EFFICIENCY OF SEED BIO-PELLETING AND FOLIAR SPRAY BY USING *Bacillus subtilis* 168-2 TO CONTROL LEAF SPOT DISEASE ON LETTUCE

KODCHAPHON TONPHO

A Thesis Submitted in Partial Fulfillment of the Requirements for the
Degree of Master of Science Program in Crop Science
Suranaree University of Technology
Academic Year 2022

ประสิทธิภาพของการพอกเมล็ดพันธุ์และการฉีดพ่นทางใบด้วย *Bacillus subtilis* 168-2 เพื่อควบคุมโรคใบจุดของผักกาดหอม

นางสาวกชพร ต้นโพธิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาพืชศาสตร์
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2565

EFFICIENCY OF SEED BIO-PELLETING AND FOLIAR SPRAY BY USING *Bacillus subtilis* 168-2 TO CONTROL LEAF SPOT DISEASE ON LETTUCE

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

Thesis Examining Committee

(Asst. Prof. Dr. Arak Tira-umphon)
Chairperson

(Asst. Prof. Dr. Natthiya Buensanteai)
Member (Thesis Advisor)

(Asst. Prof. Dr. Piyanath Pagamas)
Member

(Dr. Wanploy Jinagool)
Member

(Assoc. Prof. Dr. Chatchai Jothityangkoon)
Vice Rector for Academic Affaire and Quality Assurance

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

กชพร ต้นโพธิ์ : ประสิทธิภาพของการพอกเมล็ดพันธุ์และการฉีดพ่นทางใบด้วย *Bacillus subtilis* 168-2 เพื่อควบคุมโรคใบจุดของผักกาดหอม (EFFICIENCY OF SEED BIO-PELLETING AND FOLIAR SPRAY BY USING *Bacillus subtilis* 168-2 TO CONTROL LEAF SPOT DISEASE ON LETTUCE) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.ณัฐธิญา เบือนสันเทียะ, 91 หน้า.

ผักกาดหอม/การพอกเมล็ดพันธุ์/การฉีดพ่นทางใบ/โรคใบจุด/*Bacillus subtilis* 168-2

เมล็ดพันธุ์ผักกาดหอมมีรูปร่างขนาดเล็ก อาหารสะสมในเมล็ดน้อย การพอกเมล็ดพันธุ์เป็นวิธีการเพิ่มคุณภาพของเมล็ดพันธุ์ที่สามารถทำให้เพาะกล้าได้ง่ายขึ้น วัสดุพอกเป็นองค์ประกอบที่สำคัญที่จะทำให้การพอกเมล็ดพันธุ์ประสบความสำเร็จได้ และโรคใบจุดเป็นหนึ่งในสาเหตุที่ทำความเสียหายให้แก่ผักกาดหอม การพอกเมล็ดพันธุ์และการฉีดพ่นทางใบด้วย *Bacillus subtilis* 168-2 สามารถควบคุมโรคใบจุดและส่งเสริมการเจริญเติบโตของผักกาดหอมได้ ดังนั้นงานวิจัยนี้มีวัตถุประสงค์เพื่อทดสอบการพอกเมล็ดพันธุ์ด้วย *B. subtilis* 168-2 ในการส่งเสริมการเจริญเติบโตและตรวจสอบกลไกการชักนำความต้านทานโรคใบจุด *Alternaria alternata* โดยการพอกเมล็ดพันธุ์และการฉีดพ่นทางใบด้วย *B. subtilis* 168-2 จากผลการตรวจสอบในสภาพห้องปฏิบัติการพบว่า การพอกเมล็ดพันธุ์พอกด้วย calcium sulfate ร่วมกับ *B. subtilis* 168-2 ความเข้มข้น 10^8 cfu/ml (SeedBio 3) เมล็ดพันธุ์ผักกาดหอมสามารถขึ้นรูปเมล็ดพอกได้ง่าย มีความกร่อนต่ำ และสามารถละลายน้ำได้ดี เมื่อตรวจสอบคุณภาพเมล็ดพันธุ์หลังการพอก พบว่า SeedBio 3 ทำให้เมล็ดพันธุ์มีความงอกและความเร็วในการงอกสูงที่สุดเมื่อเปรียบเทียบกับกรรมวิธีอื่นๆ นอกจากนี้ยังพบว่า การพอกเมล็ดพันธุ์ทำให้ราก และลำต้นของต้นกล้าผักกาดหอมมีความยาวมากกว่าเมล็ดพันธุ์ที่ไม่ผ่านการ ส่วนการทดสอบในสภาพโรงเรือนได้คัดเลือก SeedBio 3 และฉีดพ่นด้วยสิ่งกระตุ้น 4 ครั้ง พบว่า การพอกเมล็ดพันธุ์(SeedBio 3) และการฉีดพ่นด้วย *B. subtilis* 168-2 ความเข้มข้น 10^8 cfu/ml (กรรมวิธีที่ 5) สามารถลดการเกิดโรคใบจุดผักกาดหอมได้ 47.3% เมื่อเปรียบเทียบกับกรรมวิธีควบคุม และมีน้ำหนักสดสูงที่สุด 86.56 กรัม/ต้น และมีน้ำหนักแห้งสูงที่สุด 8.93 กรัม/ต้น ซึ่งมากกว่ากรรมวิธีควบคุมอย่างมีนัยสำคัญทางสถิติ จากนั้นคัดเลือกตัวอย่างใบผักกาดหอม มาทำการตรวจสอบกลไกการปกป้องตัวเองได้แก่ ปริมาณกรดซาลิไซลิก (SA) และปริมาณการสะสม superoxide dismutase (SOD) ตรวจสอบปริมาณการสะสม indole-3-acetic acid (IAA) ผักกาดหอมที่พอกเมล็ดและฉีดพ่นด้วยกรรมวิธีที่ 3 (SeedBio 3 และฉีดพ่นด้วย *B. subtilis* 10^8 cfu/ml) สูงสุดที่ 10.68 ไมโครกรัมต่อน้ำหนักสด รองลงมาคือ กรรมวิธีที่ 4 (SeedBio 3 และฉีดพ่นด้วย Mancozeb) ที่ 9.67 ไมโครกรัม

ต่อน้ำหนักสด ตามลำดับ และกรรมวิธีที่ 3 สามารถชักนำภูมิต้านทานโรคโดยกระตุ้นให้สะสม SOD สูงสุดที่ 17.76 ไมโครกรัมแคททีคอล/มิลลิกรัมโปรตีน ซึ่งมากกว่าและแตกต่างกันในทางสถิติเมื่อเปรียบเทียบกรรมวิธีที่ 4 (15.60 ไมโครกรัมแคททีคอล/มิลลิกรัมโปรตีน) และมีประสิทธิภาพในการส่งเสริมการเจริญเติบโตของผักกาดหอมโดยกระตุ้นให้สะสม indole-3-acetic acid ในกรรมวิธีที่ 3 เท่ากับ 12.67 ไมโครกรัม/มิลลิกรัมน้ำหนักสด ที่ 0 ชั่วโมงหลังการปลูกเชื้อ (HAI) ซึ่งสูงมากกว่ากรรมวิธีอื่นอย่างมีนัยสำคัญทางสถิติ และมีแนวโน้มการสะสม IAA เพิ่มขึ้นในช่วงเวลา 24 HAI เท่ากับ 16.98 ไมโครกรัม/มิลลิกรัมน้ำหนักสด ซึ่งแตกต่างกันอย่างมีนัยสำคัญทางสถิติ นอกจากนี้เมื่อนำใบผักกาดหอมที่ฉีดพ่นด้วยสิ่งกระตุ้นและภายหลังการปลูกเชื้อ 24 ชั่วโมง มาตรวจสอบการเปลี่ยนแปลงสารชีวเคมีด้วยเทคนิค Synchrotron FT-IR microspectroscopy พบว่า การพอกเมล็ด (SeedBio 3) และฉีดพ่นด้วย *B. subtilis* 168-2 ความเข้มข้น 10^8 cfu/ml (กรรมวิธีที่ 3) ส่งเสริมให้ผักกาดหอมมีการสังเคราะห์สารในกลุ่มเพคติน C=O ester และกรดอะมิโน เอไมด์ I และ เอไมด์ II ในเนื้อเยื่อชั้นมีโซฟิลล์เพิ่มขึ้นที่ 0.166± 0.023, 0.112± 0.012 และ 0.077± 0.015 ตามลำดับ ซึ่งมากกว่าและแตกต่างกันอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับกรรมวิธีควบคุม จากการทดลองนี้แสดงให้เห็นว่าการพอกเมล็ดและการฉีดพ่นด้วยกรรมวิธีที่ 3 มีประสิทธิภาพในการควบคุมโรคใบจุดผักกาดหอมโดยมีประสิทธิภาพในการยับยั้งการเจริญของเชื้อราสาเหตุโรคโดยชักนำให้ผักกาดหอมเกิดกระบวนการปกป้องต้นเองให้ต้านทานต่อเชื้อราสาเหตุโรคเสริมสร้างความแข็งแกร่งของผนังเซลล์ผักกาดหอมให้มีความต้านทานต่อการเข้าทำลายของเชื้อก่อโรค การพอกเมล็ดพันธุ์ (SeedBio 3) และฉีดพ่นด้วย *B. subtilis* 168-2 ความเข้มข้น 10^8 cfu/ml (กรรมวิธีที่ 3) จึงเป็นแนวทางที่เหมาะสมในการนำไปใช้ในระบบการผลิตผักกาดหอมอินทรีย์ ลดการใช้สารเคมีในการควบคุมโรค และยกระดับคุณภาพของเมล็ดพันธุ์ ดังนั้นในธุรกิจเมล็ดพันธุ์จึงอาศัยเทคโนโลยีการพอกเมล็ดพันธุ์ (Seed pelleting) เพื่อเพิ่มคุณภาพเมล็ดพันธุ์ส่งเสริมการงอก ต้นกล้าแข็งแรง ลดโรคในระยะต้นกล้า รวมไปถึงเป็นการสร้างเอกลักษณ์บนผิวของเมล็ดพันธุ์เพื่อป้องกันการปะปนของเมล็ดพันธุ์ ซึ่งสามารถประยุกต์ใช้ในเชิงพาณิชย์และทำให้เกษตรกรมีเมล็ดพันธุ์ที่ดีมีคุณภาพในระบบการเพาะปลูก

สาขาวิชาเทคโนโลยีการผลิตพืช ลายมือชื่อนักศึกษา .....

ปีการศึกษา 2565 ลายมือชื่ออาจารย์ที่ปรึกษา.....

KODCHAPHON TONPHO : EFFICIENCY OF SEED BIO-PELLETING AND FOLIAR SPRAY BY USING *Bacillus subtilis* 168-2 TO CONTROL LEAF SPOT DISEASE ON LETTUCE. THESIS ADVISOR : ASST. PROF. NATTHIYA BUENSANTEAI, Ph.D., 91 PP.

LETTUCE/SEED PELLETING/FOLIAR SPRAY/LEAF SPOT DISEASE/*Bacillus subtilis* 168-2

Lettuce seeds are small and have less food accumulated in the seed. Seed pelleting is a way to raise the quality of the seeds that can make them suitable for planting. Filter materials are key elements to making seed pelleting successful. Leaf spot disease is one of the most serious problems in lettuce production. Seed pelleting and foliar spraying with *Bacillus subtilis* 168-2 are able to control leaf spot disease and promote lettuce growth. Therefore, this research aims (1) to examine the potential of lettuce seed pelleting with *B. subtilis* 168-2 to enhance plant growth and control lettuce leaf spot disease, and (2) to investigate the induced resistance mechanism against *Alternaria alternata* in lettuce plants after seed pelleting and foliar spraying with *B. subtilis* 168-2. From the results of the examination in laboratory conditions, it was found that seed pelleting with calcium sulfate and *B. subtilis* 168-2 at a concentration of 10^8 cfu/ml (SeedBio 3) can be easily shaped. These pelleted seeds had low friability and dissolved in water very well. The quality test of the pelleted seeds found that these pelleted seeds (SeedBio 3) also had the highest germination rate and the highest speed of germination when compared with the other treatments. Also, the result pointed at that the seedling of pelleted seeds had longer root length and shoot length than most of the seeds unpelleted. Under greenhouse conditions, the pelleted seeds (SeedBio 3) were selected for plating and sprayed with elicitors 4 times before inoculating with *A. alternata*. The results showed that the treatment of seed pelleted (SeedBio 3) combined with spraying *B. subtilis* 168-2 at a concentration of 10^8 cfu/ml (Treatment 5) significantly reduced the severity of lettuce leaf spot disease by 47.3% compared with the control treatment. Moreover, Treatment 5 maximized fresh weight 86.56 g/plant and dry weight 8.93 g/plant, which was significantly higher than those of the control treatment. Then, samples of lettuce leaves were selected to examine the defense mechanisms including salicylic acid (SA), superoxide dismutase (SOD) and plant growth regulator indole-3-acetic acid (IAA). Treatment 3 (SeedBio 3 and sprayed with *B. subtilis* 168-2 10^8) increased the

SA accumulation to the maximum at 24 hours after inoculating (HAI) with 10.68 µg g^{-1} fresh weight, followed by Treatment 4 (SeedBio 3 and sprayed with Mancozeb) which had 9.67 µg g^{-1} fresh weight. The SOD activity was maximum in Treatment 3 with 17.76 µg catechol /mg^{-1} protein, which was significantly higher than Treatment 4 (15.60 µg catechol /mg^{-1} protein). The accumulation of IAA in treatment 3 was 12.67 µg/mg^{-1} fresh weight at 0 HAI, which was significantly higher than those in the other treatments. After that, the IAA accumulation of Treatment 3 increased to 16.98 µg/mg^{-1} fresh weight at 24 HAI. In addition, the lettuce leaf samples at 24 HAI were investigated for their biochemical changes by the Synchrotron Fourier-transform infrared (FT-IR) microspectroscopy technique. The results showed that the integral area of pectin C=O ester group and amino acid, amide I and amide II increased at the levels of 0.166 ± 0.023, 0.112 ± 0.012, and 0.077± 0.015, respectively. The amount of pectin, amide I, and amide II in the mesophyll tissue of Treatment 3 was significantly higher than those in the control. Our results indicated that the seed pelleting (SeedBio 3) and spraying with *B. subtilis* 168-2 at a concentration of 10^8 cfu/ml (Treatment 3) were effective in controlling lettuce leaf spot disease by inducing plant resistance, and can play a role in plant cell wall reinforcement against plant pathogen. This Treatment 3 is therefore an appropriate approach to be used in the organic lettuce production system as it reduces chemical fungicide use and improves the quality of seeds. Therefore, in the seed business, seed pelleting technology should be used to increase seed quality, to promote germination and strong seedlings, to reduce disease in the seedling stage, as well as to create a unique identity on the seed to prevent seed contamination. Also, it can be applied for commercial use and enable farmers to have quality seeds in the cultivation system.

School of Crop Production Technology
Academic Year 2022

Student's Signature Kodchaphon Tongho
Advisor's Signature .....

ACKNOWLEDGMENTS

On the accomplishment of the present study, I would like to express my sincere thanks to my thesis advisor, Asst. Prof. Dr. Natthiya Buensanteai who critically reviewed the work and for her tireless guidance, encouragement, and assistance in making this successful dissertation, not only the research methodologies but also many other methodologies in life. I also owe sincere thanks to, Asst. Prof. Dr. Piyanath Pagamas and Dr. Wanploy Jinagool for broadening my academic seed pelleting and plant growth regulating chemicals, I am most grateful for his teaching and advice.

In addition, I would like to thank all my friends in Plant Pathogen Laboratory, Synchrotron Light Research Institute, The Center for Scientific and Technological Equipment 3, Suranaree University of Technology for their useful input and kind help.

Finally, I most gratefully acknowledge my parents and my sister for all their support throughout the period of this research.

KODCHAPHON TONPHO

TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF TABLES

Table Page

LIST OF TABLES (Continued)

Table Page

LIST OF FIGURES

Figure Page

LIST OF FIGURES (Continued)

Figures Page

LIST OF ABBREVIATIONS

ANOVA	=	Analysis of variance
CV	=	Coefficient of variation
DAI	=	Days after inoculation
DAT	=	Days after transplanting
FT-IR	=	Fourier-transform infrared
H	=	Hour
HAI	=	Hours after inoculation
IAA	=	Indole-3-acetic acid
ISR	=	Induced systemic resistant
PCA	=	Principle component analysis
PDA	=	Potato dextrose agar
SA	=	Salicylic acid
SOD	=	Superoxide dismutase
µg catechol /mg^{-1} protein	=	Microgram catechol per milligram protein
µg/mg^{-1}	=	Microgram per milligram

CHAPTER I
INTRODUCTION

Lettuce (*Lactuca sativa* L.) is one of the most important fresh vegetables around the world, especially in Thailand. It has low calories and high nutrition values such as fat and sodium. Additionally, lettuce is a good source of vitamin C, zinc, fiber, iron, folate, and various health-beneficial bioactive ingredients (Baslam et al., 2013; Kim et al., 2016). In previous studies, it was shown that anti-diabetic activities, cholesterol-lowering, and anti-inflammatory were attributed to the bioactive compounds in lettuce (Ferreres et al., 1997; White and Broadley, 2009; Kim et al., 2016). In 2016, Thailand exported 10.35 tons of lettuce seeds worth more than 7.68 billion baht to several countries (Office of Agricultural Economics, 2016). In order to achieve a good quality of lettuce, seedling is an essential cultivation process due to the small and flat shape of lettuce seeds, lacking accumulated nutrients, and diseases infection resulting from the tropical climate leading to quickly severe invasion and destruction of diseases. The major diseases are root rot (*Pythium* spp.), downy mildew (*Peronospora* spp.), and leaf spot (*Cercospora* spp., *Septoria* spp., *Curvularia* spp. *Alternaria* spp.) causing serious yield loss in lettuce production. The leaf spot symptoms on lettuce are small spots or dark brown to black – overlapped spots on the lettuce leaf. This disease outbreak can damage more than 50%, especially in the rainy season with high humidity (Rimmer et al., 2006; Rotniam, 2009). The epidemic of leaf spot disease is increasing every year. Chemical fungicides are always used to prevent plant diseases from the seedling stage to the harvesting season subsequently increasing production costs by 5 times (Kangsopa and Siri, 2015a). Also, Lettuce seeds are small and expensive, it always has a low germination rate due to seed-borne fungal contamination (Tumniarattanapong, 2005; Kangsopa and Siri, 2015b). In this case, the fungal pathogen originates on the lettuce seed surface and it can cause seedling leaf spots. seed treatments can terminate the foliar application of fungicides after the planting season.

Seed treatment is the physical, chemical, and biological agents and techniques applied to protect plant seeds and improve crop health (Ramzan et al., 2016; Kaewkham, 2017; Prakash et al., 2018). Seed priming of lettuce seeds has been boosted to prevent plant disease epidemic caused by seed-borne fungal pathogens (Baker et al., 1972; Schmitt et al., 2009; Schaerer, 2012). Moreover, seeds could be enhanced through priming, soaking, coating, and pelleting for a uniform crop.

Seed pelleting is one of the processes and techniques of adding active and inert ingredients into seeds to increase their weight, size, shape, and improve disease tolerance. Moreover, the seed pelleting can increase more plant nutrients and beneficial microorganism/biocontrol agents which are advantageous to the germination, the growth development of seedlings, and plant disease control (Kaufman, 1991; Avelar, 2012; Siri, 2015). As seed bio-pelleting is an essential technology for seed modification in order to enhance size and weight upon the requirement was beneficial for organic agriculture (Jeffs and Tuppen, 1986; Kaufman, 1991; John et al., 2000). Moreover, seed bio-pelleting can indirectly improve seed germination which macro and micronutrient pelleting enrich the rhizosphere region that triggers the vegetative seedling growth in addition to enhancement of microbial community around the root zone (Ellis, 2004; Suma, 2005; Doijode, 2006). Microbial antagonists or plant growth-promoting rhizobacteria/fungi (PGPR/PGPF) can be used to control many soil-borne pathogens in an eco-friendly method. Biological control agents or PGPR such as *Azospirillum*, *Azotobacter*, *Pseudomonas*, *Serratia,* and *Bacillus* could promote plant growth and induce disease resistance (Kloepper et al., 1980; Buensanteai et al., 2008; Akhtar et al., 2012; Sivasakhti et al., 2014). These beneficial microorganisms could be produced organic acids, amino acids, plant growth regulators as IAA to increase shoot and root growth in several economic crops (Lakshminarayana et al., 1992; Buensanteai et al., 2008; Oteino et al., 2015; Jiraporn et al., 2017). Application of biological control agents or PGPR, especially *Bacillus*, as seed priming could suppress the root-infecting fungi pathogens (Tumniarattanapong, 2005). In previous, the pelleting seeds have been successful with plant nutrients (Rotniam, 2009; Adhikari et al., 2016). It was found that pelleting seeds with Zn at the rate of 50 mg showed that corn, soybeans, pigeon peas, and ladies fingers higher drier weight than uncoated seeds at 22.35, 10.73, 14.60, and

9.47 grams, respectively. Moreover, research in 2016 by Adhikari et al. found that seeds of mungbean pelleted with different biocontrol agents to determine their effect on plant growth and colonization of roots by root-infecting fungi, *Fusarium solani*, *Macrophomina phaseolina*, *Pythium aphanidermatum*, *Rhizoctonia solani* and *Sclerotium rolfsii*. Treatments of mungbean seeds with fungal antagonists showed more shoot and root length compared to bacterial antagonists, whereas seeds treated with bacterial antagonists showed maximum shoot and root weight. *Trichoderma harzianum* and *B. subtilis* were the best among all the biocontrol agents since they provided the most plant growth and greater reduction in root colonization by all root-infecting fungi. *B. cereus, T. virens, P. fluorescens* and *Micrococcus varians* were also effective against root-infecting fungi but to a lesser extent. The *T. harzianum, T. virens, B. subtilis* and *P. fluorescens* were also found to be the highest effective among all biocontrol agents (Ramzan et al., 2016). However, there is no research focusing on lettuce seed bio-pelleting and its defense mechanism against leaf spot infection.

Therefore, the purpose of this research will focus on (1) to examine of lettuce seed pelleting with *Bacillus subtilis 168-2* to enhance plant growth and control lettuce leaf spot disease, and (2) to investigate the induced resistance mechanism using *Bacillus subtilis 168-2* against *Alternaria alternata* after seed pelleting and foliar application in lettuce.

CHAPTER II
LITERATURE REVIEW

2.1 The importance and characteristics of lettuce

2.1.1 Importance of lettuce

Lettuce (*Lactuca sativa* Linn.) is one of the Asteraceae (Compositae) species, originally born in the Mediterranean and Asia. It is well-known for consumption and normally grown in many cold and tropical countries; there is high consumer demand throughout the year, especially, during special occasions and it is very popular among health lovers. This is because it is a kind of vegetable with high nutritional values, a source of vitamins and minerals that are essential to the human body with various types of antioxidants. It can be freshly consumed or used as an ingredient in various menus. In Thailand, there is approximately 23,437 rai of lettuce farm yielding 32,000 tons which are equivalent to 0.3 % and 0.1 % of Thailand respectively (FAO, 2013). Lettuce is considered to be one of the important economic vegetables of Thailand serving the growing trend of demand. It is the perfect crop for this backward gardening exercise because it grows well even when soil and night temperatures are in the range of 18-25 ℃ and 10-15 ℃, respectively. However, the high temperature reduces its quality and yield.

2.1.2 Characteristics of lettuce

- The root of lettuce, which is the taproot system with a strong taproot, grows quickly, especially when grown in sandy, loamy soil with sufficient moisture.

- The leaves have a bitter taste and a fast influx of flowers. The leaves break out from the surrounding trunk. The leaves color ranges from light green, and yellowish green to green. Some varieties are red or brown, semi-red, bronze, or greenish brown.

- Lettuce flowers are a bouquet called panicle, consisting of a group of flowers that are clustered at the top. Each cluster consists of 15-25 flowers or more. The inflorescence stem is about 2 feet long.

- Lettuce seeds are a single seed type (achene), which grows from one ovary. The seeds will have some seed coats. The seed shell will not crack when the seeds are dried. The seeds of lettuce are flat in length. The head of the spear is spear-shaped, with a small, long, stain along the long side of the seed on the seed coat surface. The seeds are creamy gray. The length of the seed is about 4 mm and about 1 mm wide.

The commonly grown lettuce varieties include green oak, red oak, red coral, butterhead, highland, Filey iceberg, and cos (Kusol, 2008).

2.2 Important diseases of lettuce

2.2.1 Root rot disease

The root rot disease caused by *Pythium* spp. The leave affected has a pale yellow leave starting from the midrib and spreading from the leave base to the tip. Then the leaves curled when exposed to sunlight in the daytime, or withered when dehydrated (Figure 2.1). This cause fallen leaves and dry branches, and disease spreads out, which causes perennial death. For disease epidemic and prevention, when the plant roots were weak the fungus easily destroys. This disease often spread during the summer. The prevention can keep the infected leaves out of the area and adjust the pH to 6.5-7.0 to repair the destroyed roots and give fertilizer to plants or use the Trichoderma to eliminate the pathogen and help regenerate the strong roots (Koike, 2015).

