, 296.87, 281.50 and 250.51 μg g^{-1} fresh weight, respectively). The green shallot leave treated by the biosurfactants from *B. subtilis* strain D604 contained relatively high amounts of PC at 352.71 μg g^{-1} fresh weight at 24 h. Overall, these results suggested that green shallot may have some effect on modulating the endogenous phenolic compounds for induce resistance after treatment (Table 4.7).

Table 4.7 Effect of phenolic compound (PC) in leaves of green shallot with the foliar application and after inoculation with *C. gloeosporioides*.

Treatment[1/]	Phenolic compound (μg g^{-1} fresh weight)		
	0 HAI[2/]	**24 HAI**	**48 HAI**
Biosurfactants from *B. subtilis* strain D604	243.24a[3/]	352.71a	269.68a
B. subtilis strain D604	255.28a	281.50cd	260.96a
B. subtilis commercial	231.50b	296.87bc	235.74b
Prochloraz (positive control)	241.87a	316.87b	266.42a
dH_2O (negative control)	232.33b	250.51d	246.04c
F-test	*	**	**
CV (%)	3.03	6.06	5.01

[1/] Phenolic compounds were evaluated at after 7 days foliar treatments, Prochloraz fungicide or distilled water (control) and at 0, 24 and 48 hours after challenging with *C. gloeosporioides*.; [2/]HAI: hours after inoculated; [3/]each value represents a mean of three replicate plants with two leaves per plant. Mean in the column followed by the same letter are not significantly different according to the DUNCAN test (α= 0.05).

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4.8 Assay of defense enzymes

4.8.1 Chitinase analysis

The results showed that chitinase activities in the green shallot leaf after treated with biosurfactants from *B. subtilis* strain D604 had the highest chitinase activity at 1.65 µmol mg^{-1}protein at 0 h. It was while the leave treated by *B. subtilis* strain D604, *B. subtilis* commercial, Prochloraz (positive control), and dH_2O (negative control) showed the contents of 1.53, 1.47, 1.47, and 1.44 µmol mg^{-1}protein, respectively, and significantly different at the statistical analysis. Subsequently, at 24 h after challenge inoculation with *C. gloeosporioides*, the green shallot leaves treated with biosurfactant from *B. subtilis* strain D604 showed the highest chitinase activity of 4.11 µmol mg^{-1}protein. Next, Prochloraz (positive control), *B. subtilis* strain D604, and *B. subtilis* commercial were 3.19, 2.52, and 2.46 µmol mg^{-1}protein respectively, significantly different at the statistical when compared with the dH_2O (negative control). After challenge inoculation at 48 h, each treatment had a liable decrease in the levels of the chitinase (Table 4.8). These results pointed out the different role of chitinase in green shallot had signaling pathways helping the plants effectively respond to the pathogen infection.

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4.8.2 Phenylalanine ammonia-lyase (PAL) analysis

Analysis of PAL activity showed a wide variation in the level of PAL activity in different green shallot leaves. At 0 h after challenge inoculation, the highest levels were 1.79 µmol mg^{-1}protein after treated with biosurfactant from *B. subtilis* strain D604. While Prochloraz, and *B. subtilis* commercial showed 14.40, 1.40, and 1.31 µmol trans-cinnamic acid min^{-1} mg^{-1}protein, respectively, differently significant in statistics when compared with the control. The same pattern of change in PAL

activity was observed at 24 h after challenge inoculation. The green shallot treated with biosurfactant from *B. subtilis* strain D604 appeared the highest PAL activity of 15.39 μmol trans-cinnamic acid min^{-1} mg^{-1}protein, followed by the *B. subtilis* strain D604, *B. subtilis* commercial, and Prochloraz showing 9.93, 7.36, and 6.35 μmol mg^{-1}protein respectively. Then after challenge inoculation at 48 h. The results showed that the PAL activity each treatment had a liable decrease in the levels of the PAL (Table 4.9).

Table 4.8 Effect of biosurfactant on the accumulation of chitinase in green shallot.

Treatment	Chitinase (μmol mg^{-1} protein)		
	0 HAI[1/]	**24 HAI**	**48 HAI**
Biosurfactant from *B. subtilis* strain D604	1.65a[2/]	4.11a	2.97a
B. subtilis strain D604	1.53b	2.52c	2.19c
B. subtilis commercial	1.47bc	2.46c	1.16e
Prochloraz (positive control)	1.47bc	3.19b	2.52b
dH_2O (negative control)	1.44c	1.88d	1.50d
F-test	**	**	**
CV (%)	2.95	3.52	2.38

[1/]HAI: hours after inoculated; [2/]each value represents a mean of three replicate plants with two leaves per plant. Mean in the column followed by the same letter are not significantly different according to the DUNCAN test (α= 0.05).

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Table 4.9 Effect of biosurfactant on the accumulation of Phenylalanine ammonia-lyase in green shallot.

Treatment	**Phenylalanine ammonia-lyase (µmol mg^{-1} protein)**		
	0 HAI[1/]	**24 HAI**	**48 HAI**
Biosurfactant from *B. subtilis* strain D604	1.79a[2/]	15.39a	4.80c
B. subtilis strain D604	1.40b	9.93b	3.97d
B. subtilis commercial	1.31b	7.36c	5.77b
Prochloraz (positive control)	1.40b	6.35d	8.43b
dH_2O (negative control)	1.07c	5.55e	4.34d
F-test	**	**	**
CV (%)	3.91	3.75	4.59

[1/]HAI: hours after inoculated; [2/]each value represents a mean of three replicate plants with two leaves per plant. Mean in the column followed by the same letter are not significantly different according to the DUNCAN test (α= 0.05).