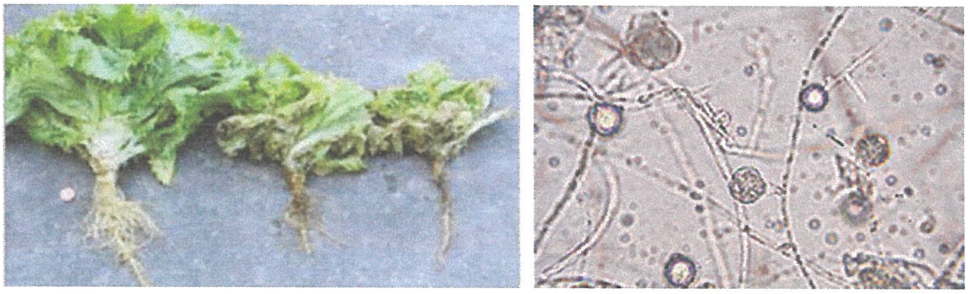


Figure 2.1 The symptom of lettuce root rot disease (left) and *Pythium* spp. (right)

2.2.2 Downy mildew disease

The downy mildew disease was caused by the *Peronospora* spp. In the leave affected by fungus, a small group of black spots occurred under the leave. There were powder light grey molds spread over the leaves. When the fungus spreads out and covers all the leave area, the leave fall or dries (Figure 2.2). For the disease epidemic and prevention, this disease can be found from the seedling stage to the mature stage, the disease causes losses of quantity (weight or volume). The prevent can trim or prune: remove the affected leaves (Koike, 2015).

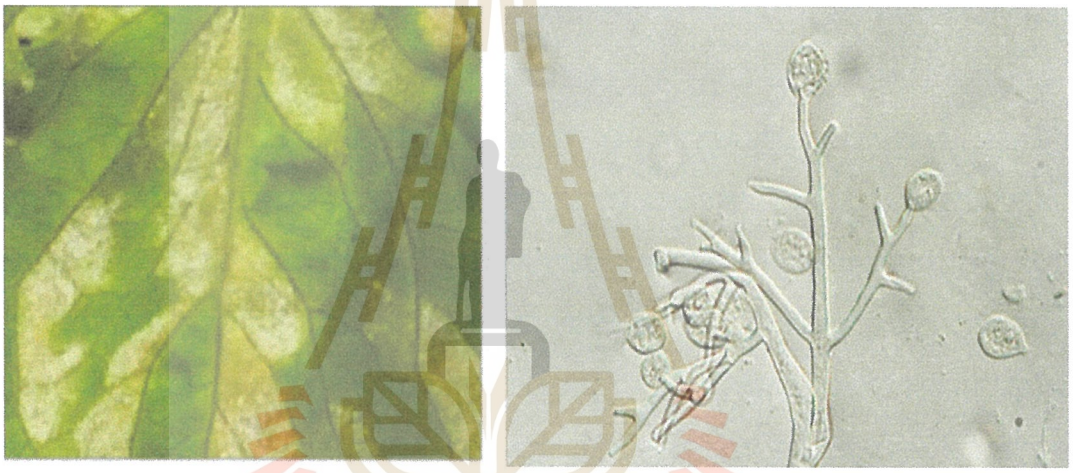


Figure 2.2 The symptom of lettuce downy mildew disease (left) and *Peronospora* spp. (right)

2.2.3 Leaf spot disease

The leaf spot disease is the increase in serious damage and diversity of pathogens. This disease is caused by *Cercospora* spp., *Septoria* spp., *Curvularia* spp. and *Alternaria* spp. These fungi can easily propagate by blowing away in the wind, sticking with insects, rainwater, or water used in spraying systems. This can cause the epidemic to spread to other areas. The spores germinate on older leaves because older leaves have weaker immune systems than the other parts. The fungus can penetrate through natural openings in the plant, or it can get in through wounds on the plants surface. Interestingly, the lettuce grown with the hydroponics system tends disease

resistance less than in the soil because the hydroponics system was genetically uniform causing plants less adaptation to the environment and easy disease infection. In addition, there are few beneficial microorganisms in hydroponic plants causing a lack of natural protection (Campbell, 1989). The leaf spot disease can occur every season in Thailand, especially in the rainy season with high humidity. However, it can occur in summer as well, mostly from excessive water spraying and poor ventilation in the planting area. During the rainy season in 2004, the spread of leaf spot disease in vegetables was found in celery, and in Butterhead, Batavia, Green Oak, and Red Oak, for foreign salad leaves. From the end of 2005 until now, it was found that the epidemic has expanded by finding symptoms of leaf spot disease on lettuce in small and medium-scale farms in Samut Prakan, Chachoengsao, Chonburi, and Rayong provinces, etc. (Khuhakan, 2011). The leaf spot disease is considered a high damage level that growers have to be alerted with the four main disease-caused fungal pathogens include *Cercospora* spp., *Septoria* spp., *Curvularia* spp., and especially *Alternaria* spp.

2.2.3.1 *Cercospora* spp.

The characteristics of the Cercospora leaf spot lesion are diverse based on the type of plant. But, the center of the wound is light gray to white or light brown to white, and around the wound is brown or reddish-brown like a frog. If serious symptoms, the wound binds together causing burns, and occurring with young leaves or distorted (Figure 2.3) (Campbell, 1989).

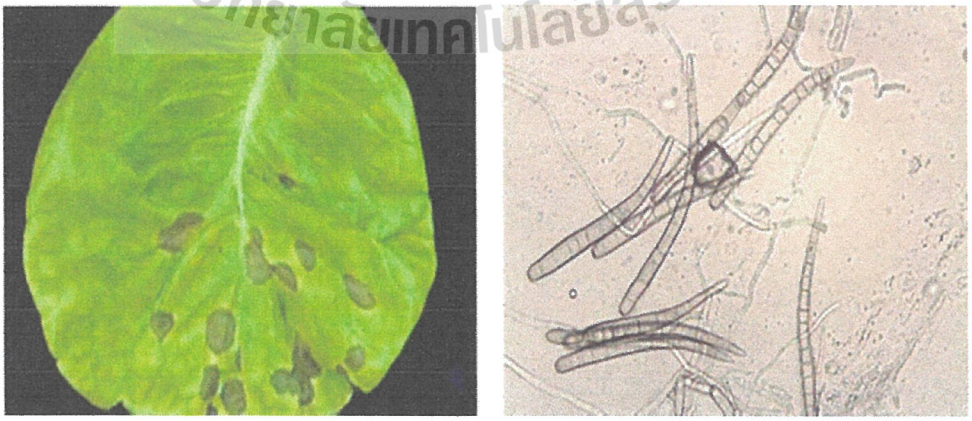


Figure 2.3 The symptom of lettuce Cercospora leaf spot disease (left) and *Cercospora* spp. (right)

2.2.3.2 *Septoria* spp.

The symptoms start from a small yellow spot and enlargement; the wound shape is unstable. The wound color is greenish olive brown, with black spots and a lot of spores. If the symptoms are very severe, the wound area tore off, causing the tattered leaves and eventually death. (Figure 2.4) (Sak, 1994).

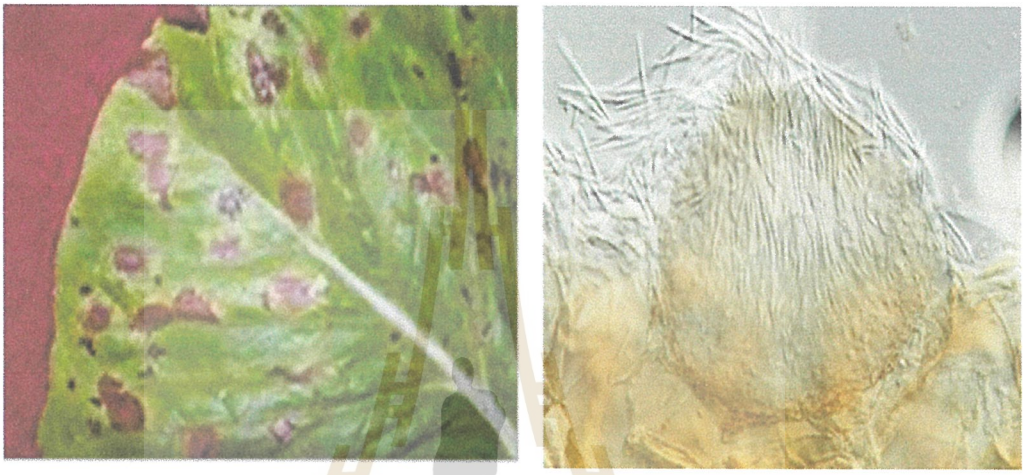


Figure 2.4 The symptom of lettuce Septoria leaf spot disease (left) and *Septoria* spp. (right)

2.2.3.3 *Curvularia* spp.

The symptoms are shown on older leaves. In the first phase, there was a small spot of needle size in light green color. Then the middle of the spot is dry with gray or light brown color, surrounded by a reddish-brown ring, which eventually, turns to burnish, and a yellow halo around the wound (Figure 2.5) (Sak, 1994).

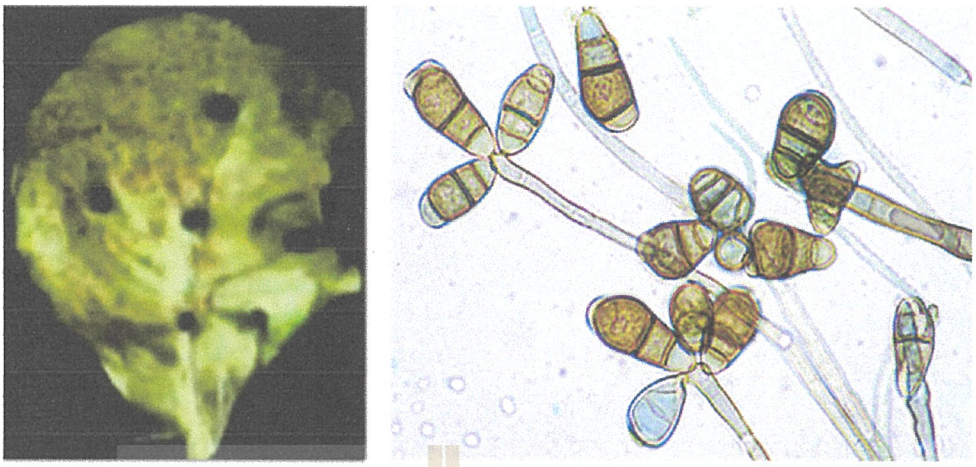


Figure 2.5 The symptom of lettuce Curvularia leaf spot (left) and *Curvularia* spp. (right)

2.2.3.4 *Alternaria* spp.

The initial symptom usually originates in old and lower leaves of the plant with small yellow spots on their leaves gradually growing up to be rather round dried brown bands, stacked together in several circles which are clearly visible at the top of the leave. The black spots which are a group of fungal spores produced for the propagation may appear in the wound area.

2.3 Alternaria leaf spot on lettuce

The Alternaria leaf spot on lettuce caused by *Alternaria alternata*. The size of the wound could be just a small spot or even to 2-3 inches in diameter on a leaf. In case of severe symptoms, the tissue between the veins would eventually die. The epidemic is caused by the spores of the fungal pathogen blown in wind, insects, animals, or water. When the spores enter the plant, the fungal pathogen germinates and grows into fibers growing inside the plant through the stomata and is consistently destroyed.

In healthy plants, it takes about 8-10 days for the disease infection, but in a susceptible plant, the pathogen takes about 4-5 days to grow up.The temperature range is 25-30 °C and has high humidity in the air, especially in the rainy season, causing the disease to spread quickly (Figure 2.6). The classification of *Alternaria* spp. can be explained as follows (Agrios, 1997):

Kingdom: Fungi
Division: Eumycota
Subdivision: Deuteromycotina
Class: Hyphomycetes
Order: Hyphales (Moniliales)
Family: Dematiaceae
Genus: *Alternaria*

Figure 2.6 Leaf spot disease (*Alternaria alternata*) (left) and the symptom of lettuce Alternaria leaf spot (right)

2.3.1 Disease cycle of *Alternaria* spp.

The *Alternaria* spp. fungal pathogen is propagated by spores or conidia, an ovoid, obclavate, or cylindrical shape. Some species have a tip extending into a beak called rostrate pale to greenish brown, some species are short or very long like filiform and the walls are smooth or rough (verruculose). The spores have long and transverse walls divided into sub-cells up to the beak. The shape of single or multiple cells connected in a chain or a catenulate on the conidiophore. The conidiophore shape unstable form or branches out into light brown to dark brown conidiogenous cells (cells that produce spores). Spores can be formed in the inner wall of the conidiogenous cell the outer wall like a balloon (enteroblasts). When spores are removed from the mother's cell, they remain marks left as a small hole in the wall. Sometimes new cells grow under the marks, ready to continuously create newborn spores from the area above the original origin (Figure 2.7) (Sak, 1994; Agrios, 1997).

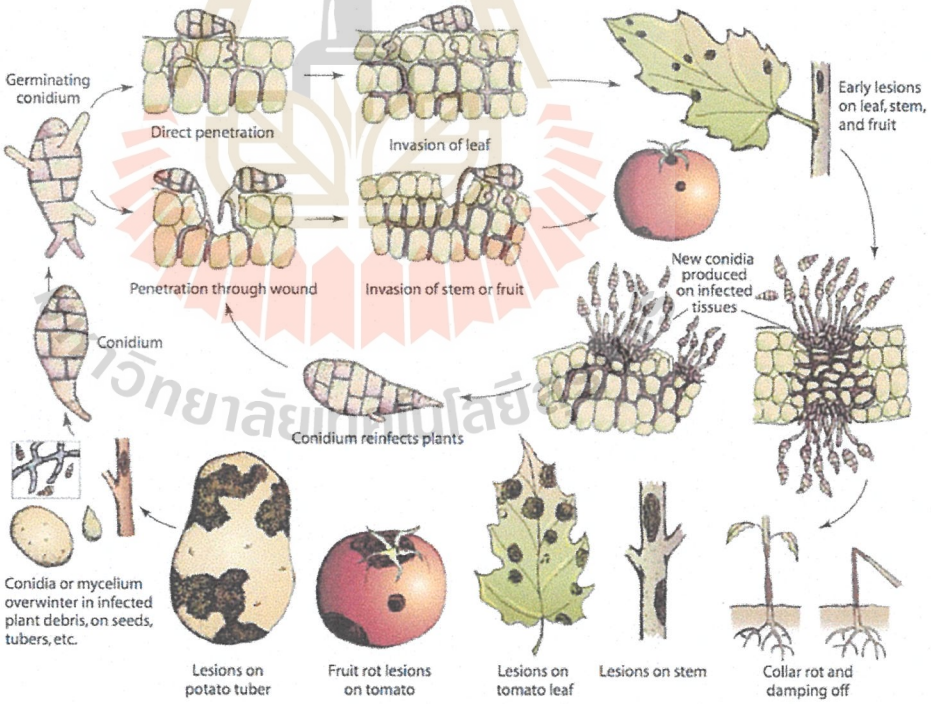


Figure 2.7 Disease cycle of *Alternaria* spp. (Agrios, 1997)

2.3.2 Alternaria disease control

Leaf spot disease was often controlled using combination methods including cultural practice, chemical methods, and biological control.

2.3.2.1 Cultural practice

It means choosing a plantation area from studying geography, choosing a plot that has never been spread and is not suitable for disease, destruction of plants and secondary habitats that are the source of infection, diseases, disease-related diseases or seed treatment, such as seed soaking, seed coating and seed pelleting, etc. (Taylor et al., 1998). The seeds used to grow are important factors that affect the quality and quantity of products potentially obtained in the future.

2.3.2.2 Chemical method

It is one of the effective methods that growers use because they potentially obtain results in the short term and it is convenient for use but it may cause side effects to the user and the product.

2.3.2.3 Biological control

This method has been used to replace the chemical methods with the principle of the biological control using microorganisms that are widely used as plant probiotics. As these beneficial microorganisms have the growth promotion ability and resistance induction, this method allows plants to build their immune system to withstand resistance. The infestation of pathogenic pathogens in which microorganisms are qualified as probiotics, such as beneficial microorganisms or substances produced by microorganisms. These microorganisms can stimulate the plant's resistance to reduce pathogens infection. This method is an alternative to replace chemicals for disease control by inducing plants to protect themselves and the ability to resist the pathogen by using microorganisms qualified as plant probiotics. This can induce the process of self-defense from the invasion of pathogens in different ways (Buensanteai et al., 2008).

2.4 Induced resistance in plant disease

Inducing resistance in plants is a stimulating process for self-prevention from various plant pathogens. It is important to limit the extent of pathogens in areas that are infected such as the reaction of HR which is the biochemical stimulation of the plant cell area destroyed by germ cells causing sudden death. The infected area was restricted to food and eventually died for inhibits the spread of infection to the neighboring cells. However, we can divide the pattern of induction or activation of plant resistance into two ways including 1) Systemic Acquired Resistance (SAR) is a characteristic that induces plants to stimulate resistance. That is a broad-spectrum resistance to pathogens throughout the whole plants, not only in the infected area. When localized necrosis lesions occur, there will be a Hypersensitive Response (HR) because the plant receptor will be aware of the danger and give signals for creating reactive oxygen species such as superoxide radicles, hydrogen peroxide and hydroxyl radicle, which are free radicals they will combine with biomolecules within the cell and fight against invasive molecules that cause HR response in the cells. Except for programmed cell death within the cell, the plant receptor will also send signals to various molecules that increase the generation and transmission of salicylic acid (SA) to stimulate the expression of pathogenesis-related protein genes (PR genes) PR-proteins, a group of proteins, that are important in the SAR process helping prevent the destruction of pathogens before their releasing into liquid within plant cells and without enzyme properties. This protein is not usually found in normal plants but only in plants after being infected by pathogens. It is used for inhibiting the infestation of plant pathogens. It produces PR-proteins in the area within plant cells, the gap between cells and plant cell walls (Sticher et al., 1997; Hammerschmidt, 1999), 2) Induced Systemic Resistance (ISR), formed by plant growth promoting rhizobacteria bacteria, such as *Pseudomonas fluorescens*, can produce elicitors stimulating plant cells to resist resistance without inducing HR reactions and plants do not produce substances. ISR-induced resistance by PGPR, the plant produces jasmonic acid to stimulate the plant to create defensins and proteinase inhibitors. The ISR process occurs when there are stimuli such as *Bacillus* sp. or *Trichoderma* sp. It causes protein metabolism changes in oxidative enzyme content and phytoalexins to against the pathogen's infection.

However, these changes occur only sometimes (Figure 2.8) (Hunt and Ryals, 1996).

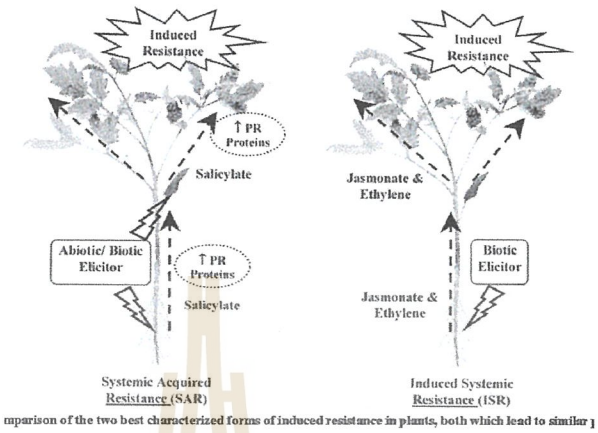


Figure2.8 A pictorial comparison of the two best characterized forms of induced resistance in plants, Systemic acquired resistance (SAR) (left) and Induced systemic resistance (ISR) (right) (Vallad and Goodman, 2004).

2.4.1 Mechanisms

Reactive oxygen species (ROS) essentially promote the oxidation of lipids, proteins, chlorophyll bleaching, and nucleic acids, which results in cell death, however plants may reduce ROS by producing a variety of antioxidants that can be split into two groups: 1) Enzymatic antioxidants such as glutathione reductase (GR), catalase, monodehydroascorbate reductase (MDHAR), ascorbate peroxidase (APX), and dehydroascorbate reductase (DHAR) (CAT). 2) Non-enzymatic antioxidants such tocopherol, ascorbate, carotenes, flavonoids, and glutathione (GSH). These antioxidants have specific stress-reduction mechanisms, such as SOD, CAT, APX, peroxidase, and glutathione reductase. Furthermore, MDHAR plays an important role in lowering the plant's superoxide and hydrogen peroxide levels and converting hydrogen peroxide to water and oxygen, among other things (Ashraf, 2009).

The ascorbic acid (AA) or vitamin C on the plants is an antioxidant, which is essential in oxidants toxicity reduction during stress receipt in the plants. The most common stress in plants is oxidative stress, which affects cells in various positions, such as DNA mutations, protein carbonylation and lipid peroxidation. Lipid is one of the

components in membrane and a biomolecule sensitive to oxidation which can be called as a reactive oxygen species (ROS) such as H_2O_2 (hydrogen peroxide) and released in a large quantity. The H_2O_2 is a ROS that causes stress from the pathogen. This compound will seriously damage various systems in plant cells accumulated over a long period of time. The plant will construct a mechanism to respond by accelerating the ROS decomposition using AA, an antioxidant that plays an important role in ROS elimination in plants. Actually, the plant has its own ROS elimination system (Asada, 1992). For example, superoxide dismutase will catalyze the sensitive O_2^- and change it to H_2O_2 and it will be eliminated by combining with AA and converting it into the water while AA will be converted into monodehydroascorbate (MDA) by the ascorbate peroxidase (APX). The MDA will be reduced by MDA reductase (MDAR) to AA in order to maintain the AA levels in cells; however, some MDA will be changed to dehydroascorbate (DHA). At this stage, DHA reductase (DHAR) will catalyze DHA back to AA again.

APX enzymes act as isoenzymes and antioxidants preventing oxidative stress caused by the formation of organic are stimulated by the disease causing the amount of active oxygen species (AOS) containing H_2O_2, O_2^- and nitric oxide in plants to be higher. This increasingly stimulates cell death resulting from the stimulation or infection of the disease. The disease pathogen will act as a signal molecule to stimulate self-defense responses at the area of the cell next to the area where the pathogen is destroyed. Consequently, the enzyme APX is responsible for controlling oxidative stress caused by H_2O_2 produced from photosynthesis in plants and the activity of superoxide dismutase (SOD) activity. This molecule is toxic by itself causing oxidations and reduction of glutathione (GSH/GSSSG), NADPH and AA. In addition, higher levels of H_2O_2 in cells stimulate cell death in response to the disease destruction. H_2O_2 at low concentrations can make plants resistant to stress. H_2O_2 plays a role in signaling molecule that increases when the plant is in a stressful state (Bor et al., 2003).

It was found that the antioxidants are the collaboration of various species of enzymes. Based on the literature review, it was found that when plants receive AA from outside, plants will increase their stress resistance. Athar and Ashraf (2008) have investigated the effects of AA on wheat under conditions of salt stress by using salt-

tolerant wheat and salinity-sensitive wheat species planted in a nutrient solution containing sodium chloride salt. It was found that salt stress affects both wheat species causing their lower growth rate and increasing the accumulation of sodium and chloride ions, but decreasing potassium ions. However, when AA was passed through the roots of both wheat varieties, it was found that giving AA at 100 mg/l in saline resistant strains damaged by stress, salt decreased and the plant had a better growth rate, higher photosynthesis rate with more potassium ions and calcium ion accumulation.

2.4.2 Elicitors for induced resistance plant disease

Controlling the pathogenesis of various plants using various types of elicitor is the response stimulator substance, by stimulating various methods of the self-defense system in plants for further signal transmission, increasing resistance from the destruction of pathogens or various stress conditions. There are two types of elicitors including abiotic elicitors or non-living elicitors such as plant hormones, heavy metal ions and biotic elicitors, substances coming from infection or the reaction between plants and diseases which can encourage plants to grow healthily and increase immunity. The use of this elicitor is an alternative choice to increase product quality, reduce diseases, used chemicals, and can be appropriately applied in the plant manufacturing system for different species effectively (Saengchan, 2014).

2.4.3 Abiotic elicitors

The abiotic elicitors are non-living compounds including chemical and physical that have a role for stimulating plants to respond to more resistance for self-protection such as plant hormones, salts of various heavy metals, ultraviolet radiation, low-temperature exposure, or various free radicals. According to Epperlein et al. (1986), phytoalexin was vary depends on the cotyledons of soybeans, peanuts, and peas, round seed pods (pea) such as peas and chickpeas. Silver nitrate is used for releasing free radicals to stimulate resistance mechanisms to enter the pattern of plant pathogens. Chen et al. (1993) reported that SA could induce resistance of SAR in plants by merging SA with O_2^- to inhibit the activity of catalase, but seizures will be accumulated H_2O_2 which is related to self-protection activities of dangerous plants. Wong et al. (2002) studied the external SA's contribution to the activation of SAR

systems by using various chemicals such as potassium cyanide, antimycin A and SA to induce viral resistance (*Turnip vein-clearing virus*, *TVCV*) in *Arabidopsis thaliana*. It was found that all 3 chemicals can induce resistance, but only SA can stimulate the occurrence of SAR system (induce the expression of PR-proteins gene).

2.4.4 Biotic elicitors

Biotic elicitors are elicitors from living organisms that are derived from part of life. The first elixir that has been identified is monacolin A, a polypeptide derived from *Monilinia fructicola*, causing the rot disease of stone fruit, which is necessary to induce phaseolin in bean pods, such as beans, bean lentils that are not plants where pathogens live. It has been reported that many species of polysaccharides can be isolated from the cell walls of fungi, such as *Phytophthora megasperma* and *Phytophthora megasperma* var. *sojae*. *Phytophthora* pathogens of soybean are used as a capacitor element as well as chitin. The role is an elixir, generating the process of producing lignin in wheat, which is a mechanism of resistance to use this type of elicitors. It can be considered as a biological control of disease by antagonistic bacteria having five mechanisms for inhibiting or controlling the pathogenesis of plant pathogens as follows: 1) competition when antagonistic bacteria can compete with plant pathogens in areas such as the more efficiency of nutrient use, air, space occupancy. This causes plant pathogens not to grow or live in an area where antagonistic bacteria live, 2) antibiosis, antagonistic bacteria selected for use in biological control of plant diseases will emphasize the most destructive properties of pathogens with this antagonist, the ability to produce substances that inhibit or destroy pathogens such as toxin or antibiotic 3) parasitism, parasite-dependent bacteria destroying other organisms which are very rare. The use of plant disease control has not been as successful as a life-threatening reaction. 4) Induced disease resistance is a mechanism produced by microorganisms such as fungi or bacteria which used to be germs. When they are brought to impair their ability of causing diseases, they can induce or stimulate plants to create resistance to the destruction of pathogens, and 5) Plant growth Promoting Rhizobacteria: PGPR, antagonistic bacteria, in addition to induced plant resistance to the destruction of pathogens, they can also promote plant growth as well. There are several types of bacteria including *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azospirillum*, and *Bacillus*.

It has been reported that PGPR can promotegrowth and productivity in many plants including oranges, mulberries (mulberry), sweet cherry plums, and raspberries. For the area, PGPR's habitat is mainly in the root system. If there is an increase in the number and gather together more roots, the plants themselves will have various responses especially, the response to certain substances released by the PGPR through the SAR system. The important substances produced from this plant related to self-defense include ethylene, jasmonic acid (JA) and salicylic acid (SA) organized in groups playing a role in stimulating growth and resistance to plant diseases (Glick et al., 1999).

Phytohormone productivity is a key mechanism that raises PGPR effectiveness for plant growth (Mayak et al., 2004). The Pseudomonas putida strain GR12-2 has been found to be able to increase the amount of ethylene in green beans by a process involving a bacterial species that can create Indole-3 acetic acid (IAA), which stimulates the creation of ethylene in order to restrict root growth. Overall, the deposition of PGPR on the developing roots or seed surface can minimize the amount of ethylene in plants. The IAA is then created using the amino acid tryptophan, which is taken from the seeds or exudates of the root. The long-term growth of the plants is promoted if PGPR produces the right quantity of IAA, and certain plants will also add ACC (1-aminocyclopropane-1-carboxylic acid), which is a precursor for the production of ethylene. As a result of PGPR using ACC as a source for nitrogen, less ethylene will be produced. Normally, JA induces the production of ethylene and the genes responsible for protecting plants from plant diseases, such as defensins, thionios, and proteinase inhibitors (Buensanteai, 2012).

Pilet and Saugy (1985) have studied the concentration of IAA with corn root length. It was found that the corn root length significantly correlated with the quantity of IAA used for testing.

Loper and Schroth (1986) have studied microorganisms that can produce IAA for promoting sugar beet root length, dried weight of plants, upper part above soil surface, and root system of sugar beet seedlings. The mentioned microorganisms are *Pseudomonas syringae* pv. *savastanoi,* and *Bacillus* sp. showed the ability for enhancing plant growth. Many species were found in the field condition of urban agriculturists in Berkele, California United States of America.

Inthasan et al. (2017) studied the effect of bacteria-producing indole-3-acetic acid (IAA) on the growth and nutrient content of chili (*Capsium annuum* L.). Fifty-seven isolates were found and only fifteen isolates could produce indole-3-acetic acid (IAA), with the range of IAA production at 20-126 µg/ml. The result showed that isolate 1 (*Brevibacillus agri*) provided the highest IAA value at 126 µg/ml. Compared to other treatments, isolate 1 significantly increased the number of leaves, shoot height, root length, fresh weight, and dry weight of the shoot and root portions. The maximum nitrogen, phosphorus, and potassium uptake in the shoot (3.68%N, 0.30%P, and 3.65%K) and root (2.08%N, 0.15%P, and 2.49%K) were also attributed to bacteria isolate 1. In contrast to bacteria producing low levels of IAA, those producing high levels of IAA had an impact on all growth and physiological measures.

2.4.4.1 ***Bacillus subtilis***

B. subtilis is classified into the PGPR group and helps induce plant resistance to the destruction of plant pathogens as shown in Table 2.1. Bacterium in the *Bacillaceae* family, rod shape saprophytes or almost straight with a size of 0.3-2.2 x 1.2-7.0 micrometers. It is an aerobe or facultative anaerobe with endospore. That is a gram-positive bacterium with peritrichous flagella and can be naturally found in both soil, water, air, and various parts of plants, such as leaves, stems, and areas around the plant roots. It can grow well for approximately 24-48 hours at the normal temperature with a pH range of 5.5-8.5 and can produce the hydrolytic enzyme to break down polysaccharides, nucleic acid, and lipids. It also produces approximately 168 kinds of peptide-type antibiotics inhibiting or destroying plant pathogens with no effect or pathological conditions on humans, animals, or plants as these bacteria can be isolated and fed on culture media. There are many studies investigating about the use of *B. subtilis*, especially in the control of disease by biological methods and antagonistic to various plant pathogens which are important in applying in the agriculture to reduce chemical usage in plant production. Brian and Deborah (2002) studied mutations using mutagenic to acquire *B. subtilis* M04 to create inhibitors against *Botrytis cinerea* (gray mold), *Ralstonia solanacearum* (bacterial wilt), and *Erwinia carotovora* pv. *carotovora* (bacterial soft rot) and found antibiotics when testing them with chemicals. The properties of antibiotics found that extracellular substances can be heat resistant and

dissolved in methanol. This substance absorbs the light wavelength of 212 nm, which has properties similar to the cyclic antibiotic lipopeptides such as Iturin A2. It was found that *B. subtilis* strain BacB isolated from the stem of sugar beet at the density of 1×10^6 cfu/ml or a higher density was significantly reduced sugar beet leaf spot disease (*C. beticola*) in the plot condition when compared with control (Collins and Jacobsen, 2003).

Okigbo and Osuine (2003) isolated *Bacillus* sp. from soil samples under the mango tree by soil dilution spread plate method and selected effective strains to control fungal pathogens caused mangoes leaf spot disease including (*Pestalotiopsis mangiferae*, *Botryodiplodia theobromae* and *Maerophoma mangiferae* by dual culture method until five days. The results showed that *B. subtilis* strain NCIB 3610 could inhibit the growth of all three types of fungal mycelium by 57, 61, and 58%, respectively.

Karakurt et al. (2009) performed determine the effects of 4 PGPR strains including *B. subtilis* OSU - 142, *Bacillus megaterium* M - 3, *Burkholderia cepacia* OSU – 7, *Pseudomonas putida* BA - 8 and their combinations on fruit setting, pomological and chemical characteristics as well as vegetative growth of sour cherry trees (*Prunus cerasus* L. cv. *Kütahya*). The tested bacterial strains or some of their combinations have a great potential to increase, especially fruit setting and plant vegetative growth, and indirectly affect fruit pomological and chemical characteristics. They are considered biofertilizers for fruits, vegetables, and ornamental plant production in sustainable and ecological agricultural systems.

Liang et al. (2011) studied the ability of *B. megaterium* strain L8 a PGPR for induced resistance cucumbers against soil rot disease caused by *Pythium aphanidermatum*. It was found that cucumber treated with *B. megaterium* L8 could significantly reduce damping-off disease by 83.45% at 28 days after planting compared with control was 31.68%.

Mohamed and Gomaa (2012) investigated the potential role of plant growth promoting bacteria (PGPB) in alleviating soil salinity stress during plant growth under greenhouse conditions. It was found that the increasing salinity in soil lead to decrease plant growth, photosynthetic pigments content, phytohormones contents including indole-3-acetic acid (IAA), gibberellic acid (GA3) and mineral uptake compared to soil

without salinity. Seeds of radish inoculated with *Bacillus subtilis* and *Pseudomonas fluorescens* cause significantly an increase in fresh and dry masses of roots and leaves, photosynthetic pigments, proline, total free amino acids and crude protein contents compared to noninoculated ones under salinity. The bacteria also increased phytohormones contents (IAA and GA3) and the contents of N, P, K+, Ca^{2+}, and Mg^{2+} but decreased abscisic acid (ABA) contents and Na^{+} and Cl^{-} content which may contribute in part to activation of processes involved in the alleviation of the effect of salt.

Srimai and Akarapisarn (2014) also showed that *B. subtilis* strain LBF02 can control *Cercospora* spp. causing leaf spots in vegetable salads.

Abeer et al. (2015) studied the role of a salt-tolerant PGPR, B. subtilis, in the alleviation of salinity stress during the growth of Indian Bassia (*Bassia indica* [Wight] A.J. Scott), under controlled growth chamber conditions regarding seed inoculation, physiological parameters. It was discovered that salt adversely affected physiological parameters and decreased shoot and root length by 16 and 42%, and dry weight by 37 and 23%, respectively. In comparison to uninoculated plants, the B. subtilis inoculation of unstressed and salt-stressed Indian Bassia significantly improved root and shoot growth, total lipid content, the phospholipid fraction, photosynthetic pigments (chlorophyll a and b, and carotenoid contents), and also increased oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids in plant leaves compared to uninoculated plants.The salt-tolerant PGPR, *B. subtilis*, may work in concert with other auxin (IAA) sources to increase the growth and fitness of Indian bassia plants under salt stress and to improve salt stress resistance by lowering stress ethylene levels.

Table 2.1 List of *B. subtilis* induced plant resistance to the destruction of phytopathogens.

Bacteria	Disease – control efficiency / Stimulates plant growth	Reference
B. subtilis KS03	Rice nose attack disease	Cho et al., 2003
B. subtilis RB14	Tomato root rot disease	Asaka and Shoda, 1996
B. subtilis RB14-C	Tomato root rot disease	Asaka and Shoda, 1996
B. subtilis NSRS 89-24	Dry leaf sheath disease	Leelasuphakul et al., 2006
B. subtilis CaSUT007	Cassava leaf blight disease	Buensanteai et al., 2008
	Cucumber root rot disease	
	Tomato root rot disease	
	Chili anthracnose	

2.5 Application of *Bacillus subtilis* for plant disease control

B. subtilis can be used both in the form of cells and endospores to prevent and control plant diseases directly. *Bacillus* has been developed to promote plant growth as well as raise seed quality and stimulate the immune system in plants to be able to use in many forms as follows:

- Foliar spraying

Arananarod et al. (2008) was studied controlling rice disease by the use of *B. subtilis* No.33 at concentration of 1.6 x 10^8cfu/ml spraying rice plants together with plant disease prevention agents carbendazim + epoxiconazole 25% SC or propiconazole 25% EC or propiconazole + difenoconazole 30% EC at the rate of the active ingredient half of the recommended rate or spraying only *B. subtilis* No.33 (1.6 x 10^9cfu/ml). In the phase of rice, close to grains and rice fields, 5%, it is effective to control seed disease and increase rice yield, as well as the use of plant protection substances carbendazim + epoxiconazole 25% SC or propiconazole 25% EC or propiconazole + difenoconazole 30% EC at the rate of active ingredient rate recommended and better than control method at a statistical significance.

Chantarat et al. (2009) prepared the bacterial bioavailability of *B. amyloliquefaciens* DGg13 by preparing two formulas of biochemistry. The first formula used 2 ml of suspended solids mixed with 200 g soil powder, incubated at room

temperature for 48 hours. The second formula used 700 ml suspended cell, mixed with 1 kg of talcum powder, mixed and crushed. After storing for six months, it was found that in the formula that was mixed with soil powder, the number of bacteria remained constant at $2.5x10^{11}$ cfu/ml, close to that of the first month ($4x10^{11}$ cfu/g). It was different from the formula using talcum powder in which the bacteria decreased according to the storage period. In the 6^{th} month, the number of bacteria reduced to $2x10^{8}$ cfu/ml from the original $6.3x10^{12}$ cfu/ml and found that when using both formulas for chili, the disease could be reduced from *Colletotrichum* spp. to 86.97 and 84.99 %, respectively but the biopharmaceuticals both formulated when mixed with water and then sprayed on plants, there is a problem of clogging the nozzle resulting white stains on chilis and their leaves.

- Seed treatment

Seed treatment such as seed soaking, seed coating and seed pelleting is a method to improve seed quality to be of better quality than the original such as changes physical, physiology of seeds, etc. for use in cultivation (Taylor et al., 1998; Kaewkham, 2017).

- Seed soaking

Seed soaking involves immersing seeds in a solution with low water potentials, such as polyethylene glycol, to allow them to absorb enough moisture to start the germination process but not enough to root out so that each seed develops germination at the same or similar level (Heydecker and Coolbear, 1977; and McDonald, 2000).

- Seed coating

Seed coating is the buildup of the substance in a thin, homogeneous layer that is tightly adhered to the seed and does not fall off, preserving the seed's shape (Tongpamnuk, 1997). The tool and procedures have been developed in the pharmaceutical coating industry using polymers that are tough and have ingredients of various active inner that are well-known in the seed business. The substances used for coating must not deform the seeds and are often used with directly affected seeds (Copeland and McDonald, 1995). It is also utilized in conjunction with plant hormones (Greipsson, 2001). The type of material depends on the coating's intended use.

- Seed pelleting

Seed pelleting is to make the seed being wrapped in various materials to change the seed structures to have a more appropriate shape than ever and also to increase the weight of the seed (Zenk, 2004). The use of components having properties of more volume, toughness and higher concentration will result in acquiring the round seeds. In addition, the particles coated on the seed surface also help increase the efficiency of water and air suction as well (Tongpamnuk, 1997). Seed pelleting is the use of knowledge in science, engineering, physics, chemistry, art and agriculture (Kubik, 2002). The seed pelleting used will look like a large dish like a pan for molding granular fertilizer to gather with the use of mortar tank developed as a seed forming tank as a pelleting (Hill, 1999). Adhesive materials that are suitable for the pelleting and growth of plant seeds have to be studied. Therefore, pelleting the seed thereby enhances the work of agricultural machinery to be easy, convenient and fast for crop cultivation (Smith and Miller, 1987; Hill, 1999). The studies have been developed in different ways of seed pelleting to determine the suitability of each type of plant seeds. The successful seed pelleting process depends on the filler and the adhesive materials. The filler materials must be appropriate because the pelleted material is the important factor to change the shape, size, and weight of seeds. Thus, good pelleting materials should have the ability to mold the seed pelleting easily with a uniform particle size, not interfering with the permeation process of water, oxygen. Importantly, it must not be toxic or adversely affects the quality and seed germination (Hill, 1999). In general, the pelleted materials popularly used to wrap seeds including talcum, calcium carbonate, vermiculite, pumice, gypsum, bentonite, dolomite and zeolite etc. (Taylor and Harman, 1990). The active ingredients were chosen based on the purpose of the pelleted materials. The well-known active ingredients include fungicides, insecticides, nutrients, hormones, growth inhibitors and microorganisms that promote plant growth which are often used in association with sticky polymers used as a binder for the attachment of active ingredients with the seed very well (Sikhao, 2016).

2.5.1 Pelleting materials

2.5.1.1 Adhesive material

The materials used for coating should be flawless since the type of adhesive utilized determines the physical integrity of the coating, which has a significant impact on how the pellets are handled, transported, and planted. Numbers of materials have been used as binders and fillers. The binders (adhesives) used for pelleting include gum arabic, gelatin, starch, methylcellulose, polyvinyl alcohols, polyvinyl acetate, polyoxyethylene glycol based waxes and carboxymethyl cellulose, methyl ethyl cellulose, plastic resins and some of the cheap and low-cost binding materials are rice gruel, maida gruel and starch gruel. The concentration recommended for methylcellulose is 3% w/v, methyl ethyl cellulose is 5% w/v, gum arabic is 45% and nitric coat is 4.3% w/v solution in water. The low-cost adhesives such as rice gruel, maida gruel etc. are used at the concentration of 5% or 10% depending upon filler material used for pelleting. The selection of adhesive is also based on selective purposes. Plastic resins, polyvinyl acetate and insoluble poly electrolyte complexes are used to bind pesticides to seed, polyethylene oxide are to prevent erosion of surface sown seeds, polyurethane to bind lime in a way that resists coat abrasion, blends of polyvinyl alcohol and polyvinyl acetate to bind vermiculite and poly electrolytes or dextran to aggregate soil around the seeds thereby improving the aeration of sown seeds (Krishnasamy, 2005; Veena, 2007).

2.5.1.2 Filler material and active ingredient

The commonly used filler for pelleting is calcium carbonate, calcium sulfate, limestone, gypsum, activated clay, dolomite, talc, vermiculite, diatomaceous earth, montmorillonite, kaolin clay, and bentonite. Different active ingredient biofertilizers viz., rhizobium, phosphobacteria, *Azospirillum*, *Azatobacter* and *Bacillus* sp. are used as filler materials, which are fixed to the seed with the help of an adhesive. The common botanicals used for pelleting are arappu (*Albizia amara*), pungam (*Pongamia pinnata*), notchi (*Vitex negundo*), prosopis (*Prosopis juliflora*) and neem (*Azadirachta indica*). These leaf powders contain an auxin-like substances, which regulate the growth and initial establishment (Krishnasamy, 2005; Veena, 2007).

The research of Evlakova (1985) on cotton seed pelleting (*Gossypium hirsutum* L.) using vermiculite as a pelleting material, resulted in a 24.50% increase in germination compared to the seed not passing the pelleting.

Veena (2007) studied the pelleting techniques in onion and revealed that polyvinyl acetate (60 ml/kg of seed) is the best binding material compared to gum arabic and sawdust (10 g/kg of seed) alone is the most suitable filler material compared to clay and bentonite as these caused deleterious effects on seed quality.

Kangsopa and Siri (2013) introduced a tobacco seed pelleting with 150 grams of pumice and binder: Hydroxypropyl methylcellulose, 4% by weight per 3 grams of tobacco seeds. In addition, Kangsopa and Siri (2014) reported the quality and shelf life of tobacco seeds after pelleting seeds together with anti-fungal substances, it was found that when the seeds were stored for four months, the pelleted tobacco seed that was added together with anti-fungus had the decrease in germination from 30 to 50%. Additionally, the quality of seed paste combined with metalaxyl, captan, and pyraclostrobin. It is able to maintain its quality for about 2-3 months.

Kangsopa and Siri (2014) reported that when anti-fungal agents are used at too high concentrations will reduce the amount of protein within the seed but will increase the amount of free fatty acids which has an effect on activation of lipoxygenase enzymes and increases lipid peroxidation, causing enzymes to be inhibited for protein degradation resulting in the suspension of the cell membrane extension causing damage to the seeds viability and the physiology of tobacco seedlings.

According to Trachoo and Siri (2015), the tobacco seed pelleting with talcum together with pumice makes the pelleted seed form easy and makes the highest yield of seed germination.

Moreover, Trachoo et al. (2016) also performed pelleting tobacco seed with 150 grams of pumice and 40 ml of 4% hydroxypropyl methylcellulose (HPMC) as adhesive materials. The seeds were pelleted with 2 types of plant nutrients included magnesium sulfate ($MgSO_4$) and potassium chloride (KCl). The concentration of the nutrients was divided into 4 levels including 0.5, 1, 2 and 3 grams/seed with 3 grams of tobacco seed by using SKK11 machine. After that, the seed quality was inspected and found that pelleted tobacco seed with 1 gram of KCl can accelerate seed germination by 96% and

12.84 plants/day respectively. When inspected in the laboratory condition, the seed germination was 86% and 9.97 plants/day respectively while inspected in the greenhouse condition. It was significantly different when compared with the unpelleted seeds. According to the comparison between the tobacco seeds that are pelleted with both types of plant nutrients. There was no statistical difference when checking the quality in the laboratory condition but when examining it in the laboratory condition, there was a statistical difference. In addition, it was found that 0.5 grams of KCl pelleted tobacco seed is the strongest seed when examined by means of accelerated seed germination.

Cipriano et al. (2016) was studied the use of 12 strains of affecting dissolving phosphate and producing hormones that are important for promoting the growth of lettuce.

Olivera et al. (2016) reported that coating seed wheat together with arbuscular mycorrhiza can increase potassium concentrations (K), sulfur (S) and zinc (Zn) in the soil. In addition, it can also control root rot disease as well as promote plant growth such as mungbean cultivation (Ramzan et al., 2016).

Kangsopa and Siri (2018) assessed seed treatment with microorganisms promoting plant growth in lettuce seeds and tracked germination, seedling growth and cultivation in the hydroponics system by experimenting at the Agriculture and Agri-Food Canada (AAFC) in Canada. The experiments were divided into 2 groups namely, Experiment 1, selecting 2 types of microorganisms: *Pseudomonas fluoresces* 31-12 and *Bacillus subtilis*, each of which typically used to make seed treatment consisting of methods: seed soaking, seed coating, and seed pelleting. Then the germination and the growth of seedlings were investigated. According to the results of laboratory tests, it was found that seed germination was not statistical difference. Seed coating and seed pelleting together with *P. fluorescens* 31-12 had the best root length equal to 41.16 and 41.23 mm, respectively and seed pelleting with *B. subtilis* had the best root length of 124.26 mm. In terms of the investigation in the greenhouse, it was found that coating and pelleting seeds with *P. fluorescens* 31-12 had the best initial length (60.10 and 57.85 mm, respectively) and the best fresh stem weight (747.72 and 743.06 mg, respectively). The seed coating with *P. fluorescens* 31-12 had the best dry stem weight

was 28.83 milligrams and was statistical difference when compared to untreated seed. The second experiment selected the coating method and seed coating together with *P. fluorescens* 31-12. to cultivate in the system of leaf fresh weight, root fresh weight, dry leaf weight, and dry root weight and there were significantly different when compared to untreated seeds.

2.6 Application of Fourier Transform Infrared (FTIR) microspectroscopy for detecting biochemical components associated with induced resistance against plant disease

Fourier transform infrared (FTIR) microspectroscopy creates molecular microscopic pictures. Infrared light absorption is detected within a microscopic tissue segment using FTIR microspectroscopy, and the infrared absorption spectrum is calculated pixel-by-pixel (Saarakkala et al., 2010; Le Thanh, 2017). Synchrotron based Fourier Transform Infrared (SR-FTIR) microspectroscopy has been developed as a rapid, direct, non-destructive and bio analytical technique (Yu, 2004; Thumanu et al., 2015). It can analyze samples at the micron level or micro-sample areas. Advantages of synchrotron light brightness than a conventional global source and has a small effective source size, and is capable of exploring the molecular chemistry within the microstructures of samples with a high signal-to-noise ratio (Yu, 2004; Kastyak-Ibrahim et al., 2012; Wang et al., 2015). In order to comprehend the interactions between plant microorganisms and plant diseases, SR-FTIR microspectroscopy is a novel technique for examining plant cell walls. It is discriminating enough to enable the identification of functional groupings of plant cellular components (Kenneth and Lawrence, 2005; Yu, 2008; Thumanu et al., 2015; Thumanu et al., 2017). Few reports have been published on the application of FTIR in plants. McCann et al. (1992) demonstrated that FTIR spectroscopy can detect large conformational changes in pectic polymers on removal from the cell wall and on drying of both single onion (*Allium cepa*) cell walls. FTIR spectroscopy provides a powerful and rapid assay for wall components and putative cross-links by identifying polymers and functional groups. Yu (2008) used synchrotron light sourced FTIR microspectroscopy to characterize the protein molecular structure of plant tissue. The cluster analysis and principal component analysis did not show

clear differences between the yellow and brown canola seed tissues in terms of protein amide I structures, indicating that they are related to each other. Both yellow and brown canola seeds contain the same proteins but in different ratios. Thumanu et al. (2017) have investigated the resistance mechanisms of chili induced by the *Bacillus subtilis* strain D604 using SR-FTIR. The result revealed that the changes of the biochemical component in the cell were observed in the C=O ester from lipids, lignin, or pectin (1770-1700 cm^{-1}) as well as polysaccharides (1200-900 cm^{-1}) in the treated samples of D604 and distilled water and then challenge inoculation with chili anthracnose pathogen, *C. acutatum.*

CHAPTER III

MATERIALS AND METHODS

3.1 The fungal pathogen isolation and pathogenicity test in lettuce

3.1.1 Sample collection and isolation of *A. alternata*

Lettuce leaves 3 samples from Suranaree university of technology Farm show symptoms and signs of leaf spot disease and were collected to isolate the pathogens by tissue transplanting method in water agar (WA). Lettuce leaf spot samples were washed thoroughly under tap water and allowed to dry under laminar flow. After that, the diseased area leaves were separated with healthy tissue. Then, the samples were sterilized with 10% Clorox solution for 1-2 minutes depending on the tissue size. The excess traces of Clorox on the tissue was removed by washing 2 times in sterile distilled water and then transferred to WA medium. The Petri dishes were subsequently incubated at room temperature (25 °C) for 2 days. The pure fungal colonies developed from the infected tissues were isolated single spores and transferred onto potato dextrose agar medium (PDA) (Phialathounheuane et al., 2012).