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CHAPTER V

DISCUSSION AND CONCLUSION

This experiment studied to evaluate the efficiency of biosurfactants produced by *B. subtilis* on growth promotion and induced resistance in green shallot under laboratory and greenhouse conditions.

5.1 Isolation of pathogen by tissue transplanting technique had efficacy to isolate anthracnose pathogen in green shallot

The pathogen used in this study was provided under the name *C. gloeosporioides* which causes anthracnose disease on green shallot (Liu et al., 2014 and Abdelrahman et al., 2018). The pathogen isolates used in this study were in green shallot tissues, grown on WA medium at room temperature, and subcultured to PDA medium (Connell et at., 2004). The important principles for isolating the pathogens such as young green shallot leaves that look like fresh wounds were over the outside of the bulb or leaves with dark green or black colored spots, about 2-3 mm in diameter, and show ring (Ciba plant protection vegetables, 1911; Compendium of onion and garlic disease, 1995). When selected the sample rapid to isolation in laminar flow after sub-cultured to PDA medium at 7 old days, the fungi can well grow up. It corresponds to the morphology and conidia of the *C. gloeosporioides.* The growth pattern was either circular, with the mycelia showing a uniform growth pattern and radial in a ring-like pattern. Subsequently, one representative isolate was selected for further study.

5.2 Pathogenicity and virulence test by detaching leave assay

Tested 6 isolates of *C. gloeosporioides* at the concentration $1_{\times}10^{6}$ CFU mL^{-1} in green shallot leaves found that all isolates were aggressive, but each isolate showed varying degrees of virulence. Among these isolates, PSKR-SUT 10 had the most virulent. The symptoms and sign of this disease the outside of the leave with small white sunken spots and eventually the disease will form a concentric ring pattern covered with mycelium and orange spore mass of *C. gloeosporioides*. This result indicated that artificial inoculation of green shallot with *C. gloeosporioides* to occur anthracnose disease had to adjust 3 factors such as pathogen, host plant, and environment to suitable as a disease incidence. Plant diseases their occurrence and severity result from the impact of 3 factors because the severity of anthacnose disease depends on the plant susceptible, a virulent pathogen, and conducive is the existing environmental conditions in supporting disease and pathogen spread. Besides, invasion of anthracnose disease the *C. gloeosporioides* which is a hemibiotrophic ascomycete. the genus *Colletotrichum* contains proteases and carbohydrate-active enzymes of any fungus. They also produce secondary metabolites, which are commonly phytotoxic, in both their biotrophic and necrotrophic phases by playing a role in a biotrophic phase where it secures a relationship with the host plant and switching to a necrotrophic phase to obtain nutrients and colonize the tissue to kill cells and progress through the plant causing necrotic lesions and plant death, resulting in a 50-80% yield loss in green shallot.

Moreover, pathogenicity and virulence test by detaching leave assay in green shallot can be used as a rapid screening technique to pathogenicity test and virulence test in a short time with minimal cost. Similarly, Sharma et al. (2005) reported that the

assay of the detached leaf can be used as a rapid screening technique to evaluate germplasm, segregating breeding materials, and mapping populations for resistance to *H. armigera* in a short time. Also, Browne and Cooke (2003) used a detached leaf assay for pre-screening resistance to *Fusarium* head blight in wheat.

5.3 Efficacy of *B. subtilis* as a biological control agent against *C. gloeosporioides*

Screening of *B. subtilis* as a biological control agent against *C. gloeosporioides* 14 isolates (CaSUT007, CaSUT007-1, CaSUT008, CaSUT008-2, CaSUT111, D604, D604-1, D501, D501-1, SUNB1, SUNB2, 37-4, 37-5 and 37-6) by dual culture method under laboratory to study direct mode of action of *B. subtilis* to control anthracnose disease. Found that *B. subtilis* 5 strains including D604, CaSUT008-2, SUNB2, CaSUT007-1, and CaSUT007 with strong antagonistic activity to *C. gloeosporioides* isolate PSKR-SUT 10 was observed. The highest percentage of inhibition of *C. gloeosporioides* was 49.55, 46.00, 45.66, 44.50, and 43.78% respectively. This study corresponds to Wongchalee (2015) tested *Bacillus* for antagonistic activities against *Colletotrichum* sp. by using the dual culture technique. Found that *Bacillus* strains D604, D502, and CaSUT007 showed high activity against *Colletotrichum* sp. Similar to Awang et al., 2015 *Bacillus* strain CBF, YCA0098, and YCA5593, were tested against *C. gloeosporioides* the result showed that all *Bacillus* sp. significantly reduce the mycelia growth and spore germination of the *C. gloeosporioides*. In 2014 Ashwini and Srividya found that *B. subtilis* showed high antagonistic activity against *C. gloeosporioides*, and Srividya et al., 2012 report that *B. subtilis* produced appreciable levels of enzymes such as

chitinase, glucanase, and cellulase and showed broad-spectrum antagonism against fungal phytopathogens.

5.4 The efficiency biosurfactant produced by antagonistic *B. subtilis* strain D604 to inhibit the mycelium growth of *C. gloeosporioides*

The efficiency of biosurfactant by *B. subtilis* was carried out by the method of agar well method. The results found that the biosurfactant *B. subtilis* strain D604 at 200 µl had efficacy to inhibit the growth of anthracnose disease caused *C. gloeosporioides* higher 73.48% and the biosurfactant at 150 µl had inhibition percentage of efficacy to pathogen at 70.81%. Moreover, the biosurfactant from *B. subtilis* strain D604 showed the highest inhibition percentage of mycelium growth. The efficiency of concentration biosurfactant from *B. subtilis* strain D604 to control anthracnose disease. The result found that biosurfactants at the concentration of 25% could control anthracnose disease in green shallot. The results are in line with Pyoung et al. (2009). These authors reported that three biosurfactant lipopeptides produced by *B. subtilis* CMB32 such as iturin A, fengycin, and surfactin A. Strain CMB32 had the strongest antagonistic effect to inhibit *C. gloeosporioides*, and also had antifungal activity against other fungal plant pathogens, including *F. solani* KCTC 6328, *B. cinereal* KACC 40573, *F. oxysporum* KACC 40037, *R. solani* KACC 40151, and *P. capsici* KACC 40157. In 2012, Andersson reported secondary metabolites from the bacterial biocontrol agent *P. brassicacearum* MA250 can inhibit the growth of the fungal pathogen *Microdochium nivale* at concentrations down to 180 µM, which indicates that they may contribute to the biocontrol effect of *P. brassicacearum* MA250 on *M. nivale*. Sarwar et al (2018) extracted lipopeptides from bacteria to assess their

antifungal activities. The results showed that *B. amyloliquefaciens* FZB42, *Bacillus* sp. NH 217 and *B. subtilis* NH-100 exhibited excellent biosurfactant and displayed good antifungal activity against plant disease pathogens, such as *Fusarium moniliforme*, *F. oxysporum*, and *F. solani.* Paramanandham et al. (2017) reported a secondary metabolite produced by some microorganisms, that acts as a general inhibitor to avoid competition and predation between pathogen and biocontrol agent. It was considered to be toxic to the pathogen and increased the death rate of the pathogen in the rhizosphere environment. In 2015, Mnif et al. evaluated *B. subtilis* SPB1 lipopeptides antifungal agent against *F. solani* infestation. The results showed that the treatment of *F. solani* by SPB1 lipopeptides generated excessive lyses of the mycelium and destroyed the related spores together with a total inhibition of spore production. The preventive treatment appeared as the most promising as after 20 days of fungal inoculation, rot invasion was reduced by almost 78 %, in comparison to that of non-treated one. When treating infected tomato plants, disease symptoms were reduced by almost 100% when applying the curative method. The results of this study were very promising as it enabled the use of the crude lipopeptide preparation of *B. subtilis* SPB1 as a potent natural fungicide that could effectively control the infection of *F. solani* in tomato and potato tubers at a concentration similar to the commercial fungicide (hymexazol). Ongena et al. (2007) studied surfactin and fengycin lipopeptides of *B. subtilis* as elicitors of induced systemic resistance in plants. The results found that surfactin and fengycin lipopeptides of *B. subtilis,* a novel class of compounds, can be perceived by plant cells as signals to activate defense mechanisms. Moreover, experiments conducted on bean and tomato plants showed that overexpression of both surfactin and fengycin biosynthetic genes in the producer *B. subtilis* strain 168 was

associated with a significant increase in the potential of the derivatives to induce resistance. The lipopeptides are considered as virulence factors and display a strong phytotoxic activity centered on an ability to form pores in plant plasma membranes, thereby inducing cell death (Dalla Serra et al., 1999). In 2008, Ongena and Jacques reported the biocontrol of plant diseases using the three families of *Bacillus* lipopeptides including surfactins, iturins, and fengycins for the antagonistic activity for a wide range of potential pathogens of bacteria and fungi. The lipopeptides can also influence the ecological fitness of the producing strain in terms of root colonization and also have a key role in the beneficial interaction of *Bacillus* sp. with plants by stimulating host defense mechanisms.

5.5 The efficacy of biosurfactant produced by antagonistic *B. subtilis* strain D604 to plant growth promotion

The biosurfactant produced by *B. subtilis* strain D604 tested in this study exhibited the potential to increase plant height with 30.34 cm, the weight of shoot with 119.96 g, and the dry weight of shoot with 32.69 g respectively, and increase contents of chlorophyll a, b, and total chlorophyll was high at 19.78, 6.07, and 25.86 µg mL^{-1}. Moreover, the indole-3-acetic acid content in green shallot leaf increase after treated with biosurfactants from *B. subtilis* strain D604 highest amount of 14.76 µg g^{-1} fresh. These biosurfactants are important parameters for a beneficial association with the plant roots and can improve the growth of the plant. Further, these biosurfactants can increase the bioavailability of hydrophobic molecules which may serve as nutrients.

Biosurfactants provide wettability to soil and support the proper distribution of chemical fertilizers in the soil thus assisting plant growth promotion. The results are

corresponding to Dusane et al. (2010). These authors reported that the biosurfactant produced by *Pseudomonas* spp. regulated the process of quorum sensing (cell to cell communication). In 2005, Haas and Défago reported the lipopeptide biosurfactants produced by *Pseudomonas* sp. and *Bacillus* sp. had been implied in biocontrol due to their potential positive effect on competitive interactions with organisms and supported plant growth. Akinrinlola et al. (2018) reported *B. megaterium* and *B. safensis* increased the growth of soybean and wheat. Magdalena et al. (2016) reported that bacteria exhibit features of plant growth-promoting bacteria (PGPB) and can increase the biomass of plants via several mechanisms that include: the production of phytohormones (such as indoleacetic acid, IAA), siderophores, and biosurfactants to promote plant growth. Xiafang et al., (2008) reported that A biosurfactant-producing *Bacillus* sp. can promote the plant growth, produce indole acetic acid (IAA), and siderophores. Reviewing the functions of biosurfactant indicates the essential role of these green compounds for sustainable agriculture.