3.1.2 Pathogenicity and virulence test of *A. alternata*

Pathogenicity and virulence tests were conducted to detect the highest virulence isolate causing leaf spot disease on lettuce by Koch's postulation technique. Healthy leaves of lettuce were surface sterilized in 10% Clorox solution and rinsed in 3 changes of running tap water and allowed to dry. Single spore suspensions of representative fungal isolates were counted by using the Haemacytometer and adjusted to a concentration of 10^7-10^8 spores/ml and inoculated by spraying on healthy lettuce leaves (Sompong et al., 2012). In another set, instead of using a single spore suspension, sterile distilled water was sprayed to serve as a negative control. The inoculated lettuce leaves were kept in moist chamber under room temperature conditions, and observation of the disease on the leaves was recorded by evaluating the severity and virulence of the disease with a score of 1-5 (modified by Wokocha et al., 2010) as follows 1 = No symptoms

2 = The virulence is found less than 25 % of the leaf area

3 = The virulence is found 26-50 % of the leaf area

4 = The virulence is found 51-75 % of the leaf area

5 = The virulence is found more than 75 % of the leaf area

The disease severity of leaf spot was calculated by the following formula:

Disease severity (%) = Sum of numerical disease scoring/Total plant numbers × The highest disease scoring X 100

The highest virulence isolate was selected for further study. After that, the highest virulence isolate was cultured on potato dextrose broth (PDB) and shaken at 120 rpm at room temperature for 4 days. The single spore suspended solids was filtered into a microcentrifuge tube as 1 ml/tube, then centrifuge at 8,000 rpm for 10 minutes, pour the pellucid part and add PDB culture medium containing 60% glycerol at a ratio of 1:1. Then dissolve the spore with a vortex mixer and store at -80 °C (Gonzalesel et al., 2006).

3.2 Lettuce seeds pelleted using *Bacillus subtilis* 168-2

3.2.1 Preparation of *Bacillus subtilis* 168-2

Bacillus subtilis 168-2 was derived from the storage stock of Plant Pathology & Biopesticide Laboratory, Suranaree University of Technology. The storage stock was cultured in 150 ml molasses diammonium phosphate and yeast extract (MDY) medium in a shaking machine at 150 rpm, 30 °C for 48 hours. The *B. subtilis* 168-2 was obtained with the amount of bacterial cell 10^8 cfu/ml at OD_{600nm} = 0.2 by spectrophotometer (Nikaji, 2016; Kangsopa and Siri, 2018).

3.2.2 Preparation of lettuce seeds pelleted

Lettuce seed from organic vegetable community enterprise, sufficiency economy settlement Wang Nam Khiao District was pelleted with *B. subtilis* 168-2 adjusted to a concentration of 10^5, 10^8 and 10^9 cfu/ml mixed with 250 grams of calcium sulfate and 0.3 % of carboxymethyl cellulose (CMC) as a binder by pelleting method (Buakaew and Siri, 2016). The experiment was carried out in a completely randomized design (CRD), 5

treatments, 4 replications with 10 g of lettuce seed per replication (Table 3.1). The lettuce seeds were pelleted in a rotary drum (Buakaew and Siri, 2016), then remove the pelleted seeds to dehumidified using a dehumidifier (humidified content of seeds not more than 7 %). After that, the sample was examined for seed quality such as weight, friability, dissolved seed pelleted, and survival of *B. subtilis* 168-2 was examined 6 months after pelleting and shelf life.

Table 3.1 List of treatments in preparing lettuce seeds pelleting

Treatments	Applications		
	Active ingredient	Filler materials (Calcium sulfate)	Adhesive materials (CMC)
SeedBio 1	Unpelleted (control)	-	-
SeedBio 2	*B. subtilis* 168-2 at 10^5 cfu/ml.	250 g	0.3 %
SeedBio 3	*B. subtilis* 168-2 at 10^8 cfu/ml.	250 g	0.3 %
SeedBio 4	*B. subtilis* 168-2 at 10^9 cfu/ml.	250 g	0.3 %
SeedBio 5	Commercial *B. subtilis* (positive control)	250 g	0.3 %

3.2.3 Pelleted seed quality

The lettuce seed pelleted of each treatment was randomized to test their quality. The quality of pelleting seeds was examined in 4 properties as follows:

1) Forming of seed pelleted during the duration of pelleting seeds, the adhesion of pelleted materials and completely and easily covering the seed in a regular round shape was observed. The criteria of assessment are 1-5. In forming seed pelleted, the scores are determined as follows: 1 = very difficult, 2 = difficult, 3 = fair, 4 = easy, and 5 = very easy (Buakaew and Siri, 2016).

2) Weight of 1,000 seeds pelleted was randomized for 4 replications and weighed to find the weight of pelleted seeds. The weight of 1,000 lettuce seeds without pelleting is 0.81 g (Buakaew and Siri, 2016).

3) Friability of seed pelleted was conducted by selected randomly 100 seeds pelleted for 4 replications and weighing them before testing by taking into Tablet Friability Tester at 25 rpm for 4 minutes (100 rounds) and then weighing the weight left after testing. After that, calculating the value of friability (adapted from Yanisa et al., 2013)

$$\% \text{ friability} = (\text{weight loss/before testing weight}) \times 10$$

4) Dissolution of pelleted seed adapted from Anderson et al. (1969) method. Random sampling 10 seeds, 4 replications, soak in 10 ml of water by soaking each seed one by one then measure the filler materials dissolution time.

3.2.4 The germination of pelleted lettuce seeds

The germination test in the laboratory was examined by randomly sampling 5 treatments with 4 replications, 100 seeds/replication. The test seeds were germinated by the Top of Paper (TP) method and kept in the germination growth chamber at temperature 20 °C. The first count was observed at 4 days and the final count was examined at 7 days, the results were expressed in percentage of normal seed and abnormal seed, hard seed, fresh seed and dead seed by evaluating the germination test and germination speed (Maguire, 1962; ISTA, 2019). The seed germination percentage was calculated using the following formula (ISTA, 2019):

$$\text{Germination \%} = (\text{Number of germinated seeds/Total number of seeds}) \times 100$$

The speed of germination was calculated by the following formula given by Czabator (1962):

$$\text{Speed of germination} = n1/d1+n2/d2+n3/d3$$

Where n = Number of germinated seeds

d = Number of days

3.2.5 Data analysis

The data of pelleting together with *B. subtilis* 168-2 were analyzed according to a Completely Randomized Design (CRD). The seed germination percentage was transformed for statistical analysis using Arcsine transformation and the mean of pelleting was compared by Duncan's New Multiple Range Test (DMRT).

3.3 Efficiency of seed pelleting and spraying with *B. subtilis* 168-2 to control leaf spot disease on lettuce under greenhouse conditions

Selected four treatments of pelleting seed by *B. subtilis* 168-2 of different concentration with the best germination rates were from experiment 3.2.4 and spray every 7 days (4 times) with *B. subtilis* 168-2, Commercial *B. subtilis*, Mancozeb and water were investigated the enhance plant growth and control the leaf spot disease in lettuce under the greenhouse condition, compared with control treatments as spraying lettuce with water and seeds that does not pelleting. The lettuce seeds of each treatment were cultivated into a 25 cm diameter pot using SUT soil and planting distance between seedlings 30 × 30 cm. The experiment was carried out in CRD with 10 treatments, 4 replications, with 10 plants per replication (Table 3.2). The plants were inoculated with *Alternaria* spp. at 29 DAT by spraying suspension at 10^8 spore/ ml. Then, the lettuce plant was kept in a greenhouse with high humidity conditions by covering plastic.

Table 3.2 List of treatments on assessing the efficacy of bio-pelleting and foliar spray using *B. subtilis* 168-2 under greenhouse condition.

Treatments	Applications[2/]	Foliar spraying at 7, 14, 21 and 28 DAT[1/]
T1	Unpelleted (control)	Water
T2	Pelleted with calcium sulfate 250 g and *B. subtilis* 168-2 at 10^5 cfu/ml	*B. subtilis* 168-2 at 10^5 cfu/ml
T3	Pelleted with calcium sulfate 250 g and *B. subtilis* 168-2 at 10^5 cfu/ml	Water
T4	Pelleted with calcium sulfate 250 g and *B. subtilis* 168-2 at 10^5 cfu/ml	Mancozeb
T5	Pelleted with calcium sulfate 250 g and *B. subtilis* 168-2 at 10^8 cfu/ml	*B. subtilis* 168-2 at 10^8 cfu/ml
T6	Pelleted with calcium sulfate 250 g and *B. subtilis* 168-2 at 10^8 cfu/ml	Water
T7	Pelleted with calcium sulfate 250 g and *B. subtilis* 168-2 at 10^8 cfu/ml	Mancozeb
T8	Commercial *B. subtilis* 10^8 cfu/ml pelleted seeds (Positive control)	Commercial *B. subtilis* 10^8 cfu/ml
T9	Commercial *B. subtilis* 10^8 cfu/ml pelleted seeds	Water
T10	Commercial *B. subtilis*10^8 cfu/ml pelleted seeds (Positive control)	Mancozeb

[1/] DAT: Days after transplanting

[2/] SeedBio2= Pelleted with calcium sulfate 250 g and *B. subtilis* 168-2 at 10^5 cfu/ml, SeedBio3= Pelleted with calcium sulfate 250 g and *B. subtilis* 168-2 at 10^8 cfu/ml, SeedBio5= Commercial *B. subtilis* 10^8 cfu/ml pelleted seeds

The disease severity of leaf spot was observed every day until 1 week by evaluating the disease score as experiment 3.1.2. Then the disease reduction was calculated based on the disease severity. The disease reduction was calculated by formula:

Disease reduction (%) = [(Disease severity of T1 – Disease severity of the others treatment)/ Disease severity of T1] x 100

The plant growth parameters including plant height, fresh weight, dry weight and canopy width were recorded 45 days after planting (Bradford, 1986; Heydecker and Coolbear, 1997; Bewley et al, 2013). Selected 5 treatments to investigate the mechanism of lettuce against leaf spot disease for salicylic acid analysis, superoxide dismutase analysis and plant growth regulating chemicals was Indole-3-acetic acid analysis. After that, selected 3 treatments from 5 treatments to investigate biochemical changes in lettuce using the synchrotron FT-IR microspectroscopy technique. The data of pelleting and spraying with *B. subtilis* 168-2 were analyzed according to a Completely Randomized Design (CRD).

3.4 Investigate the mechanism of induced resistance using *Bacillus subtilis* 168-2 against *A. alternata* after seed pelleting and foliar spray in lettuce plant

3.4.1 Defense mechanism of lettuce against leaf spot disease

3.4.1.1 Salicylic acid (SA) analysis

Lettuce leaves of the 3rd lettuce leaves from the top of the plant were collected 3 times including 1) immediately after spraying the pathogen (0HAI), 2) after 24 hours of spraying the pathogen, and 3) after 48 hours of inoculation the pathogen. The collected samples were homogenous ground in 90% (v/v) methanol with the ratio of 1 g per 2.5 ml. Then the mixtures were centrifuged at 15,000 rpm for 15 min and removed the precipitation. Then remaining supernatant from the first centrifuge will be extracted in 100% (v/v) methanol and centrifuge again at 15,000 rpm for 15 minutes. After that, the supernatant of 100 µl was mixed with 100 µl of 0.02 M of ferric ammonium sulfate and incubated at 30 °C for 5 min. The absorbance was read at 530 nm by a

spectrophotometer (Bio-Tek, USA). The amount of salicylic acid (μg/g^{-1} fresh weight) was calculated by comparing to the standard curve y = 0.0293x + 0.1191, R^2 = 0.8133. (Raskin et al., 1989). The data of salicylic acid were analyzed according to a Completely Randomized Design (CRD).

3.4.1.2 Superoxide dismutase (SOD) analysis

Three grams of the 3rd lettuce leaves from the top of plant were investigated at 0, 24, 48, 72 and 96 HAI *A. alternata*, which was ground in the 3 ml of cold homogenization buffer (2 mM EDTA, 150 mM NaCl, 50 mM Tris-Cl (pH 7.5), 0.5 mM DTT (dithiothreitol) prepare a 0.5 M stock solution of DTT in cold H2O; store froze. Add the DTT to the chilled buffer at the indicated concentration) and centrifuge at 10,000 rpm, 4° C for 15 min. Then, 200 μl of the supernatant solution was collected and mixed with 800 μl of riboflavin solution, which was placed under 15-watt fluorescent for 10 min. After that, the mixture was measured absorbance at the wavelength of 550 nm by using a spectrophotometer (Bio-Tek, USA) (Hammerschmidt et al., 1982). The amount of SOD was calculated by comparing with standard curve and analyzed according to a Completely Randomized Design (CRD).

3.4.2 Plant growth regulating chemicals

3.4.2.1 Indole-3-acetic acid (IAA) analysis

One grams of the 1st lettuce leaves from the top of plant was investigated at 0, 24, 48, 72 and 96 HAI *A. alternata*. The sample was ground in 1 ml of homogenization buffer chilled (0.1 M Tris-HCl buffer with pH 7, 0.1 M KCl, 1 mM PMSF, 1 μg/ml leupeptin, 1% Triton X-100, and 3% PVPP) and centrifugation at 10,000 rpm, 4° C for 15 min. Then 200 μl of lettuce extract was mixed with 400 μl of Salkowski's reagent, which was incubated at room temperature for 20 min. Then, the mixture was measured absorbance at a wavelength of 535 nm (Bio-Tek, USA) (Hammerschmidt et al., 1982; Duangkaew et al., 2015). The measurement was based on the principle of color intensity obtained from the interaction between the IAA and perchloric acid (colorimeter). The amount of IAA was calculated by comparing with standard curve and analyzed according to a Completely Randomized Design (CRD).

3.5 Investigation of biochemical changes of lettuce after seed pelleted and sprayed with *B. subtilis* 168-2 used Synchrotron FT-IR microspectroscopy technique

Lettuce leaves were selected from experiment 3.3 at 24 h after inoculation *A. alternata*. The analysis proceeded in a completely randomized design (CRD) within three replications. The samples were subsequently cut to 6 × 9 mm size and embedded in the OCT compound (optimal cutting temperature), followed by Quick-freezing in liquid nitrogen. Then, the samples were stored at -80°C as long as to do cryo-sectioning process. Subsequently, each of the Lettuce leaves samples was cut crosswise to 30 µm using a microtome of cryostat (Leica 3050 S, Germany) and stick on BaF2 window size 22 × 1 mm for FTIR microspectroscopy.

The determination was operated with a mapping by using a size of aperture at 10 × 10 µm with a resolution of 4 cm^{-1}, with 64 scans by used spectra were received with FTIR spectrometer (Vertex 70 from Bruker Optics, Ettlingen, Germany) together with an IR microscope (Hyperion series 2000, Bruker) with MCT detector refrigerated in liquid nitrogen through the determination range from 4000 to 800 cm^{-1}. Spectral equipment control was carried on by OPUS 7.2 software (Bruker Optics Ltd, Ettlingen, Germany) at the Synchrotron Light Research Institute (SLRI).

Spectra from each cluster were assayed using PCA for differentiating of biochemical elemental of leaves tissue. The process was using 2nd derivative and vector normalized (Savitzky-Golay method, 3rd polynomial, 9 smoothing points) from the Unscrambler software version 9.7 (CAMO, Norway) (Thumanu et al., 2017).

3.6 An investigation of the shelf-life of *B. subtilis* 168-2 after pelleted

One hundred seeds of SeedBio 3 pelleted were stored in a refrigerator at 4 °C were placed in NA agar plate (adapted from Wongchalee, 2015). The seeds pelleted were incubated for 48 h, and colonies of *B. subtilis* stain 168-2 were observed around the lettuce seeds then, a loop was applied to the area containing *B. subtilis* stain 168-2 colonies and dispersed in 10 ml of autoclaved distilled water and diluted to 9 levels (10^1 -10^9) concentration. Each level of dilution of 100 µl was spread on Nutrient Agar medium (NA) and incubated at room temperature (25 °C) for 24 h. Surviving of *B. subtilis*

stain 168-2 colonies were counted and calculated by quantification of bacteria after 0, 1, 2, 3, 4, 5, and 6 months of storage. (Phakdeepan et al., 2009)

$$\text{The survival of } \textit{B. subtilis} \text{ 168-2} = N \times DF \times V_0 \times 1000 / V_a \times W_0$$

Where N = Countable colonies

DF = Dilution factor

V_0 = The amount of suspension that is stock default

V_a = The amount of suspension used in the spread plate

W_0 = Weight of pelleted seed

Unit = cfu/g

3.7 Contamination test of seed pelleted

Lettuce seeds pelleted with *B. subtilis* stain 168-2 (SeedBio 3) were treated as in 3.6. Then apply each concentration of dilution of 100 µl to spread on NA medium for 3 replications per concentration, incubate for 24 hours. Count and calculate contaminated bacterial colonies after 0, 1, 2, 3, 4, 5, and 6 months' storage in refrigerated at 4°C and was used to calculate the contamination rate within the seeds pelleted (adapted from Kositchaiyong, 2010), which was calculated from:

$$\text{Contamination rate of other microorganisms within seeds pelleted} = A/B \times 100$$

Where A = Number of other contaminated

B = The total number of colonies

CHAPTER IV

RESULTS

4.1 The fungal pathogen isolation and pathogenicity test in lettuce

4.1.1 Sample collection and isolation of *Alternaria alternata*

The Green Oak lettuce leaves from the SUT farm of the Suranaree University of Technology were collected for the isolation of pathogens. The fungal pathogen was isolated by tissue transplanting method and cultured in a potato dextrose agar (PDA) medium. It was found that 3 isolates of *A. alternata* were isolated from lettuce leaf spot samples, which were GA01, GA02 and GA03. The colony turns black-brown when hyphae grow and touch the Petri plate. The spores of *A. alternata* were examined under a microscope. It was found that conidiophores produced spores called conidia. It has a straight or slightly curved cylindrical shape and a rounded tip with a septum (Figure 4.1)

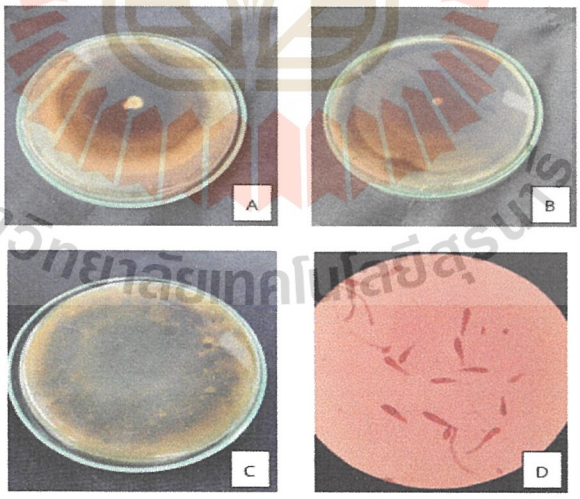


Figure 4.1 The characteristics of *A. alternata* colony isolated from Green Oak lettuce on potato dextrose agar (PDA) at 14 days. GA01 isolate (A), GA02 isolate (B), GA03 isolate (C) and spores of *A. alternata* (D).

4.1.2 Pathogenicity and virulence test of *A. alternata*

The 1.5 ml of spore suspension of each *A. alternata* isolate at a concentration of $1x10^8$ spores/ml was sprayed onto surface of healthy lettuce leaves. The severity of the disease was assessed at 3 days after inoculation under laboratory conditions. It was found that the three isolates of *A. alternata* were significantly different in pathogenicity ($P \leq 0.01$), with the GA02 isolate showed the highest virulence at 4 units and the highest percentage of leaf disease at 51.1 %. The GA01 and GA03 isolates had disease severity of 20.5 and 22.6 %, respectively (Table 4.1 and Figure 4.2). Therefore, the GA02 isolate was selected for the study.

Table 4.1 The disease severity and virulence of leaf spot disease in Green Oak lettuce at 3 days after inoculation *A. alternata*

Isolate	Disease severity (%)	Virulence of disease score [2/]
Control (water)	0.0c[1/]	0
GA01	20.5b	2
GA02	51.1a	4
GA03	22.6b	2
F-Test	**	-
CV%	15.19	-

[1/]The mean followed by the same letter in each column was not statistically different by DMRT (α=0.01).

[2/] The virulence of the disease with a score of 1-5 (modified by Wokocha et al., 2010) as follows: 1 = no symptoms, 2 = the virulence is found less than 25 % of the leaf area, 3 = the virulence is found 26-50 % of the leaf area, 4 = the virulence is found 51-75 % of the leaf area, 5 = the virulence is found more than 75 % of the leaf area.

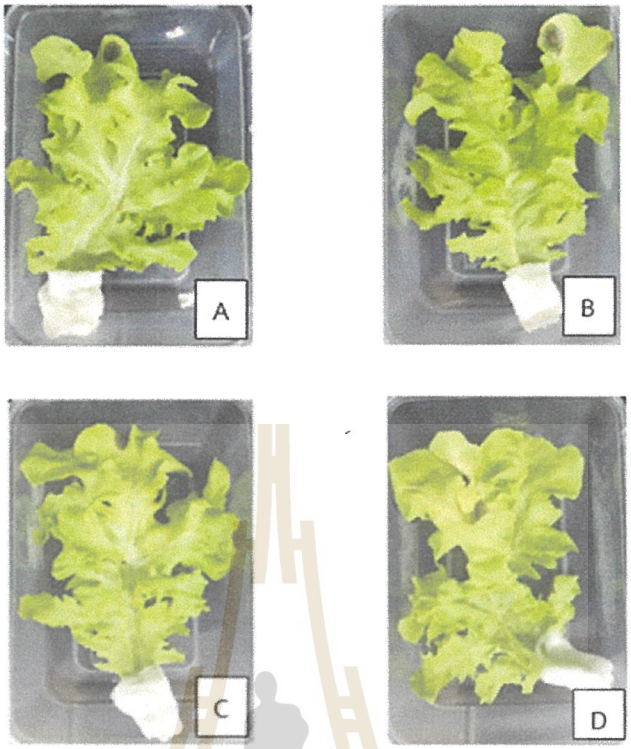


Figure 4.2 The symptom of leaf spot disease in Green Oak lettuce at 3 days after inoculation *A. alternata*, GA01 isolate (A), GA02 isolate (B), GA03 isolate (C) and control (water) (D)

4.2 Lettuce seeds pelleting by using *Bacillus subtilis* 168-2

4.2.1 Preparation of lettuce seeds pelleting

The experiment seeds pelleting varied materials and different concentrations of *B. subtilis* 168-2 could easily form the pelleted seed. The pelleted lettuce seeds in every treatment gain weight from about 30 - 40 times the size of the shape. The seed pelleting *B. subtilis* 168-2 at a concentration of 10^8 cfu/ml combined with calcium sulfate (SeedBio 3) was the lowest percentage of friability at 0.42% because these pelleting materials absorb moisture well, they were easily dissolved in water (Table 4.2 and Figure 4.3). These aid in seed surface spreads that are smooth, easy, and convenient to use with increased machinery and when testing the friability of pelleted seed.

Table 4.2 Physical properties of lettuce seeds pelleted with different concentrations of B. subtilis 168-2 and calcium sulfate.