5.6 The efficacy of biosurfactant produced by antagonistic *B. subtilis* strain D604 to reduce anthracnose disease and induce resistance in green shallot

The efficacy of biosurfactant from *B. subtilis* strain D604 was used to investigate anthracnose disease control in green shallot under greenhouse conditions. The results demonstrated that biosurfactant from *B. subtilis* strain D604 when applied as a foliar treatment every 7 days and after inoculated with the fungal for 7 days, the results showed that anthracnose disease severity at 58.80%, equivalent to Prochloraz fungicide at 64.70% significantly different with negative control (dH_2O). This result

indicated that biosurfactant from *B. subtilis* strain D604 can induce resistance in green shallot. Corresponding to Touré et al, (2004) found that lipopeptides produced by *B. subtilis* GA1 were very effective at reducing disease incidence during 5 days following *B. cinerea* inoculation and an 80% protection level were maintained over the next 10 days. and a disease reduction of about 40% was conserved after 21 days of incubation on apple. In the same year, Touré et al, report that the cell-free filtrate, extracted lipopeptides, and crude of *B. atrophaeus* CAB-1 display a high inhibitory activity against various *B. cinereal*. Next In 2015 Mnif et al. evaluated the efficiency of a lipopeptide biosurfactant derived from *B. subtilis* SPB1 at concentrations 0.1, 1, and 3 mg/ml against the hyphal growth of *F. solani*. The result demonstrates that total inhibition of hyphal growth and spore production when treating *F. solani* with 3 mg/ml of lipopeptides biosurfactant.

5.7 Mechanisms of induced resistance against anthracnose in green shallot

The efficacy of biosurfactant from *B. subtilis* strain D604 to study mechanisms of induced resistance against anthracnose disease in green shallot under greenhouse condition. This experiment was performed in CRD with 5 treatments using 4 replications. The green shallot foliar spray 4 times with 25% biosurfactant produced from *B. subtilis* strain D604. Distilled water was served as a negative control and Prochloraz fungicide was served as a positive control. After that study defense mechanisms were observed by the detection of biochemical change such as salicylic acid and phenolic compound were investigated at 0, 24, and 48 hours after inoculation (HAI). The results found that the total of PC significantly increased at 24 HAI to levels

of 352.71, 316.87, 296.87, and 281.50 μg g^{-1} fresh weight from foliar treated with biosurfactant *B. subtilis* strain D604, *B. subtilis* strain D604, commercial *B. subtilis*, and Prochloraz, respectively. Likewise, the accumulation of SA significantly increased at 24 HAI to levels of 103.58, 116.83, 90.67, and 87.04 μg g^{-1} fresh weight. respectively. Then study the defense enzymes including phenylalanine ammonia-lyase (PAL), and PR-protein: chitinase (Chi). The result found that PAL activity also significantly increased at 24 HAI with levels of 15.39, 9.93, 7.36, and 5.55 μmol mg^{-1}protein, respectively. On the other hand, Chi activity significantly increased at 24 HAI with levels of 4.11, 3.19, 2.52, and 2.46 μmol mg^{-1}protein from foliar treated with biosurfactant strain D604, Prochloraz, *B. subtilis* strain D604, and commercial *B. subtilis*.

These results demonstrated that when pathogen infection. One of the earliest responses is the oxidative burst, in with levels of reactive oxygen species. It acts as a local signal for the hypersensitive response that contributes to program cell death occurs at sites of pathogen-infected. Plants have established a complex defense system consisting of various barricade layers including induced defense controlled by a multifarious and dynamic network of signaling pathways. SA and JA have a regulatory role in plant immunity and plant defense responses. This contributes active defense response and causes the direct active SAR including defense protein such as phenylalanine ammonium-lyase (PAL), polyphenol oxidase (PPO), peroxidase (POD), lipoxygenase (LOX) and producing antimicrobial compounds in plants, e.g., phenolics, flavonoids, phytoalexins, and pathogenesis-related (PR) proteins such as chitinase, and β-1,3-glucanase. This activation has a role in not only transcriptional reprogramming and induction of early defense-related genes but also in limiting pathogen infection and priming plants against future attacks (Figure 5.1). Corresponding to He and Wolyn

(2005) report SA is essential for the stimulation of defense mechanisms and induction of SAR to *C. gloeosporioides* in green shallot. SA has long been recognized to play an important role in the situation of SAR against disease because SA levels increased in plant tissues ensuing pathogen infection and exogenous application of SA can enhance the resistance to a broad range of pathogens. Also, SA has an important role in the signaling pathway leading to ISR (Induced systemic resistance) after infection, endogenous levels of SA increase locally and systemically, and SA levels increase in the phloem before ISR occurs (Hell et al., 2002). Kim and Hwang (2014), the authors reported that PAL had a crucial role in plant responses to stress. PAL (*CaPAL*1) gene, which was induced in pepper. *CaPAL*1 in pepper conferred increased resistance to disease increased ROS burst and cell death, and induction of PR1 expression and SA accumulation. *CaPAL*1 acts as a positive regulator of SA-dependent defense signaling to combat microbial pathogens via its enzymatic activity in the phenylpropanoid pathway. Also, the phenolic compound differs in the plant, in which a direct inhibited effect of pathogens by phenolics was detected (Benhamou and Be´langer., 1998). The phenolics compounds were interceded by the deposition of phenolics and callose in the wall. Simultaneously, Chi activity increased at 24 after the fungal challenged inoculation with the fungal. Corresponds to a study of Gupta et al. (2012), the induction of PR proteins in *Eruca sativa* in response to fungal pathogen *A. brassicicola* found that the induction of pathogen resulted in a much-marked increase in the activities of β-1,3-glucanase and chitinase in resistant cultivar as compared to susceptible. In 2004, Santos et al. reported plants-produced enzymes such as β-1,3-glucanase and chitinase can break down the cell wall components of pathogens. These enzymes are important determinants of the resistance of plants to fungal diseases. The

green shallot treated biosurfactant likely conducts to drastic changes in both the rigidity and the vulnerability of cells, also the differential expression of chitinases in green shallot was considered a convenient indicator of successful signaling. The green shallot is a rewarding target of SAR and ISR research because it is an important crop, which is prone to several devastating fungal diseases and requires the frequent application of fungicides. The conclusions from the inoculation studies are in tally with the relevant literature (Busam et al., 1997) and support the thought of SAR and ISR response for green shallot defense mechanism.

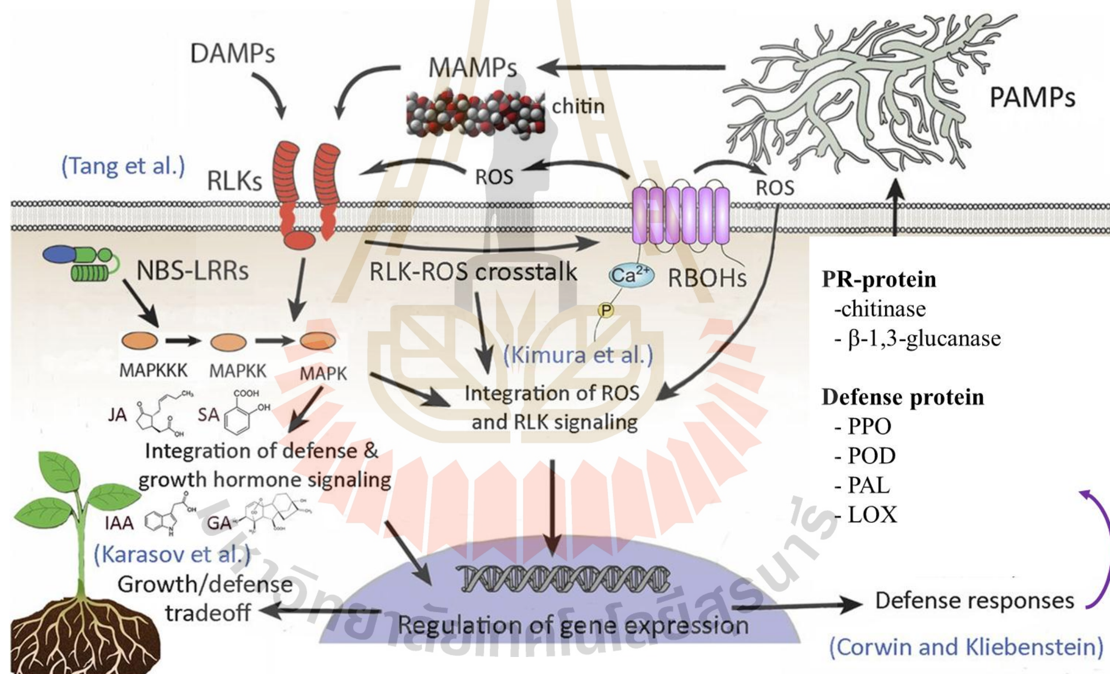

Figure 5.1 A schematic representation of the plant defense mechanism against the pathogen (Corwin and Kliebenstein, 2017; Tang et al., 2017).

Bacillus biosurfactant can induce systemic resistance against anthracnose disease of green shallot by increase the resistance intermedia compound as endogens salicylic acid, phenolic compound, Chitinase, and Phenylalanine ammonia-lyase.

These results indicated that the biosurfactant *B. subtilis* strain D604 has direct and indirect effects that could act as an antimicrobial agent and activate several plant defense mechanisms during host-pathogen interaction (Figure 5.2).