Treatments[1/]	Forming[2/]	1,000 pelleted seed weight[3/] (g)	Pelleted seed of Friability[3/] (%)	Dissolution period of pelleted seeds[3/] (min)
SeedBio 1	-	0.80c	-	-
SeedBio 2	3	39.70a	1.21c	0.17c
SeedBio 3	3	32.55b	0.42d	1.27b
SeedBio 4	3	38.92a	1.68b	0.16c
SeedBio 5	3	36.70ab	11.38a	2.00a
F-Test	-	**	**	**
CV (%)	-	0.65	23.67	40.08

**Significantly different at P ≤ 0.01.

1/ SeedBio 1= Unpelleted, SeedBio 2= Calcium sulfate 250 g, CMC 0.3 %, with *B. subtilis* 168-2 with concentration 10^5 cfu/ml, SeedBio 3= Calcium sulfate 250 g, CMC 0.3 %, with *B. subtilis* 168-2 with concentration 10^8 cfu/ml, SeedBio 4 = Calcium sulfate 250 g, CMC 0.3 %, with *B. subtilis* 168-2 with concentration 10^9 cfu/ml and SeedBio 5= Calcium sulfate 250 g, CMC 0.3 %, with Commercial *B. subtilis* with concentration 10^8 cfu/ml.

2/Forming of seed pelleted, the scores are determined as follows; 1 = very difficult, 2 = difficult, 3 = fair, 4 = easy, and 5 = very easy.

3/ Means within a column with different letters are significantly different P≤0.05 according to DMRT.

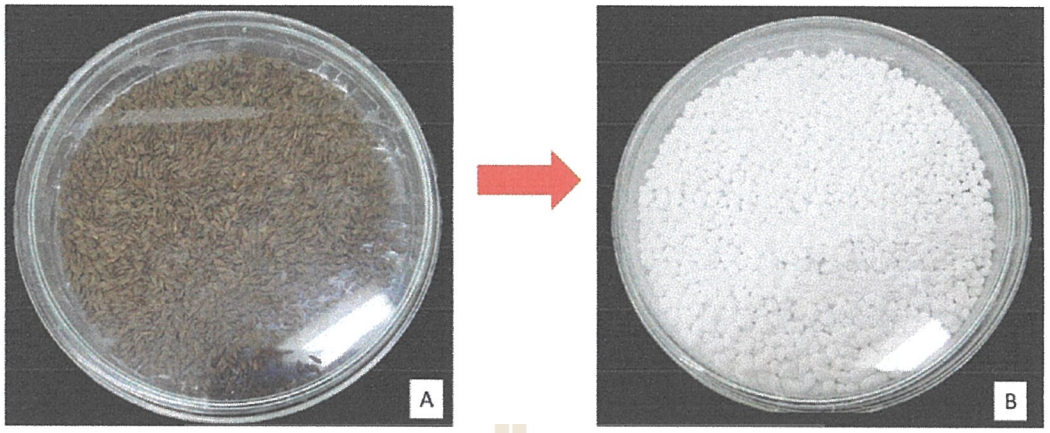


Figure 4.3 Physical appearance of lettuce seeds unpelleted (A) and pelleted with calcium sulfate as a filler and carboxymethyl cellulose (CMC) as a binder (B)

4.2.2 Pelleted seed quality

The examination of lettuce seed quality after pelleting with different concentrations of *B. subtilis* 168-2 found that the lettuce seed pelleted with *B. subtilis* 168-2 at a concentration of 10^8 cfu/ml mixed with calcium sulfate (SeedBio 3) was the highest germination at 95%, but similar with unpelleted seeds (92%). The pelleted with *B. subtilis* 168-2 with a concentration of 10^5 cfu/ml (SeedBio 2) showed a germination rate of 81%. In addition, the lettuce seed pelleted with *B. subtilis* 168-2 at a concentration 10^8 cfu/ml (SeedBio 3) showed speed germination lower than that of unpelleted seeds (SeedBio 1), but there was a non statistical difference. In addition, the seed pelleting with *B. subtilis* 168-2 at a concentration 10^8 cfu/ml (SeedBio 3) significantly maximined root length (125.23 mm) and shoot length (52.26 mm) when compared to unpelleted seeds (Table 4.3).

Table 4.3 Germination percentage, speed of germination, shoot length and root length after pelleted seeds lettuce with different concentrations of *B. subtilis* 168-2 and calcium sulfate under laboratory condition.

Treatments[1/]	Germination[2/] (%)	Speed of germination[3/] (plant/day)	Shoot length (mm)[3/]	Root length (mm)
SeedBio 1	92a	21.65a	24.02c	85.43d
SeedBio 2	81b	18.60b	35.26b	102.56b
SeedBio 3	95a	21.52a	52.26a	125.23a
SeedBio 4	74c	17.12c	29.13bc	90.02c
SeedBio 5	55d	8.31d	34.43b	104.26b
F-Test	**	**	**	**
CV (%)	4.55	4.02	7.87	9.84

**Significantly different at $P \leq 0.01$.

[1/] SeedBio 1= Unpelleted, SeedBio 2= Calcium sulfate 250 g, CMC 0.3 %, with *B. subtilis* 168-2 with concentration 10^5 cfu/ml, SeedBio 3= Calcium sulfate 250 g, CMC 0.3 %, with *B. subtilis* 168-2 with concentration 10^8 cfu/ml, SeedBio 4 = Calcium sulfate 250 g, CMC 0.3 %, with *B. subtilis* 168-2 with concentration 10^9 cfu/ml and SeedBio 5= Calcium sulfate 250 g, CMC 0.3 %, with Commercial *B. subtilis* with concentration 10^8 cfu/ml.

[2/]Data are transformed by the arcsine before statistical analysis and back transformed data are presented.

[3/] Means within a column with different letters are significantly different $P \leq 0.05$ according to DMRT.

4.3 Efficiency of seed pelleting and spraying with *B. subtilis* 168-2 to control leaf spot disease on lettuce under greenhouse conditions

Selected four treatments of pelleting seed by *B. subtilis* 168-2 of different concentrations based on the best germination rates were from experiment 4.2.2 combined with foliar spraying with *B. subtilis* 168-2 was tested under greenhouse conditions to evaluate the ability to control lettuce leaf spot disease. The result found that the combination of pelleted seed with *B. subtilis* 168-2 at 10^8 cfu/ml and foliar spraying with *B. subtilis* 10^8 cfu/ml (Treatment 5: T5) were able to reduce leaf spot disease by 47.3% compared with the control. However, the combination of foliar spraying with Mancozeb and pelleted seed with *B. subtilis* 168-2 at 10^5 cfu/ml (Treatments 4: T4), or *B. subtilis* 168-2 at 10^8 cfu/ml (Treatment 7: T7) significantly reduced leaf spot disease by 50.1%, or 52.2%, respectively. Moreover, the spraying *B. subtilis* 168-2 treatment was similar to spraying *B. subtilis* commercial (Treatment 8: T8) for reducing leaf spot disease (43.5-47.3%) compared with control (Treatment 1: T1) (Table 4.4).

On the other hand, the fresh weight and dry weight of Treatment 5: T5 were 86.56 g and 8.94 g, respectively which was significantly higher than other treatments including Treatment 1: T1 (50.25 g and 3.97 g). The canopy width was similar between Treatment 2: T2 (25.67 cm), treatment 4: T4 (28.67 cm), treatment 5: T5 (26.67 cm) and Treatment 7: T7 (24.56 cm), which were also significantly higher than other treatments. Moreover, the plant height of Treatment 5: T5 was 30 cm, which was also significantly higher than other treatments including Treatment 1: T1 (20 cm) (Table 4.5).

Table 4.4 The efficiency of seed pelleting and spraying with *B. subtilis* 168-2 to control lettuce leaf spot disease under greenhouse conditions.

Treatments[1/]	The disease reduction (%)
T1= Unpelleted + spray Water	0.0d
T2= Pelleted with *B. subtilis* 168-2 10^5 cfu/ml (SeedBio 2) + spray *B. subtilis* 168-2 10^5 cfu/ml	45.1b
T3= Pelleted with *B. subtilis* 168-2 10^5 cfu/ml (SeedBio 2) + spray Water	20.6c
T4= Pelleted with *B. subtilis* 168-2 10^5 cfu/ml (SeedBio 2) + spray Mancozeb	50.1a
T5= Pelleted with *B. subtilis* 168-2 10^8 cfu/ml (SeedBio 3) + spray *B. subtilis* 168-2 10^8 cfu/ml	47.3b
T6= Pelleted with *B. subtilis* 168-2 10^8 cfu/ml (SeedBio 3) + spray Water	21.5c
T7= Pelleted with *B. subtilis* 168-2 10^8 cfu/ml (SeedBio 3) + spray Mancozeb	52.2a
T8= Pelleted with Commercial *B. subtilis* 10^8 cfu/ml (SeedBio 5) + spray Commercial *B. subtilis* 10^8 cfu/ml	43.5b
T9= Pelleted with Commercial *B. subtilis* 10^8 cfu/ml (SeedBio 5) + spray Water	18.5c
T10= Pelleted with Commercial *B. subtilis* 10^8 cfu/ml (SeedBio 5) + spray Mancozeb	45.4b
F-Test	**
CV (%)	31.5

**: Significantly different at $P \leq 0.01$

Table 4.5 The effectiveness of seed pelleting and *B. subtilis* 168-2 spraying to promote plant growth on lettuce.

Treatments[1/]	Fresh weight (g/ plant)	Dry weight (g/ plant)	Canopy width (cm)	Plant height (cm)
T1= Unpelleted + spray Water	50.25e	3.97d	20.55b	20c
T2= SeedBio 2 + spray *B. subtilis* 168-2 10^5 cfu/ml	80.45b	7.91b	25.67a	28b
T3= SeedBio 2 + spray Water	71.34c	6.61c	19.45c	27b
T4= SeedBio 2 + spray Mancozeb	80.43b	7.85b	28.67a	23bc
T5= SeedBio 3 + spray *B. subtilis* 168-2 10^8 cfu/ml	86.56a	8.93a	26.67a	30a
T6= SeedBio 3 + spray Water	69.56c	6.70c	19.57c	28b
T7= SeedBio 3 + spray Mancozeb	72.56c	7.37b	24.56a	27b
T8= SeedBio 5 + spray Commercial *B. subtilis* 10^8 cfu/ml	73.67c	7.15b	23.44b	20c
T9= SeedBio 5 + spray Water	62.55d	6.37c	20.78b	19c
T10= SeedBio 5 + spray Mancozeb	71.11c	7.91b	23.79b	18c
F-Test	**	*	*	*
CV (%)	37.3	25.12	20.26	16.4

[1/] SeedBio2: Pelleted with *B. subtilis* 168-2 10^5 cfu/ml , SeedBio3: Pelleted with *B. subtilis* 168-2 10^8 cfu/ml, SeedBio5: Pelleted with Commercial *B. subtilis* 10^8cfu/ml; **: Significantly different at $P \leq 0.01$; *: Significantly different at $0.05 \geq P > 0.01$.

4.4 Investigate the mechanism of induced resistance against *A. alternata* in lettuce after seed pelleting and foliar spraying by *B. subtilis* 168-2

4.4.1 Defense mechanism of lettuce against leaf spot disease

4.4.1.1 Salicylic acid (SA) analysis

The treatments were selected based on the disease-reducing results in experimental 4.3. The salicylic acid (SA) was investigated at 0, 24 and 48 hours after the fungal inoculation (HAI). The result found that immediately after inoculation (0 HAI), Treatment 3: T3 (SeedBio 3 + spray *B. subtilis* 168-2 10^8 cfu/ml) was the highest SA content at 6.02 µg g^{-1} fresh weight, followed by Treatment 4: T4 (SeedBio3 + spray Mancozeb) and treatment 2: T2 (SeedBio 2 + spray *B. subtilis* 168-2 10^5 cfu/ml) at 5.90 and 4.26 µg g^{-1} fresh weight, respectively which is significantly higher than other treatments. At 24 HAI, the accumulation SA of Treatment 3: T3, Treatment 4: T4 and Treatment 2: T2 was 10.68, 9.67, and 6.15 µg g^{-1} fresh weight, respectively, which was a statistically significant difference. The accumulation SA of all treatments increased at 24 HAI when compared with 0 HAI and then decreased at 48 HAI. The result showed Treatment 3: T3 can enhance the higher level of salicylic content at 24 HAI to approximately 77.40 %, which was higher than other treatments (Table 4.6). This was accompanied by a similar change in SOD activity level (Table 4.7).

Table 4.6 Salicylic acid accumulation in lettuce leaves after seed pelleting and foliar spray with *B. subtilis* 168-2 and *A. alternata* inoculation under greenhouse conditions.

Treatments[4/]	Endogenous salicylic acid ($\mu g\ g^{-1}$ fresh weight)[1/]			Increase of SA activity (%) at
	0 HAI[2/]	24 HAI	48 HAI	24 HAI
T1= Unpelleted + spray Water	3.89d[3/]	4.32e	1.77e	11.05
T2= SeedBio 2 + spray *B. subtilis* 168-2 10^5 cfu/ml	4.26c	6.15c	3.63c	44.36
T3= SeedBio 3 + spray *B. subtilis* 168-2 10^8 cfu/ml	6.02a	10.68a	7.57a	77.40
T4= SeedBio 3 + spray Mancozeb	5.90b	9.67b	4.30b	63.89
T5= SeedBio 5 + spray Commercial *B. subtilis* 10^8 cfu/ml	3.77d	5.66d	2.01d	50.13
F-Test	**	**	**	-
CV (%)	5.67	9.89	4.64	-

[1/] Salicylic acid content after sprayed by T1, T2, T3, T4 and T5 for 4 times and after inoculation of *A. alternata* at 0, 24, 48 h.

[2/] HAI = Hours after inoculation *A. alternata*

[3/] The characters were identical with non-statistically significant differences. The Average was compared using DMRT (α=0.01).

[4/] SeedBio 2: Pelleted with *B. subtilis* 168-2 10^5 cfu/ml, SeedBio 3: Pelleted with *B. subtilis* 168-2 10^8 cfu/ml, SeedBio 5: Pelleted with Commercial *B. subtilis* 10^8 cfu/ml.

4.4.1.2 Superoxide dismutase (SOD) analysis

The accumulation of SOD in lettuce was investigated at 0, 24, 48, 72 and 96 HAI. It was found that Treatment 3: T3 (SeedBio 3 + spray *B. subtilis* 168-2 10^8 cfu/ml) was the most effective in inducing SOD accumulation of lettuce. The SOD was a substance in the plant immune system to protect the plant from infection of *A. alternata*. The accumulation of SOD at 0 HAI in Treatment 3: T3 and Treatment 5: T5 were 11.02 and 10.21 µg catechol /mg^{-1} protein, which was significantly higher than other treatments. Then, the SOD activity of Treatment 3: T3 and Treatment 4: T4 increased to 17.76 and 15.60 µg catechol /mg^{-1} protein at 24 HAI, which is significantly higher than other treatments. After that, the SOD activity of Treatment 3: T3 and Treatment 4: T4 decreased to 14.08 and 11.71 µg catechol /mg^{-1} protein at 48 HAI. Then, the SOD activity decreased at 72 and 96 HAI. The result showed Treatment 3: T3 can enhance the higher level of superoxide dismutase at 24 HAI to approximately 61.16 %, which is higher than other treatments (Table 4.7). This was accompanied by a similar change in SA activity level.

Table 4.7 Superoxide dismutase accumulation in lettuce leaves after seed pelleting and foliar spray with *B. subtilis* 168-2 and *A. alternata* inoculation under greenhouse conditions.

Treatments[4/]	Superoxide dismutase (µg catechol /mg^{-1} protein)[1/]					Increase of SOD activity (%) at 24 HAI
	0 HAI[2/]	24 HAI	48 HAI	72 HAI	96 HAI	
T1= Unpelleted + spray Water	9.25c[3/]	10.15e	7.67d	5.55d	2.76d	13.51
T2= SeedBio 2 + spray *B. subtilis* 168-2 10^5 cfu/ml	9.33c	11.24d	7.01d	4.89e	3.67c	20.47
T3= SeedBio 3 + spray *B. subtilis* 168-2 10^8 cfu/ml	11.02a	17.76a	14.08a	10.78a	6.98a	61.16
T4= SeedBio 3 + spray Mancozeb	9.97bc	15.60b	11.78b	9.67b	4.77b	56.46
T5= SeedBio 5 + spray Commercial *B. subtilis* 10^8 cfu/ml	10.21b	14.24c	10.76c	7.55c	2.01d	39.47
F-Test	**	**	**	**	**	-
CV (%)	7.26	10.89	9.77	8.05	5.54	-

[1/] Superoxide dismutase (SOD) content after sprayed by T1, T2, T3, T4 and T5 for 4 times and after inoculation of *A. alternata*.

[2/] HAI = Hours after inoculation *A. alternata*

[3/] The characters were identical with non-statistically significant differences. The average was compared using DMRT (α=0.01).

[4/] SeedBio 2: Pelleted with *B. subtilis* 168-2 10^5 cfu/ml, SeedBio 3: Pelleted with *B. subtilis* 168-2 10^8 cfu/ml, SeedBio 5: Pelleted with Commercial *B. subtilis* 10^8 cfu/ml.

4.4.2 Plant growth regulating chemicals

4.4.2.1 Indole-3-acetic acid (IAA) analysis

The accumulation of IAA in lettuce was investigated at 0, 24, 48, 72 and 96 HAI *A. alternata*. The accumulation of IAA in Treatment 3: T3 (SeedBio 3 + spray *B. subtilis* 168-2 10^8 cfu/ml) was 12.67 µg/mg^{-1} fresh weight at 0 HAI, which was significantly higher than other treatments. After that, the IAA accumulation of treatment 3: T3 increased to 16.98 µg/mg^{-1} fresh weight at 24 HAI, which was significantly higher than Treatment 4: T4 (SeedBio 3 + spray Mancozeb) was 14.80 µg/mg^{-1} fresh weight. And both Treatment 3: T3 and Treatment 4: T4 was significantly higher than in other treatments. Then, the IAA accumulation of Treatment 3: T3 decreased at 48 HAI but was still higher than other treatments, which was 9.60 µg/mg^{-1} fresh weight. The increasing IAA accumulation of Treatment 3: T3 was consistent with the growth of lettuce in the results of Table 4.5 because had better fresh weight, dry weight, canopy width and plant height and there were significantly higher when compared to other treatments. The result showed Treatment 3: T3 can enhance the higher level of Indole-3-acetic acid at 24 HAI to approximately 34.02 %, which was higher than other treatments (Table 4.8). This was accompanied by a similar change in SA activity and superoxide dismutase level.

Table 4.8 Indole-3-acetic acid (IAA) accumulation in lettuce leaves after seed pelleting and foliar spray with *B. subtilis* 168-2 and *A. alternata* challenged inoculation under greenhouse conditions.

Treatments[4/]	Indole-3-acetic acid (µg/mg-1 fresh weight)1/					Increase of IAA content (%) at 24 HAI
	0 HAI[2/]	24 HAI	48 HAI	72 HAI	96 HAI	
T1= Unpelleted + spray Water	5.87d[3/]	6.59d	4.23d	3.55c	1.60c	12.26
T2= SeedBio 2 + spray *B. subtilis* 168-2 10^5 cfu/ml	10.02b	11.98c	5.05c	4.98bc	1.98bc	19.56
T3= SeedBio 3 + spray *B. subtilis* 168-2 10^8 cfu/ml	12.67a	16.98a	9.60a	5.45b	2.67b	34.02
T4= SeedBio 3 + spray Mancozeb	11.43b	14.80b	8.09ab	6.66a	3.01a	29.48
T5= SeedBio 5 + spray Commercial *B. subtilis* 10^8 cfu/ml	9.12c	11.56c	7.09b	4.56b	1.45d	26.75
F-Test	**	**	**	**	**	-
CV (%)	5.45	10.78	8.44	6.45	4.34	-

[1/] Indole-3-acetic acid (IAA) content after sprayed by T1, T2, T3, T4 and T5 for 4 times and after inoculation of *A. alternata*.

[2/] HAI = Hours after inoculation *A. alternata*.

[3/] The characters were identical with non-statistically significant differences. The average was compared using DMRT (α=0.01).

[4/] SeedBio 2: Pelleted with *B. subtilis* 168-2 10^5 cfu/ml, SeedBio 3: Pelleted with *B. subtilis* 168-2 10^8 cfu/ml, SeedBio 5: Pelleted with Commercial *B. subtilis* 10^8 cfu/ml.

4.5 Investigation of biochemical changes of lettuce after seed pelleting and spraying with *B. subtilis* 168-2 by using Synchrotron FT-IR microspectroscopy technique

Treatment 1 (T1: unpelleted + spray water), Treatment 2 (T2: SeedBio 3 + spray Mancozeb), and Treatment 3 (T3: SeedBio 3 + spray *B. subtilis* 168-2 10^8 cfu/ml) samples were examined for biochemical changes at 24 HAI using Synchrotron FT-IR microspectroscopy. The spectrum, which was measured at a wavelength range of 4000-800 cm^{-1} was used to analyze principle component analysis (PCA) to differentiate clusters in the mesophyll layer of lettuce leaf tissue. It was found that the PC1-axis was able to separate the spectral groups of the mesophyll layer of lettuce leaf tissue between Treatment 1: T1, Treatment 2: T2 and Treatment 7: T3 with 23 % difference. Treatment 2: T2 was arranged on the vertical, positive axis, while Treatment 3: T3 and Treatment 1: T1 were arranged on the horizontal axis, with negative values (Figure 4.4). The differentiation spectra were 1735 cm^{-1} (C=O ester of pectin), 1658 cm^{-1} (amide I) and 1546 cm^{-1} (amide II) (Figure 4.5). Then the spectrum was analyzed for the area under the graph to determine the amount of biochemical change. It was found that Treatment 3: T3 had a significantly higher amount of C=O ester of pectin and branched-chain peptides of amino acids of amide I and amide II in mesophyll tissues compared with Treatment 1: T1 and Treatment 2: T2 (Table 4.9 and Figure 4.6). The amount of pectin, amide I, and amide II in mesophyll tissue of Treatment 3: T3 was significantly higher than Treatment 1: T1 by 2.0, 1.9 and 2.7-folds, Treatment 2: T2 by 1.3, 1.6 and 1.9-folds, respectively. However, the seed pelleting with *B. subtilis* 168-2 was able to direct and indirect defense by stimulating the seed to promote growth and having a higher level of resistance to pathogens than the control treatment (Treatment 1: T1). *B. subtilis* 168-2 can produce Indole-3-acetic acid (IAA) and when the seed pelleting with *B. subtilis* 168-2 and sprayed with *B. subtilis* 168-2, lettuce was able to accumulate SA, SOD and IAA, which these compounds contributed in promoting the prolongation of lettuce seedlings, growth of lettuce and strengthen the cell walls to resist pathogens invasion,

resulting in more changes in pectin and branched-chain peptides of amino acids of amide I and amide II in mesophyll tissues than the control treatment (Treatment 1:T1).

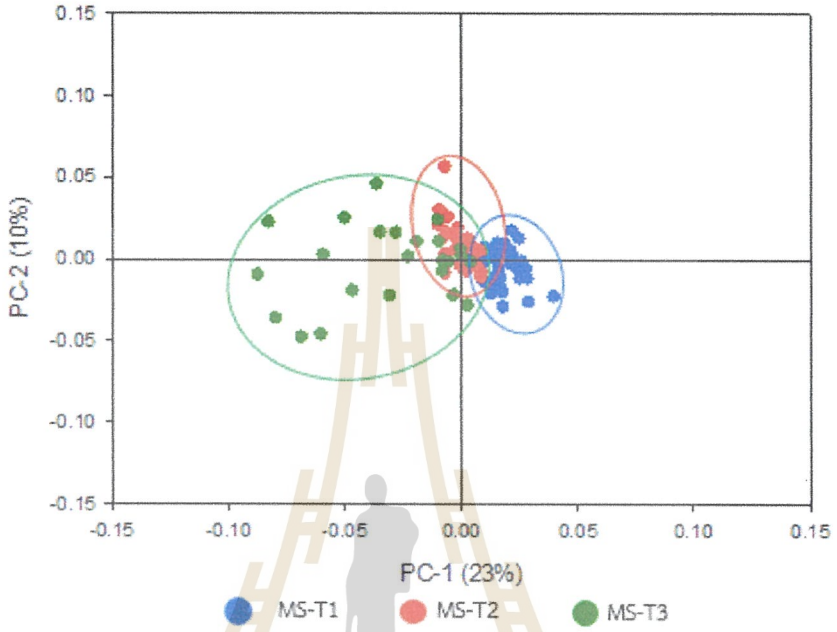


Figure 4.4 Principle component analysis (PCA) score of mesophyll tissue of lettuce leaf at 24 HAI *A. alternata*. Note: T1-blue (Treatment 1: unpelleted + spray water), T2- red (Treatment 2: SeedBio 3 + spray Mancozeb) and T3-green (Treatment 3: SeedBio 3 + spray *B. subtilis* 168-2 10^8 cfu/ml).