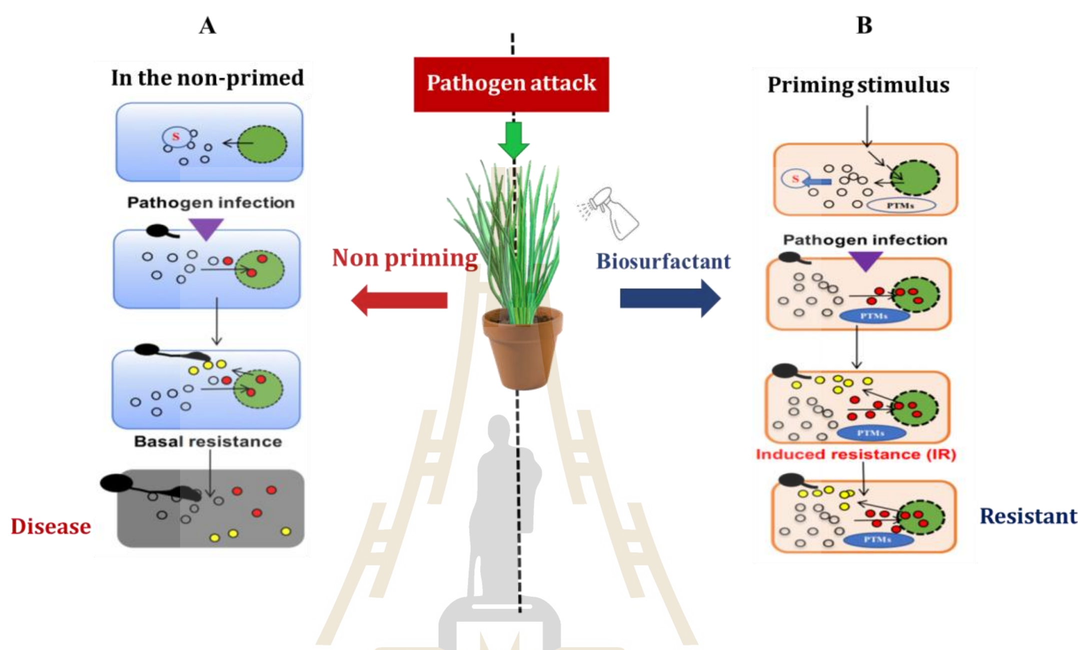

Figure 5.2 Efficacy of biosurfactant to induce systemic resistance against anthracnose disease on green shallot. (A) In the non-primed cell and (B) In the priming stimulus cell.

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5.8 Biosurfactants in agriculture

The biosurfactant produced by *B. subtilis* strain D604 tested in this study had efficacy to plant growth promotion, reduced anthracnose disease, and induced resistance in green shallot. These biosurfactants have a double effect that could act as an antimicrobial agent and activate several plant defense mechanisms during host-pathogen interaction. When applied as a foliar treatment every 7 days at the concentration of 25% can control anthracnose disease likely Prochloraz fungicide under greenhouse conditions. The

biosurfactant produced by *B. subtilis* strain D604 can be an alternative method to growth-promoting and control green shallot disease. The biosurfactant as biocontrol agents are lipopeptide and demonstrate antagonist effect on plant phytopathogens that have acquired resistance to commercial pesticides, thus initiating their use as biocontrol agents. Biosurfactant can likewise excite the immunity of the plant which is considered as an alternative approach to decrease the disease caused by phytopathogens. In the current years, surfactants have several applications in agriculture and agrochemical industries. However, there is a rare use of biosurfactants which are more environmentally friendly. The exact role of surfactant in facilitating other systems as biocontrol agents is yet not much understood and warrants investigations. These studies will help in replacing the harsh chemical there is a need to work on the production cost of green surfactants to achieve net economic gain from the application of biosurfactant in agriculture. This approach may lead to the biosynthesis of highly target-specific surfactants that indication for its potent role in sustainable agriculture. In the future, the research should be practical strategies and methods that will be adopted to develop the biosurfactant production process economically and friendly attractive: these include the use of cheapest raw materials, waste solid state, optimized and efficient bioprocesses and overproducing natural mutant *Bacillus* strains for obtaining maximum biosurfactant productivity. The application of these approaches in biosurfactant production processes, particularly those using hyper-producing natural mutant strains in the optimally controlled environment of a bioreactor, might lead towards the successful commercial production of these valuable biomolecules in near future.

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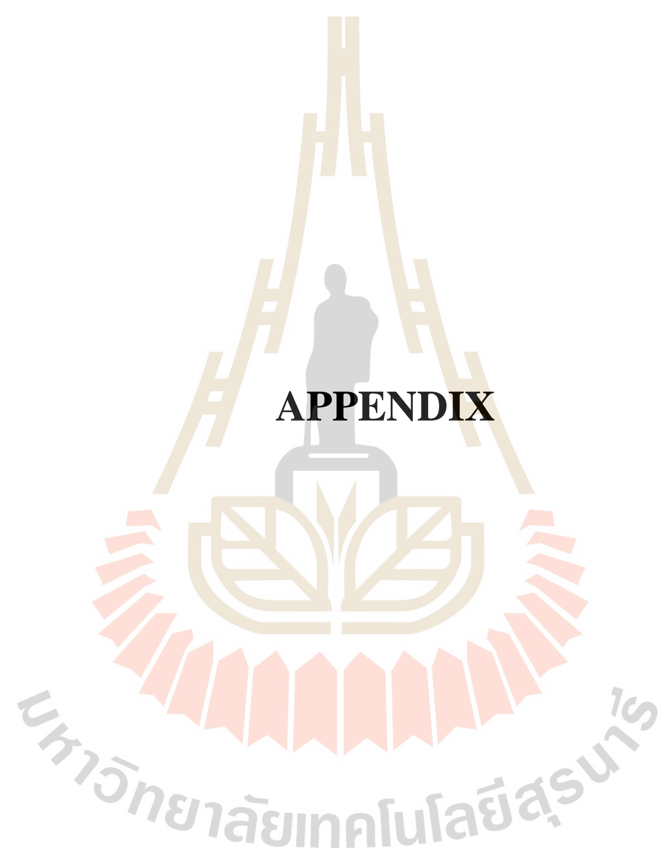
APPENDIX



I. MEDIUMS

1.1 Water Agar (WA)

Agar	15g
Water	1L

1.2 Potato Dextrose Agar (PDA)

Potato	200g
Dextrose	20g
Agar	15g
Water	1,000ml

1.3 Nutrient broth (NB) medium

Beef extract	3g
Peptone	5g
Water	1,000ml

II. CHEMICALS

2.1 0.02 M Ferric ammonium sulfate

Ferric ammonium sulfate	4.82g
Water	500ml

2.2 10% Folin-ciocalteu reagent

Folin-ciocalteu reagent	10ml
Water	100ml

2.3 7% Sodium carbonate (Na_2CO_3)

Sodium carbonate	7g
Water	100ml

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2.4 2% Potassium sorbate

Potassium sorbate	2g
Water	100ml

2.5 1 M KCl

KCl	74.56 g
Water	1,000ml

2.6 1 M phenylmethylsulfonyl fluoride (PMSF)

PMSF	174.20g
Isopropanol	1,000ml

2.7 1% Triton-x 100

Triton-x 100	1ml
Water	99ml

2.8 Salkowski's reagent

Prepare stock solution of 0.5 M FeCl3 (1.35 g in 10 ml water)

Use 1 ml of this stock to mix with 49 ml of 35% $HClO_4$

2.9 1 M Tris-HCl buffer, pH 7

Tris base	121.1g
deionization water	1,000ml

adjust pH to 7

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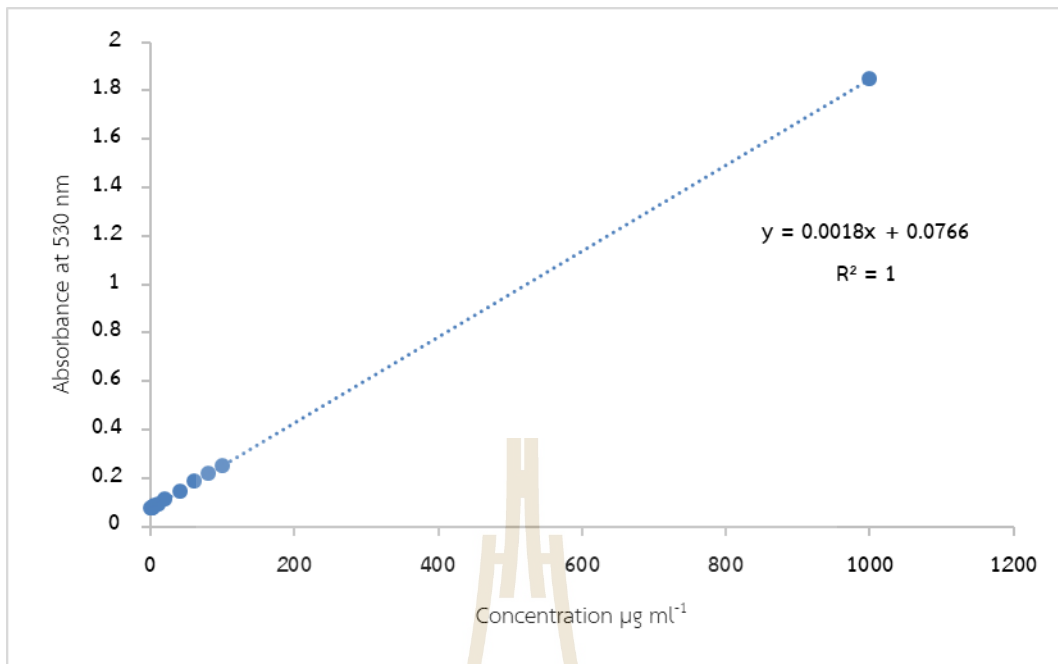
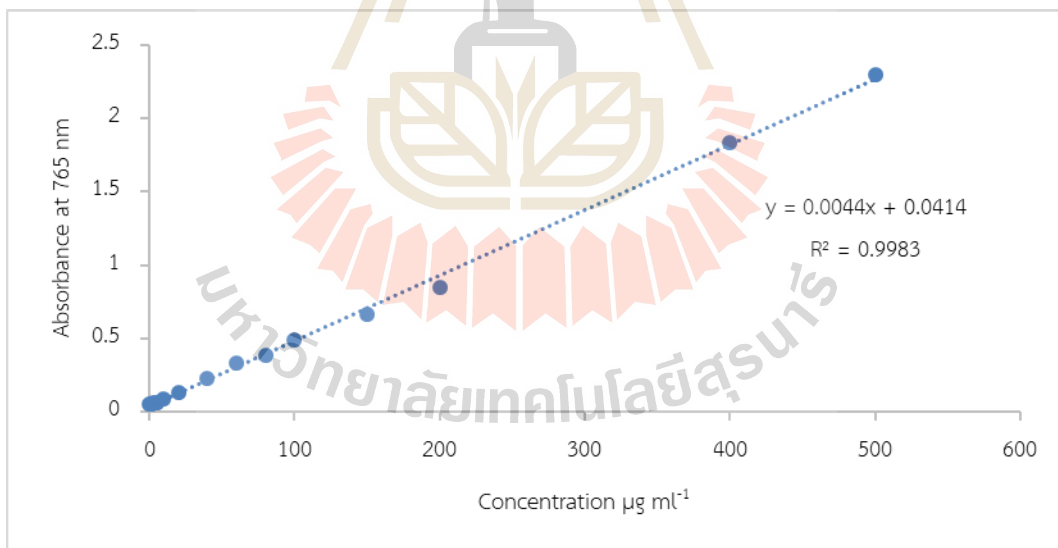


Attached figure 1 Standard curve of salicylic acid (SA) at the absorbance 530 nm.



Attached figure 2 Standard curve of gallic acid at the absorbance 765 nm.