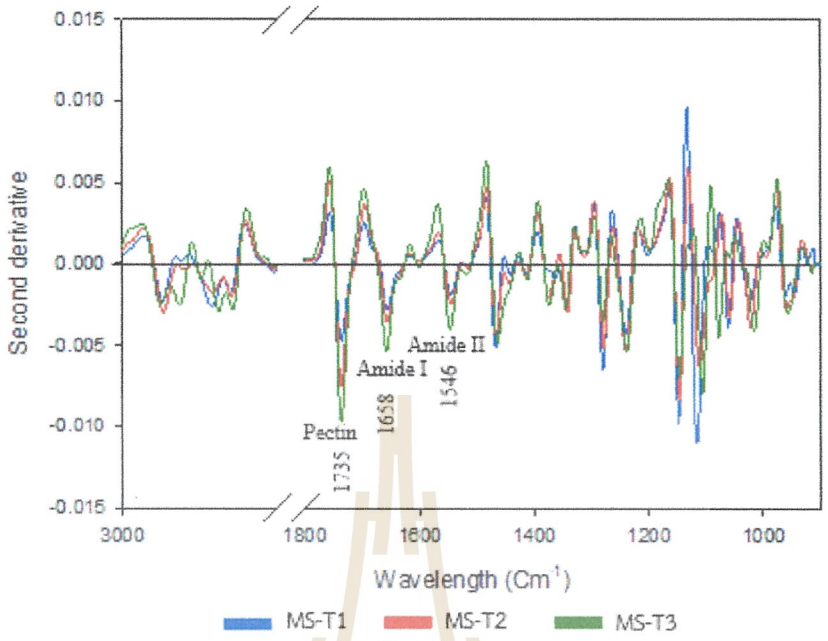


Figure 4.5 The second derivative average FTIR spectrum of mesophyll tissues of lettuce leaves at 24 HAI *A. alternata*. Note: T1-blue (Treatment 1: unpelleted + spray water), T2- red (Treatment 2: SeedBio 3 + spray Mancozeb) and T3-green (Treatment 3: SeedBio 3 + spray *B. subtilis* 168-2 10^8 cfu/ml).

Table 4.9 The integral areas of absorbance between 1740-1700 cm^{-1} (pectin), 1700-1600 cm^{-1} (amide I), 1600-1500 cm^{-1} (amide II) of mesophyll tissues of lettuce leave at 24 HAI *A. alternata*.

Treatments[4/]	The integral areas		
	Pectin	Amide I	Amide II
	1740-1700 cm^{-1}	1700-1600 cm^{-1}	1600-1500 cm^{-1}
T1= Unpelleted + spray Water	0.081± 0.005c[1/]	0.059± 0.008b	0.029± 0.005b
T2= SeedBio3 + spray Mancozeb	0.127± 0.009b	0.070± 0.012b	0.040± 0.007b
T3= SeedBio3 + spray *B. subtilis* 168-2 10^8 cfu/ml	0.166± 0.023a	0.112± 0.012a	0.077± 0.015a
F-Test	**	**	**
CV (%)	6.8	14.5	18.6

[1/] The characters were identical with non-statistically significant difference, compare averages using DMRT (α=0.01). Area under the graph was calculated by OPUS 7.5 software.

[2/] SeedBio3: Pelleted with *B. subtilis* 168-2 at 10^8 cfu/ml.

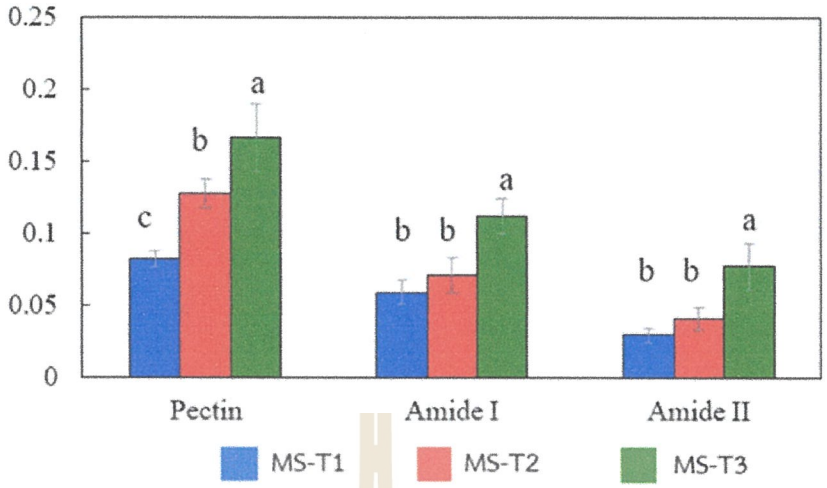


Figure 4.6 The integral areas of absorbance between 1740-1700 cm^{-1} (pectin), 1700-1600 cm^{-1} (amide I), 1600-1500 cm^{-1} (amide II) of mesophyll tissues of lettuce leaves at 24 HAI *A. alternata*. Note: T1-blue (Treatment 1: unpelleted + spray water), T2- red (Treatment 2: SeedBio 3 + spray Mancozeb) and T3-green (Treatment 3: SeedBio 3 + spray *B. subtilis* 168-2 10^8 cfu/ml).

4.6 The survival of *B. subtilis* 168-2 at 6 months after pelleted

The density of *B. subtilis* 168-2 in SeedBio 3 (calcium sulfate 250 g, CMC 0.3 %, with *B. subtilis* 168-2 with concentration 10^8 cfu/ml) at 6 months after pelleting was tested for evaluating the potential survival of bacteria in the pelleted seeds. The result found that the survival of *B. subtilis* 168-2 in pelleted seeds was 4.89±7.03 x 10^8, 3.78±6.60 x 10^8, 3.65±5.64 x 10^8, 8.56±4.16 x 10^7, 6.60±1.55 x 10^7, 8.10±0.50 x 10^6, 8.40±0.47 x 10^5 cfu/g, respectively (Table 4.10). After three months of storage, the survival of *B. subtilis* 168-2 in pelleted seeds was stable, with a slight and stable decrease at the fourth and fifth months. Following that, the *B. subtilis* 168-2 tends to decline.

Table 4.10 The survival quantity of *B. subtilis* 168-2 in pelleted seeds at 6 months after storage

Shelf life (months)[1/]	The survival quantity of *B. subtilis* 168-2 (cfu/g)
0	$4.89 \pm 7.03 \times 10^8$
1	$3.78 \pm 6.60 \times 10^8$
2	$3.65 \pm 5.64 \times 10^8$
3	$8.56 \pm 4.16 \times 10^7$
4	$6.60 \pm 1.55 \times 10^7$
5	$8.10 \pm 0.50 \times 10^6$
6	$8.40 \pm 0.47 \times 10^5$

[1/] Shelf life is used to store pelleted seeds in stored at 4 °C.

4.7 Contamination test of seed pelleted

The *B. subtilis* 168-2 in SeedBio 3 (calcium sulfate 250 g, CMC 0.3 %, with *B. subtilis* 168-2 with concentration 10^8 cfu/ml) was used to determine the degree of contamination by other microbes after 0–6 months after storage. According to the findings, the rates of bacterial and fungal contamination rate were 5.45, 5.30, 4.05, 3.89, 1.23, 1.21 and 1.63 %, respectively and 0.00, 0.02, 0.03, 0.00, 0.00, and 0.03%, respectively (Table 4.11).

Table 4.11 The lettuce seed pelleted contamination rate

Shelf life (months)[1/]	Contamination rate of other microorganisms (%)	
	Bacterial	Fungal
0	5.45	0.00
1	5.30	0.02
2	4.05	0.03
3	3.89	0.00
4	1.23	0.00
5	1.21	0.00
6	1.63	0.03

[1/] Shelf life is used to store pelleted seeds in stored at 4 °C.

CHAPTER V
DISCUSSION AND CONCLUSION

5.1 Isolation of fungal pathogen *A. alternata* by tissue transplanting method

The lettuce leaf spot samples were incubated to induce mycelium growth on the WA medium. Then the hyphae were transferred and re-cultured on the PDA medium until the fungal was purified (Rotniam, 2009). The main in the isolation of pathogens is the selection of samples that must be collected from lettuce fresh leaves containing healthy and diseased tissue. It is a dark brown to small black spot. If the lettuce leaves are sampled with a puncture wound, it is not possible to isolate the pathogen because the wound has been damaged for a long time and contaminated with other pathogens. The samples have been collected, then the sterilization should be done in a sterile cabinet immediately. The mycelium was cultured on a PDA medium for 14 days, and the fungi were able to grow well according to the morphological characteristics. The conidia formation of *A. alternata* was used in this study. This was consistent with the method recommended by Rotniam (2009). The growth characteristics of fungi on PDA medium were greenish-gray spongy fibers growing into an extended ring. The conidiophore was formed either as a single or a group, cylindrical shape, dark brown, smooth walls with partitions. The spores look club-shaped and are highly septate. Previous research has found that various fungi *Alternaria* spp. could cause leaf spot disease in plants such as *A. zinnia*, *A. helianthi* and *A. longipes* could cause leaf spot disease in sunflowers (Cosme, 1991). In addition, *A. brassicicila* and *A. brassicae* caused cauliflower leaf spot (Anan and Athinuwat, 2016), *A. porri*, caused Japanese onion purple leaf spot, *A. solani* caused blight of tomatoes, and *A. cucumerina* caused cucumber leaf spot also have been recorded (Prasongsap, 2014).

5.2 Pathogenicity and virulence test of *A. alternata*

The spore suspension of each fungal isolate was sprayed at a concentration of 1×10^8 spores/ml. It was found that the three fungal isolates were capable of causing leaf spot disease. The fungi isolate GA02 had the highest pathogenic. It is considered that the pathogenicity test of the pathogenic fungi in laboratory conditions is good, convenient to experiment and it takes a quick test time due to incubation in moist box conditions. Consistent with this Mo et al. (2007) reported that experiments in moist box conditions are a convenient, rapid and effective method for testing soybean resistance to powdery mildew and rust. Likewise, Boydom et al. (2013) could use this method to test the resistance of wheat to rust disease.

5.3 Lettuce seeds pelleted by using *B. subtilis* 168-2

In laboratory conditions, the lettuce seed was pelleted with 250 g of calcium sulfate, 0.3% CMC, and different concentrations of *B. subtilis* 168-2. It was found that SeedBio 3 (250 g of calcium sulfate, 0.3% CMC, *B. subtilis* 168-2 with concentration 10^8 cfu/ml) was the most easily forming pelleted. Pelleted lettuce seeds in every process increased their weight by about 30-50 times, resulting in the exact size and shape desired. The seed pelleted has good fluidity, easy for planting, and is easier to use with machinery (Siri, 2015; Halmer, 1987; Butler, 1993; Zenk, 2004). And Hill (1999) found that the onion seed pelleting increased the seed size approximately 6 times. After considering the method of pelleting the seeds, it was found that after seeding there was no effect on the seed germination process and can promote seed quality better than unpeeled seeds which is consistent with the experimental results of physical properties of lettuce seeds. Then, testing the friability of the pelleted seeds, it was found that the SeedBio 3 had the lowest percentage of friability (0.42%) and was statistically different from other treatments. The dissolution period test of pelleted seeds showed that it can be seen that the pelleted seed of SeedBio 3 and SeedBio 4 is the best dissolution period because this filler material has good moisture absorption properties. It is ion- exchangeable (Hirota et al., 1989).

The quality of lettuce seeds after pelleting also was tested. It was found that seed pelleting of lettuce by SeedBio 3 had the highest germination in laboratory

conditions at 95%, followed by unpelleted seed (SeedBio 1). The lettuce seeds pelleting of SeedBio 3 had a lower germination speed than unpelleted seed (Treatment 1: T1), but there was a non-statistical difference. This is consistent with the research of Coraspe et al. (1993). There was a non-statistically significant difference in the germination of lettuce seed germination in the laboratory between unpelleted and pelleted seeds. And Zink (1954) found that unpelleted lettuce seeds had a higher germination speed than pelleted lettuce seeds. And from the research of Shashibhaskar et al. (2011), the seed pelleted of tomato variety CV.PKM-1 with zinc sulfate showed that the treated seeds displayed better growth and yield than unpelleted seeds. And Soulange and Levantard (2008) reported that the fresh weight leaves of seeding of pelleted seed were higher than unpelleted seed during germination tests. In addition, Bennett (1998) reported that seed priming in combination with plant growth-promoting microorganisms can increase the germination and growth characteristics of plants. And when considering the treatment of pelleting the seeds, it was found that there was no effect on seed germination after pelleting. The seed pelleted with *B. subtilis* showed better growth than the control (T1) because the seed pelleting has a large amount of pelleting material pelleted around the seed, *B. subtilis* is better adhered to the seed compared to the soaking method and seed coating. This is because *B. subtilis* is able to synthesize phytohormone in the auxin group that plays a role in stimulating germination. It also promoted the development of seedling growth better than the unpelleted lettuce seeds (Glick, 2012). And when considering the seed pelleting method, the key principle was that the active ingredient could be attached to the seed (Taylor et al., 1998; Pedrini et al., 2017). In the pelleting seed method, the microorganisms are attached to the seed and is a combination of filler material (calcium sulfate), binder (carboxymethyl cellulose), and seed, which has strong adhesion, but is easily dissolve in water (Khorasani and Shojaosadati, 2017). So, microorganisms were able to attach to the seed pelleted in larger quantities than the seed priming and seed coating method. This resulted in the amount of bacterial cells at 10^8 cfu/ml as a rate of surviving microorganisms attached to the seed and can promote plant growth better than other methods. Therefore, the *B. subtilis* seed pelleting method showed different results when examined in laboratory

conditions for germination. The germination speed is better than other methods compared with unpelleted seeds.

5.4 Efficiency of seed pelleted and sprayed with *B. subtilis* 168-2 to control leaf spot disease on lettuce under greenhouse conditions

The efficacy of seed pelleting and spraying with *B. subtilis* to control leaf spot disease on lettuce under greenhouse conditions showed that the combination of pelleted seed with *B. subtilis* 168-2 at 10^8 cfu/ml (SeedBio 3) and foliar spraying 4 times with *B. subtilis* 168-2 at 10^8 cfu/ml (Treatment 5: T5) was able to reduce lettuce leaf spot disease by 47.3%. The timing of the use of the *B. subtilis* 168-2 is one of the factors that contribute to the success of disease control, especially at the plant weak and suitable environment. In addition, *B. subtilis* 168-2 can inhibit lettuce leaf spot pathogens and has properties as a plant growth promoting rhizosphere (PGPR) that can stimulate lettuce to produce indole-3-acetic acid (IAA) to promote growth. This was consistent with the growth of lettuce can increase fresh weight, dry weight, plant height, and canopy width in the treatment 5: T5, which is significantly higher than other treatments. Moreover, the *B. subtilis* mechanism is reported to stimulate plants to produce substances in the immune system, including salicylic acid (SA), β-1,3-glucanase, peroxidase, phenolic, guaiacol and peroxidase, as well as having a fixation nitrogen (Duangkaew et al., 2015). Some of the enzymes mentioned above are involved in the synthesis of phytoalexin and pathogenesis-related proteins, which are highly toxic to plant pathogens (Cohen et al., 1994). And the results of this research found that *B. subtilis* 168-2 can induce lettuce to accumulate SA and SOD, which are enzymes in the immune system diseases as well. Consistent with Anan and Athinuwat (2016), developed by mixing kaolin: potassium humate: glucose: FeSO4, 72:8:1:19 w/w, mixed with *B. subtilis* TU-Orga1, a volume of 20 ml/1 kg of supporter. The residual content of TU-Orga1 was approximately 10^{12} and 10^{10} cfu/ml at 12 and 24 months after being stored at room temperature (28-33 °C), respectively. This bio-formulation was effective in reducing the infestation of the causative agent of leaf blight, rot and leaf spot disease via induced immunity by stimulation of salicylic acid (SA) and superoxide dismutase (SOD) up to the highest values with 0.79-0.91 mg/g fresh weight and 13.43-16.78 mcg

catechol/mg protein, respectively, which were statistically different from the control treatment. In addition, the bio formulation also has an effect promoting the growth of kale by stimulating the accumulation of substances with the highest indole-3-acetic acid was 15.21 mcg/mg fresh weight. Moreover, the bio formulation was evaluated for controlling kale disease under field conditions in Nonthaburi province by mixing seeds before planting and spraying 6 times at 7, 14, 21, 28, 35, 42 day-old plants. The results showed that the bio-formulation product was effective in reducing the infestation of the causative agent of leaf blight, rot, wet rot and leaf spot disease, as well as increasing the yield of kale. The efficacy of bio formulation was statistically higher than copper hydroxide fungicide and nontreated control. Similar to the report of Zaidiet et al. (2006) that *B. subtilis* was able to synthesize IAA and prevent fungal infestation. In addition, nitrogen fixation activity was reported in *Bacillus* sp. OSU-142, which significantly increased the yield of wheat, sugar beet, spinach (Cakmakci et al., 2007). And *B. subtilis* CC-pg104 also increased weight gain, dry weight of lettuce after seeding treated (Rekha et al., 2007).

5.5 Investigate the mechanism of induced resistance against *A. alternata* in lettuce plants after seed pelleted and foliar sprayed by *B. subtilis* 168-2

The effectiveness of reducing leaf spot disease in lettuce was tested by seed pelleting (SeedBio 3) and spraying *B. subtilis* 168-2 at 10^8 cfu/ml (Treatment 3: T3) under greenhouse conditions. After treated with treatment 3 and inoculated *A. alternata*, the results of this research study are consistent with the previous research results. This study showed the efficacy of *B. subtilis* 168-2 in promoting growth and controlling leaf spot disease. In addition, *B. subtilis* 168-2 has the potential to stimulate plants to produce substances in the plant immune system. The treatment 3 was able to promote lettuce to accumulate salicylic acid (SA), superoxide dismutase (SOD) and Indole-3-acetic acid (IAA) higher than the control treatment. Consistent with Anan and Athinuwat (2016), it was found that *B. subtilis* TU-Orga1 was statistically different in inducing kale to accumulate SA to prevent the infestation of *Xanthomonas citri* subsp. *citri*, the causative agent of golden leaf disease, with a statistical difference at 24 HAI. It has been shown

that when resistance is induced, it is signaled and produced other biochemical to protect the plant and SA begins to decline when the plant begins to show symptoms, as well as the activity of SOD and IAA accumulated at 24 HAI and tends to increase at 48 HAI. Consistent with Preecha and Prathuangwong (2009), it was found that the beneficial bacteria *B. amyloliquafaciens* has the ability to induce plants to increase the producing SA in soybeans on the 4th day after inoculation. So, this study confirmed that the SA, SOD and IAA compounds produced by treating *B. subtilis* 168-2 in lettuce were involved in the plant induced resistance response and led to the strengthening of plant cell walls that can reduce the infestation of pathogens (Bradley et al., 1992). It also has properties as a plant growth promoting rhizosphere (PGPR) that can stimulate lettuce production including indole-3-acetic acid (IAA) to promote plant growth out of the weak stage and SA, SOD to against the infestation of plant pathogens. Furthermore, *B. subtilis* has been reported to induce plants to produce a variety of immune system metabolites, including SA, β-1,3-glucanase, peroxidase, phenolic, guaiacol and peroxidase, as well as independent nitrogen fixation mechanisms (Duangkaew et al., 2015). The enzymes mentioned above are involved in the synthesis of phytoalexin and pathogenesis-related proteins, which are highly toxic to plant pathogens (Ramputh and Bown, 1996).

In addition to its effectiveness in reducing disease through changes in SA, SOD, and IAA. When studying biochemical changes using the Synchrotron FT-IR microspectroscopy technique, it was found that lettuce leave tissues stimulated by Treatment 3 had significantly higher amounts of C=O ester of pectin, amide I and amide II compared with Treatment 2 and Treatment 1. The carbohydrates are important to the function and structure of the plant cell. Carbohydrates are found in the composition of cell walls and cell membranes in the form of cellulose, hemicellulose, chitin, lignin and pectin (Wongcharlee, 2015). Similarly, the proteins also are essential components of plants, especially biochemical processes. It can be increased in volume when stimulated by *B. subtilis* 168-2, which suggested the transformation of biochemical compounds in plant cells. It has the effect of promoting growth and stimulating plants to be vigorous and able to resist fungi that cause leaf spot disease. According to Wongchalee (2015), a Synchrotron FT-IR microspectroscopy technique was used to examine the biochemical changes of chilli leaves sprayed by *B. subtilis* D604. It was

found that the amount of hemicellulose and polysaccharide in chilli leaves tissues treated with *B. subtilis* D604 was significantly higher than water control treatment. This allows the chilli increase yields and resistance to anthracnose disease. In addition, Pimthong (2014) studied the defense mechanisms of grapes with the synchrotron FT-IR microspectroscopy technique. It was found that the CaSUT007 and chitosan treatments changed the lipids, hemicellulose and carbohydrate differed in each treatment and changed after inoculation of pathogens. Therefore, it can be concluded that when plants are infected by pathogens, there will be a change in the amount of biomolecules within plant cells. These biomolecules represent a defense system including signaling to stimulate the expression of genes for resistance disease. Therefore, the elicitors used in this experiment, *B. subtilis* 168-2 (Treatment 3: T3) has the ability to stimulate lettuce to accumulate more SA, SOD and IAA so that plants are resistant to the infestation of leaf spot pathogens and can promote plant growth. In addition, plants may produce substances after being stimulated by pathogens because many pathogens release nonspecific elicitors, such as the production of glycoproteins, various microbial enzymes including protease, cellulose and hemicellulose, etc., to digest the components of plant cell walls. Plants recognize those substances and send signals to create an immune system, such as the formation of cell walls to have more cellulose and hemicellulose makes plants resistant to the spread of the infection to other parts. The increased amount of hemicellulose (secondary cell wall) can reduce the infestation of pathogens and changes in cellulose and hemicellulose content (Hernández-Blanco et al., 2007; Eggert et al., 2014; Nafisi et al., 2014). It was also associated with the occurrence of reactive oxygen species (ROS) and phytohormones in plants (Loreto et al., 2001; Vickers et al., 2009). While the change in the C-H bending content was increased after inoculation of the pathogenic agent, which contains ethylene, which is involved in plant growth and isoprene signaling in induced systemic resistance (ISR) and is a constituent of lipid acid. It is a key component of cell membranes, making plants more resistant to pathogenic invasion (Sweeting, 1996; Monson et al., 2013; Eggert et al., 2014). Consistent with Saengchan (2014), FTIR was used to study the accumulation of substances in biochemical related to the growth-promoting mechanism after stimulation with *Bacillus* sp. isolate CaSUT007 and salicylic acid. The C-H stretching lipid groups, C=O ester lipids,

and amide I groups were found to increase but C-H bonding, C-O stretching and polysaccharide carbohydrates have a lower. This indicates that changes in biomolecules within plant cells have an effect on promoting growth and stimulate the plants to be strong and able to resist rot bacteria in Chinese green mustard. In addition, the experimental results also showed that considering the fresh weight and dry weight increased the growth of lettuce by the efficiency of *B. subtilis*, an important mechanism capable of producing phytohormone, an important organic acid is indole-3-acetic acid (IAA). The IAA has an important role in cell division and proliferation of root cells. It also increases root length and surface area so that the roots can absorb more water and nutrients (Salisbury, 1994; Patten and Glick, 2002; Ahemad and Khan, 2011). *B. subtilis* is also capable of producing enzymes. L-tryptophan-dependent growth factor, it is important to increase the number of leaves (Asghar, 2009). Furthermore, according to Garcia de Salamone et al. (2001), *B. subtilis* is also able to produce cytokinins that promote cell division, cell proliferation and plant tissue proliferation.

5.6 The survival of *B. subtilis* 168-2 at 6 months after pelleting

The survival of *B. subtilis* 168-2 (SeedBio 3) after 6 months of seed pelleting test revealed that the survival of *B. subtilis* 168-2 was stable in the initial period after storage until 2 months and there was a slight decrease in the amount of infection and remained stable in the 3 rd and 4 th months. Musiksang (2008) studied the number of microencapsulating bacteria in initial gel tablets and at 3 hours after incubation at 37 °C. It was found that the initial bacterial cell count was 10^9 cfu/ml, which decreased after 3 h incubation to 10^6 cfu/ml, an acceptable cell count. While if the number of initial bacterial cells was 10^7 and 10^8 cfu/ml, which was decreased to 10^5 cfu/ml which was too low at the end of the experiment. Their results indicated that the high number of bacterial initial cells is an important parameter in their microencapsulation or pelleted seeds. Therefore, in future, seed pelleting, the initial concentration should be used at a high rate and *Bacillus* endospores should be used instead of vegetative *Bacillus* cell. (Suárez et al., 2011; Chen et al., 2013). As a result, the stored pelleted had a low survival bacteria rate. In subsequent pelleting experiments, bacterial endospores should be used instead of the vegetative cell because *Bacillus* can produce endospore-

forming within the cell, 1 cell per 1 spore, which protect microorganism more resistant to chemicals, radiation and heat than vegetative cells lead to maintaining higher survival rate (Kloepper et al., 2004).

5.7 Contamination test of seed pelleted

Investigation of the amount of contamination of other microorganisms in the pelleted seeds (SeedBio 3) until 6 months of storage revealed that bacterial contamination was found at 5.31% at the initial time and 1.63% at the end time while the amount of fungal contamination was low, from 0.00 to 0.03% during the stored time. In the experiment and storage, it should be packed in sealed containers without leaks or damage because external moisture may enter the container, which may result in contamination of the pelleted seeds. Hill (1999) and Gregg and Billups (2010) reported that the success of seed pelleting was depending on the appropriate binder and filler materials. This is because the pelleting material plays an important role in changing shape, size, seed weight and shelf life. Therefore, the good pelleting materials should have the property of being able to form seeds easily, and have consistent particle size. It does not impede the permeability of water, oxygen gas. And most importantly, it is not easily contaminated or adversely affects seed quality and germination. Normally, the most popular poultice materials used for seed dressing are talcum, calcium carbonate, vermiculite, pumice, gypsum, bentonite, dolomite, calcium sulfate and zeolite (Taylor and Harman, 1990). According to research on cotton seed masking (*Gossypium hirsutum* L.) using vermiculite as a pelleted material, it increased germination by 24.50% and contaminated during storage by 5.50% (Evlakova, 1985). In addition, Kangsopa and Siri (2018) reported that the pelleting of lettuce seeds with calcium sulfate and carboxymethyl cellulose (CMC) made it easier to form the seeds. The seeds had the highest germination and there is a low amount of contamination from other microorganisms after storage.

5.8 Applying seed Bio-pelleted and foliar sprayed by using *B. subtilis* 168-2 in the organic farming system

From this study, it was found that seed bio-pelleting (SeedBio 3) and foliar spraying 4 times every week by using *B. subtilis* 168-2 at 10^8 cfu/ml was effective in controlling leaf spot disease by inducing resistance mechanism of lettuce against pathogenic fungi. Interestingly, the seed bio-pelleting combined spraying *B. subtilis* 168-2 (Treatment 5: T5) significantly reduced leaf spot disease lower than Mancozeb fungicide (Treatment 7: T7) but higher than in increasing fresh and dry weight yield. The seed bio-pelleting was developed and their antimicrobial activities may contribute to valuable organic farming. It is a new alternative approach for reducing the use of fungicides in organic lettuce production.

Seed pelleting can maintain the germination rate of lettuce seeds, it is also a technology for improving seed shape to be larger than the original weight and the exact shape as desired for suitability for use in cultivation and increased ease of use with machines (Zenk, 2004; Maynard and Hochmuth, 2007; Gregg and Billups, 2010; Siri, 2015). In this experiment, the filler material was calcium sulfate and the binder was carboxymethyl cellulose and the active ingredient was *B. subtilis* 168-2 (SeedBio 3) on good seed quality and germination. This allows the seeds to germinate well and consistently, reducing the severity of the disease and promoting lettuce growth.

Interestingly, when comparing the initial cost of using *B. subtilis* 168-2 as the active ingredient in the pelleted seed (SeedBio 3) with commercial pelleted seed, the cost of bio seed pelleting in this study is 500 baht per 1,000 seeds, where 1 seed is equal to 0.5 baht. While the commercial pelleted seed price was 1,000 baht per 1,000 seeds.

REFERENCES

Abeer, H., Allah E. F., Alqarawi A. A., Huqail asma A. A. L., Alshalawi S. R. M, Wirth S. and Egamberdieva, D. (2015). Impact of plant growth promoting *Bacillus subtilis* on growth and physiological parameters of *Bassia indica* (*Indian bassia*) grown udder salt stress. Pakistan Journal of Botany. 47(5): 1735-1741.

Agrios, (1997). Plant Pathology 4th Edition. New York: Academic Press.

Ahemad, M. and Khan, M. S. (2011). Toxicological assessment of selective pesticides towards plant growth promoting activities of phosphate solubilizing *Pseudomonas aeruginosa*. Acta Microbiol Immunol Hung. 58(1): 169–187.

Anan, K. and Athinuwat D. (2016). Bio-Formulation Development from *Bacillus subtilis* TU-Orga1 for Controlling the Important Diseases of Chinese Kale. Thai Science and Technology Journal. 24 (5): 794-812.

Anderson, R. A., Conway, H. F., Pfeifer, V. F. and Griffin, E. L. (1969). Gelatinization of corn grits by roll and extrusion cooking. Cereal Science Today. 14(2): 4-12.

Akhtar, N., Qureshi, M. A., Iqbal, A., Ahmad, M. J. and Khan, K. H. (2012). Influence of Azotobacter and IAA on symbiotic performance of Rhizobium and yield parameters of lentil. Journal of Agricultural Reseach. 50(1): 361-372.

Arunyanart, P., Nilpanit, N. and Dhitikiattipong, R. (2008). Using mixture powder formulation of antagonistic bacteria *Bacillus subtilis* and some chemicals to control rice seed discoloration disease. Rice Department, Ministry of Agriculture and Cooperatives.

Asada, K. (1992). Ascorbate peroxidase–a hydrogen peroxide scavenging enzyme in plants. Plant Physiology. 55(1): 235-241.

Asaka, O. and Shoda, M. (1996). Biocontrol of *Rhizoctonia solani* Damping-Off of tomato with *Bacillus subtilis* RB14. Journal Applied and Environmental Microbiology. 62(11): 4081–4085.

Ashraf, M. (2009). Biotechnological approach of improving plant salt tolerance using antioxidants as markers, Journal Biotechnology Advances. 27(1): 84-93.

Athar, H., Khan, A. and Ashraf, M. (2008). Exogenously applied ascorbic acid alleviates salt induced oxidative stress in wheat. Journal Environmental and Experimental Botany. 63(2): 224-231.

Athinuwat, D., Chuaboon, W., Buensantei, N. and Prathuangwong, S. (2014). Efficiency of new plant growth promoting rhizobacteria on corn disease control, African Journal of. Microbiology Research 8(1): 710-717.

Avelar, S. (2012). The use of film coating on the performance of treated corn seed. Revista Brasileira de Sementes. 34(3): 186–192.

Baker, K. F. (1972). Seed pathology: In Seed Biology. Vol. 2, 317-416. Academic Press, New York. 477 pp.

Baslam, M., Morale, F., Garmendia, I. and Goicoechea, N. (2013). Nutritional quality of outer and inner leaves of green and red pigmented lettuces (*Lactuca sativa* L.) consumed as salads. Scientia Horticulturae. 151(4): 103-111.

Bennett, M. A. (1998). The use of biologicals to enhance vegetable seed quality. Seed Technology. 20(2): 198-208.

Bewley, J. D., Bradford, K. J., Hilhorst, H. W. M. and Nonogaki, H. (2013). Seeds Physiology of Development, Germination and Dormancy, Third Edition. Springer, New York, NY.

Bloemberg, G. V. and Lugtenberg, B. J. J. (2001). Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Current Opinion in Plant Biology. 4(4): 343-350.

Boydom, A., Dawit, W. and Getaneh, W. (2013). Evaluation of detached leaf assay for assessing leaf rust (*Puccinia triticium* Eriks.) resistance in wheat. Journal of Pathology and Microbiology. 4(5): 1-4.

Bharathi, R., Vivekananthan, R., Harish, S., Ramanathan, A. and Samiyappan, R. (2004). Rhizobacteria-based bio-formulations for the management of fruit rot infection in chillies, Crop Protection. 23(5): 835-843.

Bradford, K. J. (1986). Manipulation of seed water relations via osmotic priming to improve germination under different field conditions. Research Journal of Agriculture and Biological Sciences. 22(6): 33-37.

Bradley, D. J., Kjellbom, P. and Lamb, C. J. (1992). Elicitor-and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. Cell. 70(1): 21-30.

Brian, G. and Deborah, F. (2002). Biological control of plant pathogens: research, Commercialization and application in the USA. Online. (Available): http://www.apsnet.org/edcenter/advanced/topics/Pages/BiologicalControl.asp

Buakaew, S. and Siri, B. (2016). Effects of seed pelleting with methylhydroxy ethylcellulose and polyvinylprrolidone as binder on seed quality of lettuce (*Lactuca sativa* L.) Khon Kaen Agriculture Journal. 45(8): 286-291.

Buensanteai, N., Yuen, G.Y. and Prathuangwong, S. (2008). The Biocontrol Bacterium *Bacillus amyloliquefaciens* KPS46 produces auxin, surfactin and extracellular proteins for enhanced growth of soybean plant. Thai Journal of Agricultural Science. 41(34): 101-116.

Buensanteai, N. (2012). Plant diseases and their control. Institute of Agricultural, Suranaree University of Technology. 204 p.

Buensanteai, N., Thumanu, K., Sompong, M., Athinuwat, D. and Prathuangwong, S. (2012). The FTIR spectroscopy investigation of the cellular components of cassava after sensitization with plant growth promoting rhizobacteria, *Bacillus subtilis* CaSUT007. African Journal of Microbiology Research. 78(6): 603-610.

Butler, R. (1993). Coatings films and treatments. Seed World October. 19-24 p.

Cakmakci, R., M. Erat, U. Erdogan and Donmez, M.F. (2007). The influence of plant growth-promoting rhizobacteria on growth and enzyme activities in wheat and spinach plants. Journal of Plant Nutrition and Soil Science. 170(2): 288-295.

Campbell, R. (1989). Biological control of microbial plant pathogens, Cambridge University Press, Cambridge. 232 p.

Cohen, Y., Niderman, T., MoKsinger, E. and Fluhr, R. (1994). b-Aminobutyric acid induces the accumulation of pathogenesis-related proteins in tomato (*Lycopersicon esculentum* L.) plants and resistance to late blight infection caused by *Phytophthora infestans*. Plant Physiology. 104(1): 59-66.

Coraspe, H. M., H. G. Idiarte and Minami, K. (1993). Avaliacao do efeito da peletizacao sobre o vigor de sementes de alface (*Lactuca sativa* L.). Scientia Agricola, Piracicaba. 50(3): 349-354.

Chantarat, T., Chamswarng, C. and Intanoo, W. (2009). Development of production and formulation of antagonistic bacteria for biological control of anthracnose disease. The 9th National Crop Protection Conference, Thai Crop Protection Honor the Buddha According to The Sufficiency Economy.

Chen, Z., Ricigliano, J. W. and Klessig, D. F. (1993). Purification and characterization of a soluble salicylic acid-binding protein from tobacco. Proceedings of the National Academy of Sciences of the United States of America. 90(9): 9533–9537.

Chen, K. N., Chen, C. Y., Line, Y. C. and Chen, M. J. (2013). Formulation of a novel antagonistic bacterium based biopesticide for fungal disease control using microencapsulation techniques. Journal of Agricultural Science. 5(3): 153-163.

Cho, S. J., Lee, S. K., Cha, B. J., Kim, Y. H. and Shin, K. S. (2003). Detection and characterization of the *Gloeosporium gloeosporioides* growth inhibitory compound Iturin a form *Bacillus subtilis* strain KS03. Journal of the Federation of European Microbiological Societies. 223(1): 47-51.

Cipriano, M. A., Lupatini, M. L., Lopes-Santos, M. J., Silva, D. A., Roesch, L. F., Destéfano, S. A., Freitas, S. S. and Kuramae, E. E. (2016). Lettuce and rhizosphere microbiome responses to growth promoting *Pseudomonas* species under field conditions. FEMS Microbiology Ecology. 92(12): 10-109.

Collins, D. P. and Jacobsen, B. (2003). Optimizing a *Bacillus subtilis* isolate for biological control of sugar beet Cercospora leaf spot. Biological Control. 26(5): 153-161.

Copeland, L. E. and McDonald, M. B. (1995). Seed Science and Technology. Champ & Hall: New York.

Czabator, F. J. (1962). Germination value: An index combining speed and completeness of pine seed germination. Forest Science. 8(9): 386-395.

Damsup, C. and Bumee, R. (2017). Determination of vitamin C in some banana by HPLC and UV-Visible technique. Research Kamphaeng Phet Rajabhat University, The 4th National Conference. 45(4): 980-985.

Doijode, S. D. (2006). Seed quality in vegetable crops. In: Basra, AS (Ed.). Handbook of Seed Science and Technology, The Haworth Press, NY, USA. 67(7): 677-702.

Doungkaew, N., Chuaboon, W. and Athinuwat, D. (2015). Beneficial bacteria induced GABA accumulation in germinated organic brown rice. Thai Journal of Science and Technology. 4(2): 123-132.

Eggert, D., Naumann, M., Reimer, R., and Voigt, C. A. (2014). Nanoscale glucan polymer network causes pathogen resistance. Scientific reports. 4(1): 1-6.

Ellis, R. H. (2004). Seed and seedling vigor in relation to crop growth and yield. Journal of Plant Growth Regulation. 11(3): 249-255.

Epperlein, M. M., Noronha-Dutra, A. A. and Strange, R. N. (1986). Involvement of the hydroxyl radical in the abiotic elicitation of phytoalexins in legumes. Physiological and Molecular Plant Pathology. 28(8): 67-77.

Evlakova, E. S. (1985). Effect of concentration of physiologically active compounds on germination of pelleted cotton seeds. Materially-republicans-koi-nauchno teoreticheskoi konferenstsil molodykh-uchenykh-i-spectsialistov tadzhikskoi SSR-sektsiya-Biology. 35(9): 50-55.

FAO, (2013). Statistical Yearbook of the Food and Agricultural Organization for the United Nations. Online. (Available): http://www.fao.org/3/i3138e/i3138e.pdf

Ferreres, F., Gil, M. I., Castañer, M. and Tomás-Barberán, F. A. (1997). Phenolic metabolites in red pigmented lettuce (*Lactuca sativa*) changes with minimal processing and cold storage. Journal of Agricultural and Food Chemistry. 45(11): 4249-4254.

García de Salamone, I. E., Hynes, R. K. and Nelson, L. M. (2001). Cytokinin production by plant growth promoting rhizobacteria and selected mutants. Canadian Journal of Microbiology. 47(5): 404-411.

Glick, B. R., Patten, C. L., Holguin, G. and Penrose, D. M. (1999). Biochemical and genetic mechanisms used by plant growth promoting bacteria. Imperial College Press, London, UK.

Glick, B. R. (2012). Plant growth-promoting bacteria: mechanisms and applications. Scientifica. doi: 10.6064/2012/963401.

González-Lamothe, R., Tsitsigiannis, D. I., Ludwig, A. A., Panicot, M., Shirasu, K. and Jones J. D. (2006). The U-box protein CMPG1 is required for efficient activation of defense mechanisms triggered by multiple resistance genes in tobacco and tomato. The Plant Cell. 18(9): 1067-1083.

Gregg, B. and Billups, L. (2010). Seed Conditioning Volume Two Technology Part-A. Science Publishers, New York.

Greipsson, S. (2001). Effects of stratification and GA3 on seed germination of a sand stabilizing grass *Leymus arenarius* used in reclamation. Seed Science and Technology. 29(8): 1-10.

Halmer, P. (1987). Technical and commercial aspect of seed pelleting and film coating. British Protection Council. 39: 191-204.

Hammerschmidt, R., Nuckles, E. M. and Kuc. J. (1982), Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*, Physiological Plant Pathology. 20(8): 73-82.

Hammerschmidt, R. (1999). Induced disease resistance: how do induced plants stop pathogens. Physiological Plant Pathology. 55(6): 77–84.

Hernández-Blanco, C., Feng, D. X., Hu, J., Sánchez-Vallet, A., Deslandes, L., Llorente, F., Berrocal-Lobo, M., Keller, H., Barlet, X., Sánchez-Rodríguez, C., Anderson, L. K., Somerville, S., Marco, Y. and Molina, A. (2007). Impairment of cellulose synthases required for Arabidopsis secondary cell wall formation enhances disease resistance. The Plant Cell. 19(3): 890-903.

Heydecker, W. and Coolbear, P. (1977). Seed treatments for improved performance-survey and attempted prognosis. Seed Science and Technology, 5, 353–425.

Hill, H.J., (1999). Advances in Seed Technology. Original of New seeds. The Haworth Press, Inc. 1(1):88-100.

Hirota, H., M. Fukuyama and Kanno, T. (1989). Improving seed pellets for grassland renovation. Conference paper Proceedings of the XVI International Grassland Congress, 4-11 October 1989. Nice, Fran.

Hunt, M. D. and Ryals, J. A. (1996). Systemic acquired resistance signal transduction. Critical Reviews in Plant Sciences. 15(7): 583-606.

Inthasan, J., Dechjirarattanasiri, C. and Boonmee, P. (2017). Effect of bacteria-producing indole-3-acetic acid (IAA) on growth and nutrient contents of bird chili (*Capsicum annuum* L.). Faculty of Agriculture, Chiang Mai University, Journal of Agriculture. 33(3): 333-344.

ISTA, (2019). International Rules for Seed Testing, Edition 2019. ISTA, Switzerland.

Jeffs, K. A. and R. J. Tuppen. (1986). Applications of pesticides to seeds. Requirements for efficient treatment of seeds. Seed treatment, British Crop Protection Council, Thornton Heath/Surrey, England. 3(7): 17-45.

Jiraporn, I., Chatprawee, D.and Pravit, B. (2017). Effect of bacteria-producing indole-3-acetic acid (IAA) on growth and nutrient contents of bird chili (*Capsicum annuum* l.). Journal of Agriculture. 33(3): 333-344.

John, C., Ferencz, S., Denes, A. and Scott, M. T. (2000). Modification of seed germination performance through cold plasma chemistry technology. Crop Science. 40(7): 1706-1718.

Kaewkham, T. (2017). Seed enhancements with plant growth promoting bacteria. Khon Kaen Agriculture Journal. 45(1): 197-208.

Kangsopa, J. and Siri, B. (2013). Effects of seed pelleting with pumice zeolite and bentonite on seed quality of tobacco (*Nicotiana tabacum* L.) Khon Kaen Agriculture Journal. 41(1): 257-262.

Kangsopa, J. and Siri, B. (2014). The influence of adhesive materials for tobacco seed pelleting on the quality of tobacco seeds. Khon Kaen Agriculture Journal. 42(2): 201-210.

Kangsopa, J. and Siri, B. (2015a) Effects of seed pelleting with chemical substances on seed quality after different storage under conditions of tobacco seed (*Nicotiana tabacum* L.). Khon Kaen Agriculture Journal. 43 (2): 331-342.

Kangsopa, J. and Siri, B. (2015b). Using potential carboxymethyl cellulose and hydroxypropyl methylcellulose as binder for seed pelleting of lettuce (*Lactuca sativa* L.) seeds. Khon Kaen Agriculture Journal. 43 (1): 268-273.

Kangsopa, J., Russell, K. and Siri, B. (2018). Effects of seed treatment with plant growth promoting bacteria on germination and growth of lettuce. Journal of Agriculture. 34(3): 385-397.

Karakurt, Y., Unlu, H., Unlu, H. and Padem, H. (2009). The in-fluence of foliar and soil fertilization of humic acid on yield and quality of pepper. Acta Agriculturae Scandinavica B. 59: 233–237.

Kastyak-Ibrahim, M. Z., Nasse, M. J., Rak, M., Hirschmugl, C., Del Bigio, M. R., Albensi, B. C. and Gough, K. M. (2012). Biochemical label-free tissue imaging with subcellular-resolution synchrotron FTIR with focal plane array detector. Neuroimage. 60(1): 376-383.

Kaufman, G. (1991). Seed coating: a tool for stand establishment; a stimulus to seed quality. Horttechnology. 89(1): 98–102.

Kenneth, M. D., Lawrence, C.D. (2005). Use of infrared microspectroscopy in plant growth and development. Applied Spectroscopy Reviews. 40: 301-326.

Keshavulu, K. and Krishnasamy, V. (2005). Effect of seed colouring on seed quality and bruchid damage in soybean. Seed Research. 33(2): 208-210.

Khorasani, A. C. and Shojaosadati, S. A. (2017). Starch and carboxymethylcellulose-coated bacterial nanocellulose-pectin bionanocomposite as novel protective prebiotic matrices. Food Hydrocolloids. 63(1): 273-285.

KhuhaKan, P. (2011). Testing of indigenous rhizobacteria on the growth and disease suppression of root rot in hydroponically grown lettuce. Naresuan Phayao Journal. 1(4): 3-9.

Kim, M. J., Moon, Y, Tou, J. C., Mou, B. and Waterland, N. L. (2016). Nutritional value, compounds and health benefits of lettuce (*Lactuca sativa* L.). Journal of Food Composition and Analysis. 89(49): 19-34.

Kloepper, J. W., Leong, J., Teintze, M. and Scroth, M. N. (1980). Enhancement of plant growth by siderophores produced by plant growth promoting rhizobacteria. Nature (London). 286(1): 885-886.

Kloepper, J. W., Ryu, C. M. and Zhang, S. (2004). Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology. 94(3): 1259-1266.

Koike, S. T. (2015). Pythium Wilt Disease of lettuce causing losses. Online. (Available): http://ucanr.edu/blogs/blogcore/postdetail.cfm?postnum=19327.

Kubik, K. (2002). Seed Pelleting an art. Online. (Available): http://goo.gl/EtwY53.

Lakshminarayana, K., Narula, N., Hooda, I. S. and Faroda, A. S. (1992). Nitrogen economy in wheat (*Triticum aestivum*) through use of *Azotobacter chroococcum*. Indian Journal Agricultural Sciences. 62(1): 75-76.

Leelasuphakul, W., Sivanunsakul, P. and Phongpaichit, S. (2006). Purification, characterization and synergistic activity of β-1,3-glucanase and antibiotic extract from an antagonistic *Bacillus subtilis* NSRS 89-24 against rice blast and sheath blight pathogens. Enzyme and Microbial Technology. 38(2): 990-997.

Loreto, F. and Velikova, V. (2001). Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes. Plant Physiology. 127: 1781-1787.

Thumanu, K., Wongchalee, D., Sompong, M., Phansak, P., Le Thanh, T., Namanusart, W., Vechklang, K., Kaewnum, S. and Buensanteai, N. (2017). Synchrotron-based FTIR microspectroscopy of chili resistance induced by *Bacillus subtilis* strain D604 against anthracnose disease. Journal of Plant Interactions. 12(1): 255-263.

Liang, J. G., Tao, R. X., Hao, Z. N., Wang, L. P. and Zhang, X. (2011). Induction of resistance in cucumber against seedling damping-off by plant growth-promoting rhizobacteria (PGPR) *Bacillus megaterium* strain L8. African Journal of Biotechnology. 10(2): 6920-6927.

Loper, J. E. and Schroth, M. N. (1986). Influence of bacterial sources of indole-3-acetic acid on root elongation of sugar beet. Phytopathology. 76(1): 386-389.

Maguire, J. D. (1962). Speed of germination aid in selection and evaluation for seeding emergence and vigor. Crop Science. 2(2): 176-177.

Mayak, S., Tirosh, T. and Bernard, R. (2004). Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. Plant Physiology and Biochemistry. 42(1): 565–572.

Maynard, D. and Hochmuth, J. (2007). Handbook for Vegetable Growers, 5 Edition. John Wiley & Sons, Inc., Hoboken, New Jersey, Canada.

McCann, M. C., Hammouri, M., Wilson, R., Belton, P. and Roberts, K. (1992). Fourier transform infrared microspectroscopy is a new way to look at plant cell walls. Plant Physiology. 100(4): 1940-1947.

McDonald, M. B. (2000). Seed Technology and its Biological Basic. Sheffield Academic Press, England. Seed Priming. 45(1): 287-325.

McDonald, M. B. and Kwong, F. Y. (2005). Flower Seed Biology and Technology. CABI Publishing, Wallingford, UK.

Mo, J. Y., Navi, S. S., Li, X., Guo, T. and Yang, X. B. (2007). Detached leaf assay: A rapid screening technique to study foliar diseases of soybean. Plant Pathology Presentations and Posters. Lowa State University. 49(1): 287-425.

Mohamed, H. I. and Gomaa, E. Z. (2012). Effect of plant growth promoting *Bacillus subtilis* and *Pseudomonas fluorescens* on growth and pigment composition of radish plants (*Raphanus sativus*) under NaCl stress. Photosynthetica. 50(3): 263-272.

Monson, R. K., Jones, R. T., Rosenstiel, T. N. and Schnitzler, J. P. (2013). Why only some plants emit isoprene? Plant, Cell & Environment. 36(3): 503-516.

Musiksang, H. (2008). Selection of lactic bacteria in chickens and increasing the survival of the infection by encapsulation. Master of Science Thesis, Biotechnology, School Prince of Songkla University, Thailand. 116 p.

Nafisi, M., Fimognari, L. and Sakuragi, Y. (2014). Interplays between the cell wall and phytohormones in interaction between plants and necrotrophic pathogens. Phytochemistry. 112: 63-71.