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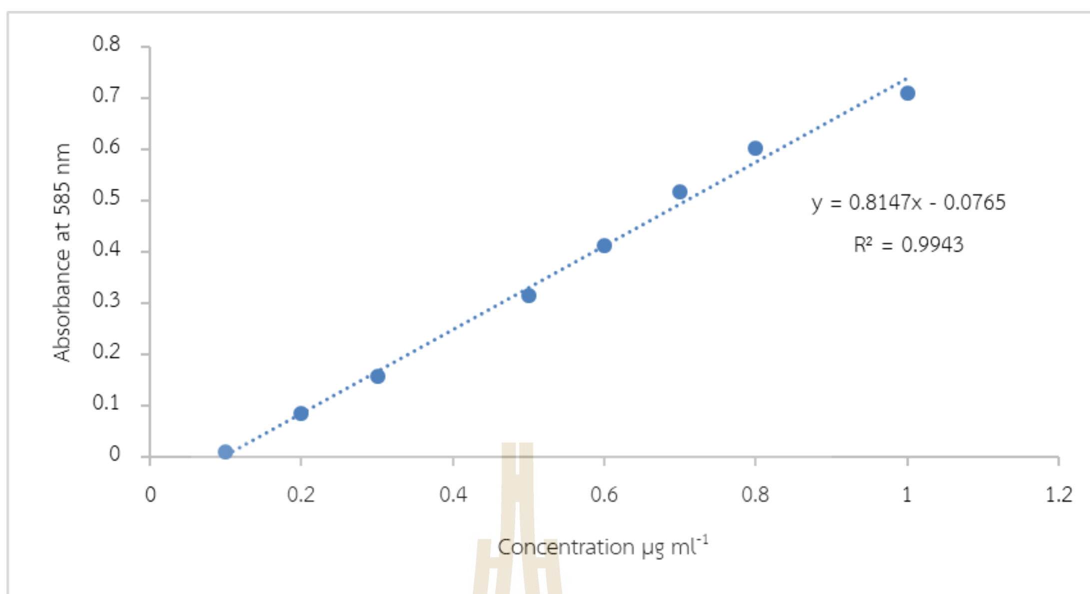
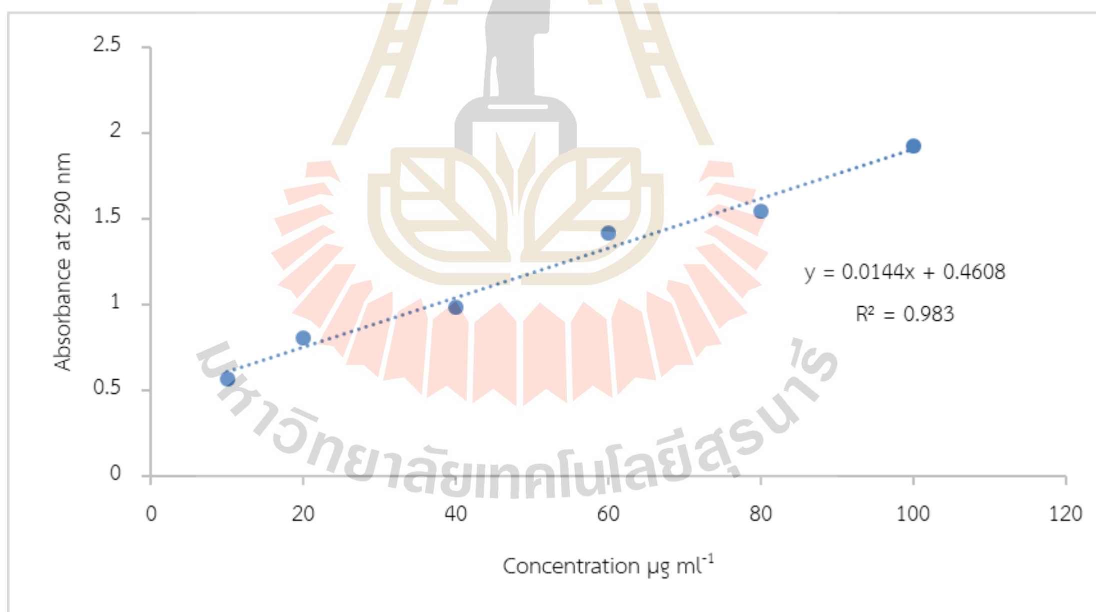


Attached figure 3 Standard curve of N-acetyl glucosamine at the absorbance 585 nm.



Attached figure 4 Standard curve of trans-cinnamic acid at the absorbance 290 nm.

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BIOGRAPHY

Miss Nattaya Thongprom was born on March 1992 in Sakon Nakhon province, Thailand. She received his Bachelor of Crop Production of Technology from the Suranaree University of Technology, Thailand in 2016.

In 2016, she won a scholarship from the External Grants and Scholarships for Graduate Students (OROG) Scholarships 2016-2018 (SUT scholarship No. 12/2559) to pursue a Master's Degree in Suranaree University of Technology, Nakhon Ratchasima, Thailand. She attends an International Workshop on Integrated Technology for Preventing and Controlling Diseases and Insect Pests of Major Crops in ASEAN Countries, Yunnan Agricultural University, China, July 1st to July 20th, 2016. And in 2019 attend the International Training Workshop on Symbiosis Farming of Rice and Aquatics for Belt & Road Countries Rice Research Institute, Academy of Agricultural Sciences, Jiangxi, Nanchang, China.

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