Nikaji, J. (2016). DevelopmenT of formulation and application of *Bacillus subtilis* for controlling soft rot disease of Chinese mustard. Master's Thesis, School of Crop Production Technology, Institute of Agricultural Technology, Suranaree University of Technology, Thailand. 100 p.

Nongnuch, K. (2008). A study on seed development and inheritance of yield components in lettuce (*Lactuca sativa* L.). Master of Science in Horticulture, Faculty of Agriculture Production, Maejo University, 89 p.

Nuengchamnong, N., Krittasilp, K. and Ingkaninan, K. (2009). Rapid screening and identification of antioxidants in aqueous extracts of *Houttuynia cordata* using LC-ESI-MS coupled with DPPH assay. Food Chemistry. 117(1): 750-756.

Office of Agricultural Economics, (2016). Thailand Trading Report. Online. (Available): http://www.oae.go.th.

Okigbo, R. N. and Osuine, M. I., (2003). Fungal leaf spot of mango (*Magifera indica* L.) in Southeastern Nigeria and biological control with *Bacillus subtilis*. Plant Protection Science. 39(2): 70-77.

Olivera, M. E., Ferrari, L., Araoz S. and Postulka, E. B. (2016). Improvements on physiological seed quality of *Festuca arundinacea* Schreb by encrusting technology: products and storage effects. Research Journal of Seed Science. 10(1): 33-37.

Oteino, N., Lally, R. D., Kiwanuka, S., Lloyd, A., Ryan, D., Germaine, K. J. and Dowling, D. N. (2015). Plant growth promotion induced by phosphate solubilizing endophytic *Pseudomonas* isolates. Frontier in Microbiology. 6(1): 745.

Patten, C. L. and Glick, B. R. (2002). Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. Applied and Environmental Microbiology. 68(8): 3795-3801.

Pedrini, S., D. J. Merritt, J. Stevens, and Dixon, K., (2017). Seed Coating: Science or Marketing Spin. Trends in Plant Science. 22(2): 106-116.

Phialathounheuane, K., Thummabenjapone, P., Hiransalee, A. and Techawongstien, S. (2012). Screening chilli cultivars for broad spectrum resistance to anthracnose. Khon Kaen Agriculture Journal. 40 (4): 41-47.

Pilet, P. E. and Saugy, M. (1985). Effect of applied and endogenous indol-3yl-acetic acid on maize root growth. Planta. 164(2): 254-258.

Prakash, M., Georgin, A., Narayanan, G., Anandan, R., Baradhan, G. and Sureshkumar, S. M. (2018). Effect of organic seed pelleting on seedling quality, gas exchange, growth, yield and resultant seed quality parameters of black gram. Agricultural Research Communication Center. 18(1) 1-7.

Preecha, C. and Prathuangwong, S. (2009). Development of *Bacillus amyloliquefaciens* KPS46 formulation for control of soybean disease, In Proceedings of the ISSAAS International Conference, February, 23-27, Bangkok.

Ramputh, A. I. and Bown, A. W. (1996). Rapid [gamma]-aminobutyric acid synthesis and the inhibition of the growth and development of oblique-banded leaf-roller larvae, Plant Physiology. 111(1): 1349-1352.

Ramzan, N., Noreen, N., Perveen, Z. and Shahzad, S. (2016). Effect of seed pelleting with biocontrol agents on growth and colonization of roots of mungbean by root-infecting fungi. Journal of The Science of Food and Agriculture. 16(1): 369-700.

Raskin, I., Turner, I. M. and Melander W. R. (1989). Regulation of heat production in the inflorescences of an arum lily by endogenous salicylic acid. Proceedings of the National Academy of Sciences USA. 86(2): 2214-2218.

Raskin, I. (1992). Role of salicylic acid in plants. Online. (Available): http://www.annualreviews./doi/abs/10.1146/annurev.pp.43.060192.002255?journalCode=arplant.2

Rekha, P. D., Lai, W., Arun, A. B. and Young, C. C. (2007). Effect of free and encapsulated *Pseudomonas putida* CC-FR2-4 and *Bacillus subtilis* CC-pg104 on plant growth under gnotobiotic conditions. Bioresource Technology. 98(2): 447-451.

Rimmer, R. S., Shattuck, V. I. and Buchwaldt, L. (2006). Compendium of Brassica Diseases, The American Phytopathological Society Press, Minnesota. 117 p.

Rotniam, W. (2009). Screening and preparation of *Bacillus subtilis* formulation for control leaf spot disease caused by *Alternaria longipes* of lettuce in hydroponic condition. Master's Thesis, Soil resources management, Prince of Songkla University, Thailand. 98 p.

Saarakkala, S. and Julkunen, P. (2010). Specificity of Fourier transform infrared (FTIR) microspectroscopy to estimate depth-wise proteoglycan content in normal and osteoarthritic human articular cartilage. Cartilage. 1(4): 262-269.

Saengchan, C. (2014). Control of bacterial soft rot disease caused by *Erwinia carotovora* pv. *carotovora* in Chinese green must and using resistance elicitors. Master's Thesis, School of Crop Production Technology, Institute of Agricultural Technology, Suranaree University of Technology, Thailand. 104 p.

Salisbury, F. B. (1994) The role of plant hormones. In Plant Environment Interactions. Ed. Wilkinson, R. E. Marcel Dekker, New York, USA. 39-81 p.

Schaerer, H. (2012). Seed treatments for healthy vegetable seedlings. Cost Action FA1105 Meeting - Organic Greenhouse Horticulture, State of the Art and Future Trends, 15–17 October, Bucharest, Romania.

Schneider-Mullar, S., Kurosaki, F. and Nishi, A. (1994). Role of salicylic acid and intracellular Ca^{2+} in the induction of chitinase activity in carrot suspension culture, Physiological and Molecular Plant Pathology. 45(2): 101-109.

Schmitt, A., Koch, E., Stephan, D., Kromphardt, C., Jahn, M. and Krauthausen, H. J. (2009) Evaluation of non-chemical seed treatment methods for the control of *Phoma valerianellae* on Lamb's lettuce seeds. Journal of Plant Disease Protection. 116(1): 200-207.

Sikhao, P. (2016). Seed coating for anti-counterfeiting seeds. Journal of King Mongkut's Agricultural, 34(3), 157-163.

Sinclair, T. R. (1998). Historical changes in harvest index and crop nitrogen accumulation. Crop Science. 38(1): 638-643.

Siri, B. (2015). Seed Conditioning and Seed Enhancements. Klungnanavitthaya Press, Khon Kaen. 239 p.

Sivasakhti, S. Usharani, G. and Saranraj, P. (2014). Biocontrol potentiality of plant growth promoting bacteria (PGPR)- *Pseudomonas fluorescence* and *Bacillus subtilis*: A review. African Journal of Agricultural Research. 9(2): 1265-1277.

Smith, A. E. and Miller, R. (1987). Seed pellets for improved seed distribution of small seeded forages crops. Journal of Seed Technology. 11(4): 42-51.

Sompong, M., Wongkaew, S., Tantasawat, P. and Buensanteai, N. (2012). Morphological, pathogenicity and virulence characterization of *Sphaceloma ampelinum* the causal agent of grape anthracnose in Thailand. African Journal of Microbiology Research.

Soontarasing, S. (1994). Diseases of Vegetables and Prevention. Kasetsart University, Department of Plant Pathology, Faculty of Agriculture, Bangkok, 390 p.

Soulange, J. G., and Levantard, M. (2008). Comparative studies of seed priming and pelleting on percentage and meantime to germination of seeds of tomato (*Lycopersicon esculentum* Mill.). African Journal of Agricultural Research. 3(10): 725-731.

Srimai, K. and Akarapisarn, A. (2014). *Bacillus subtilis* LBF02 as biocontrol agent against leaf spot diseases caused by *Cercospora lactucae-sativae* in lettuce. Journal of Agricultural Science. 6(3): 60-89.

Shahab, S., Ahmed, N. and Khan, N. S. (2009). Indole acetic acid production and enhanced plant growth promotion by indigenous PSBs. African Journal of Agriculture Research. 4(11): 1312-1316.

Shashibhaskar, M. S., Vasudevan, S. N., Nagabhushan, S. N. and Ramanjinappa, V. (2011). Effect of seed pelleting treatment on growth seed yield and quality of tomato (*Lycopersicum essulentum* Mill.) cv. PKM-1. Plant Archives. 11(6): 443-445.

Sticher, L., Mauch-Mani, B. and Métraux, J. P. (1997). Systemic acquired resistance. Annual Review of Phytopathology. 35(3): 235-270.

Suárez, M. H., Castillo, F. D. H. Morales, G. G., Saldivar, R. H. L., Herrera, R. R. and Aguilar, C. N. (2011). Biocontrol of Soil Fungi in Tomato with Microencapsulates Containing *Bacillus subtilis*. American Journal of Agricultural and Biological Sciences. 6(2): 189-195.

Suma, N. (2005). Studies of seed quality enhancement techniques in sesamum (*Sesamum indicum* L.) cv. Co 1 in sesamum. M.Sc. Thesis, Tamil Nadu Agricultural University, Coimbatore.

Sweeting, L. M. (1996). Isoprene, terpenes and natural alkenes and alkynes. Online. (Available): http://pages.towson.edu/ladon/enrich/terpene.pdf

Tatyane, S., Valdman, E., Valdman, B. and Selma, G. F. L. (2007). Salicylic acid degradation from aqueous solution using *Pseudomonas fluourescens* HK44: Parameter study and application tools, Brazilian Journal of Microbiology. 38(1): 39-44.

Taylor, A. G. and Harman, G. E. (1990). Concepts and technologies of selected seed treatments. Annual Review of Phytopathology. 28(2): 321-339.

Taylor, A. G., Allen, P. S., Bennett, M. A., Bradford, K. J., Burris, J. S. and Misra, M. K. (1998). Seed enhancements. Seed Science Research. 8(3): 245-256.

Thumanu, K., Sompong, M., Phansak, P., Nontapot, K., and Buensanteai, N. (2015). Use of infrared microspectroscopy to determine leaf biochemical composition of cassava in response to *Bacillus subtilis* CaSUT007. Journal of Plant Interactions. 10(1): 270-279.

Thumanu, K., Wongchalee, D., Sompong, M. and Buensanteai, N. (2017). Synchrotron based FTIR microspectroscopy of chili resistance induced by *Bacillus subtilis* strain D604 against anthracnose disease. Journal of Plant Interactions. 12(1): 255-263.

Tongpamnuk, P. (1997). Seed coating and pelleting and its application. Research Abstracts Conducted by University's Lecturers in Thailand during 1995-1997, Kasetsart University, Bangkok, Thailand. Research and Development. Inst. Central Laboratory & Greenhouse, 38 p.

Trachoo, S. and Siri, B. (2015). Seed pelleting with pumice talcum and green cal on seed germination and vigor of Virginia tobacco (*Nicotiana tabacum* L.). Khon Kaen Agriculture Journal. 43(1): 2313-2320.

Trachoo, S., Luangchaisri, N. and Siri, B. (2016). Seed pelleting with plant nutrients on seed quality, seedling growth and storability of tobacco seed. King Mongkut's Agricultural Journal. 34(1): 100-108.

Tumniarattanapong, T. (2005). Biology and pathogenicity of *Alternaria brassicicola* causing leaf spot of Chinese mustard and pre-harvest induced resistance against this disease. Master of Science (Agriculture), Faculty of Agriculture, Kasetsart University. 119 p.

Turan, M., Ataoglu, N. and Sahin, F. (2005). Evaluation the capacity of phosphate solubilizing bacteria and fungi on different forms of phosphorus in liquid culture. Journal of Sustainable Agriculture. 28(3): 99-108.

Udomsak, U., Kositcharoenkul, N., Phuwongphet, B. and Soakwong, W. (2013). Efficacy test of *Bacillus subtilis* for controlling *Alternaria brassicicola*, causal agent of kale leaf spot. Research and Development Results in 2013, Pest Management Groups Research, Development and Crop Protection. 56(1): 470-478.

Vallad, G. E. and Goodman, R. M. (2004). Systemic acquired resistance and induced systemic resistance in conventional agriculture. Crop Science. 44(6): 1920-1934.

Veena, M. Gouda. (2007). Studies on seed pelleting in onion (*Allium cepa* L.). Master of Science (Agriculture) Seed Science and Technology. Department of Seed Science and Technology College of Agriculture, Harvard University of Agricultural Sciences.

Volgas, G. C., Downer, R. A. and Lopez, H. B. (2003). Pesticides formulations and application system, 23rd Volume, International Standards Worldwide, Pennsylvania, 261 p.

Vickers, C. E., Gershenzon, J., Lerdau, M. T. and Loreto, F. (2009). A unified mechanism of action for volatile isoprenoids in plant abiotic stress. Nature Chemical Biology. 5(5): 283-291.

Wang, T., Tan, S. Y., Mutilangi, W., Aykas, D. P. and Rodriguez-Saona, L. E. (2015). Authentication of Whey Protein Powders by Portable Mid-Infrared Spectrometers Combined with Pattern Recognition Analysis. Journal of Food Science. 80(10): C2111-C2116.

White, P. J. and Broadley, M. R. (2009). Biofortification of crops with seven mineral elements often lacking in human diets–iron, zinc, copper, calcium, magnesium, selenium and iodine. Journal of New Phytologist. 182(1): 49-84.

Wong, C. E., Carson, R. A. J. and Carr, J. P. (2002). Chemically induced virus resistance in *Arabidopsis thaliana* is independent of pathogenesis-related protein expression and the NPR1 gene. Molecular Plant Microbe Interaction. 15(1): 75-81.

Wongchalee, D. (2015). Efficacy of an encapsulated antagonistic *Bacillus* in controlling chili anthracnose. Master's Thesis, School of Crop Production Technology, Institute of Agricultural Technology, Suranaree University of Technology, Thailand. 147 p.

Wokocha, R. C., Nneke, N. E. and Umechuruba, C. I. (2010). Screening *Colletotrichum gloeosporioides* f. sp. *manihotis* isolates for virulence on cassava in Akwaibom state of Nigeria. Agro-Science Journal of Tropical Agriculture, Food, Environment and Extension. 9(1): 56–63.

Yanisa, B., Saengrawee, S. and Padungkwan, C. (2013). A feasibility study of alcoholic-alkaline treated rice starch for disintegrant in tablet. KKU Research Journal (Graduate Studies). 13(3): 68-77.

Yodyotee, Y. (2010). Induced resistance and expression of PR-1 gene in *Hevea brasiliensis* Muell. Arg. by various stimulants. Master's Thesis, Science in Biochemistry, Prince of Songkla University, Thailand. 120 p.

Yu, P. (2004). Application of advanced Synchrotron-based Fourier Transform Infrared microspectroscopy (SR-FTIR) to animal nutrition and feed science: A novel approach. British Journal of Nutrition. 92(6): 869-885.

Yu, P. (2008). Synchrotron-based microspectroscopic analysis of molecular and biopolymer structures using multivariate techniques and advanced multi-component modeling. Canadian Journal of Analytical Sciences and Spectroscopy. 53:220-231.

Zaidi, S., Usmani, S., Singh, B. R. and Musarrat, J. (2006). Significance of *Bacillus subtilis* strain SJ 101 as a bioinoculant for concurrent plant growth promotion and nickel accumulation in *Brassica juncea*. Chemosphere. 64(6): 991-997.

Zenk, P. (2004). Seed coatings get serious. Online. (Available): http://farmindustrynews.com/mag.

APPENDIX

I. MEDIUMS

1.1 Water agar (WA)

Agar	15 g
Water	1 L

1.2 Potato Dextrose Agar (PDA)

Potato	200 g
Dextrose	20 g
Agar	15 g
Water	1 L

1.3 Nutrient broth (NB)

Beef extract	3 g
Peptone	5 g
Water	1 L

II. CHEMICALS

2.1 0.02 M Ferric ammonium sulfate

Ferric ammonium sulfate	4.82 g
Water	500 ml

2.2 1% Triton-x 100

Triton-x 100	1 ml
Water	99 ml

2.3 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% $HClO_4$

2.4 1 M Tris-HCl buffer, pH 7

Tris base	121.1 g
Deionization water	1 L
adjust pH to 7	

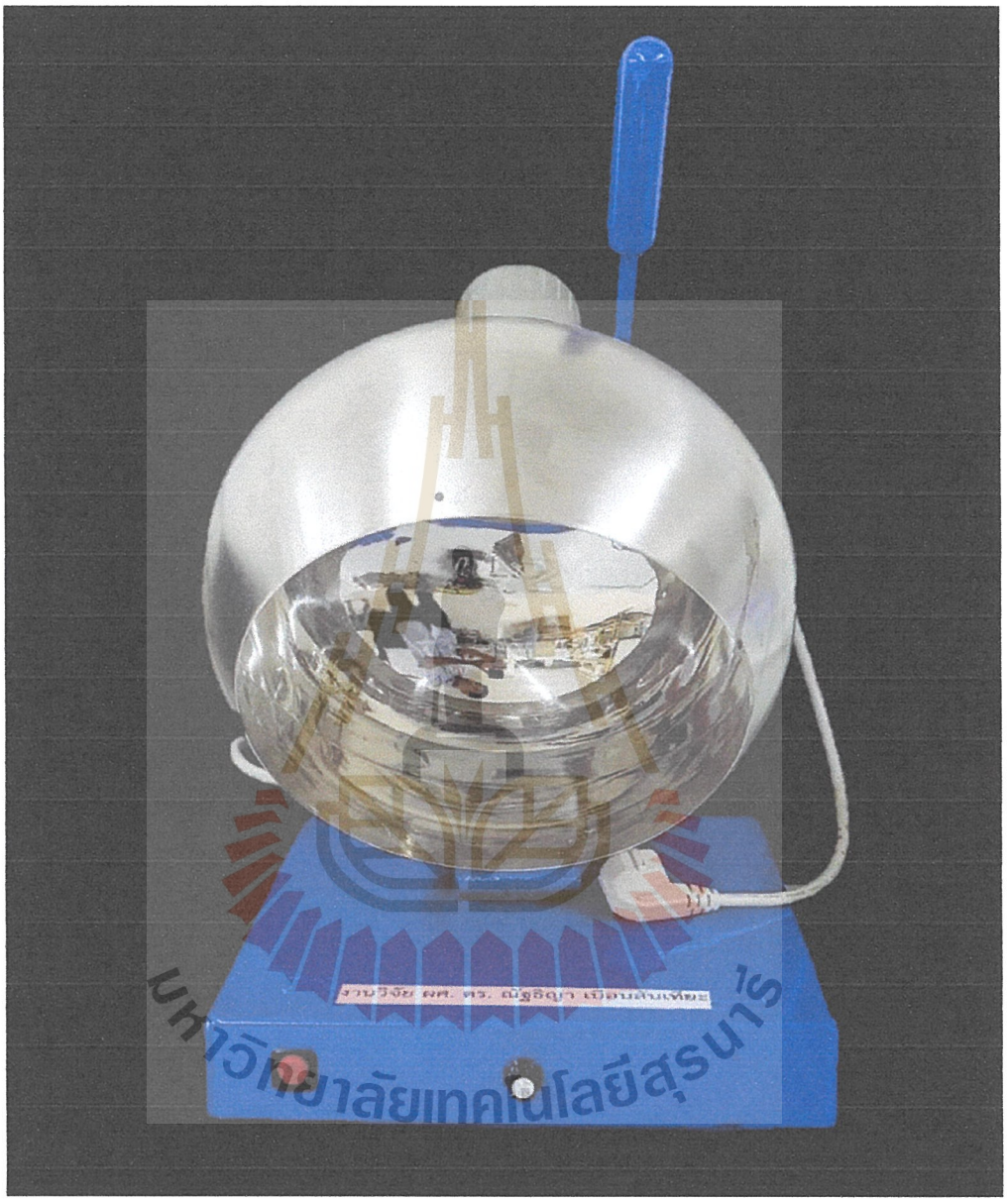
2.5 Homogenization buffer

Tris-HCl	12.1 g
KCl	7.45 g
PMSF	0.1742 g
Leupeptin	1 µg/ml
Triton X-100	10 ml
PVPP	30 g
DI Water	1 L

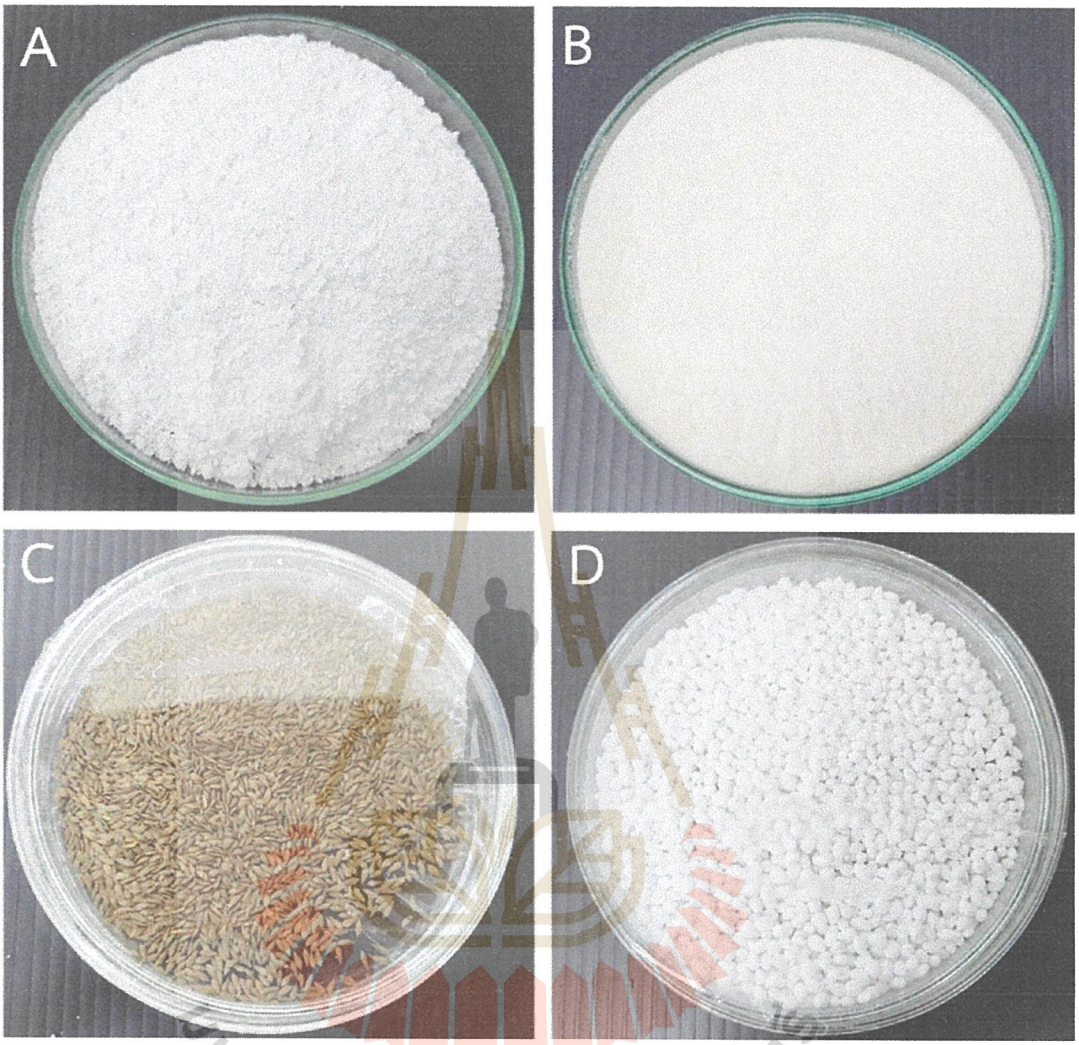
2.6 Cold homogenization buffer

EDTA	0.5844 g
NaCl	87.75 g
Tris-HCl	6.057 g
DTT (dithiothreitol)	0.0771 g
DI Water	1 L

III. ATTACHED FIGURE



Attached Figure 1. Drum coater – a seed coating equipment



Attached Figure 2. (A) Calcium sulfate – filler material, (B) Carboxymethyl cellulose – adhesive material, (C) Lettuce seed, and (D) Lettuce seed pelleted – SeedBio 3.

BIOGRAPHY

Ms. Kodchaphon Tonpho was born on December 19, 1994 at Ubon Ratchatani, Thailand. She graduated with Senior High School from Boonwattana School, Nakhon Ratchasima and stared the Bachelor Degree in Crop Production Technology at Suranaree University of Technology in 2013. she graduated in Semester 3/2016.

She obtained Master's Program School of Crop Production Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. During their study, she received she won a scholarship from the External Grants and Scholarships for Graduate Students (OROG) Scholarships 2017-2019 a teaching assistant scholarship. Then, she presented research work in the topic " Effect of Bio Seed-Pelleting with *Bacillus subtilis* to Control Leaf Spot Disease on Lettuce " in the SUT International Virtual Conference on Science and Technology (IVCST 2021), August 6, 2021, at Suranaree University of Technology.