INDUCTION OF PHYTOALEXIN BIOSYNTHESIS AND ITS ROLE AGAINST *PYTHIUM* ROOT ROT OF LETTUCE (*LACTUCA SATIVA* L.) GROWN IN HYDROPONICS

CHULALAK TALUBNAK

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENT FOR THE DEGREE OF
DOCTOR OF BIOTECHNOLOGY IN PLANT PATHOLOGY
INTERNATIONAL COLLEGE
KING MONGKUT'S INSTITUTE OF TECHNOLOGY LADKRABANG
2016

KMITL-2016-IC-D-009-001

INDUCTION OF PHYTOALEXIN BIOSYNTHESIS AND ITS ROLE AGAINST *PYTHIUM* ROOT ROT OF LETTUCE (*LACTUCA SATIVA* L.) GROWN IN HYDROPONICS

CHULALAK TALUBNAK

A THESIS SUBMITTED IN PARTIAL FULFILLMENT

OF THE REQUIREMENT FOR THE DEGREE OF

DOCTOR OF BIOTECHNOLOGY IN PLANT PATHOLOGY

INTERNATIONAL COLLEGE

KING MONGKUT'S INSTITUTE OF TECHNOLOGY LADKRABANG

2016

KMITL-2016-IC-D-009-001

หัวข้อวิทยานิพนธ์	การชักนำชีวสังเคราะห์ไฟโตอเล็กซินและบทบาทในการต่อต้านโรครากเน่า *Pythium* ของผักสลัด (*Lactuca sativa* L.) เจริญบนระบบไฮโดรโปรนิกส์
นักศึกษา	นางสาวจุฬาลักษณ์ ตลับนาค
รหัสนักศึกษา	53600101
ปริญญา	ปรัชญาดุษฎีบัณฑิต
สาขาวิชา	เทคโนโลยีชีวภาพทางโรคพืช
อาจารย์ที่ปรึกษาวิทยานิพนธ์	ผู้ช่วยศาสตราจารย์ ดร.นงลักษณ์ เภรินทวงค์
อาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม	รองศาสตราจารย์ ดร.ถนิมนันต์ เจนอักษร

บทคัดย่อ

โรครากเน่าผักสลัด เกิดจากเชื้อสาเหตุ *Pythium* sp. เป็นโรคที่มีความสำคัญและทำให้ผลผลิตลดลง โดยเฉพาะผักสลัดที่ปลูกในระบบไฮโดรโปรนิกส์ โรคนี้จะระบาดและมีความรุนแรงในช่วงฤดูร้อน การป้องกันกำจัดโดยทั่ว ๆ ไป มีการใช้สารเคมีในการป้องกันกำจัด แต่วิธีดังกล่าวมีอันตรายต่อสิ่งแวดล้อมและผู้บริโภค นอกจากนั้นยังทำให้เชื้อสาเหตุมีการปรับตัวเพื่อต้านทานต่อสารเคมี ทำให้วิธีการป้องกันนั้นไม่ได้ผล วิธีที่เป็นทางเลือกคือการชักนำให้ผักสลัดต้านทานต่อการเข้าทำลายของเชื้อสาเหตุคือ *Pythium* sp. เนื่องจากการสังเคราะห์สารไฟโตอเล็กซินเป็นดัชนีวัดความต้านทานที่เกิดขึ้นในพืช จึงทำการทดสอบประสิทธิภาพของเชื้อไม่ก่อโรค (*Pythium* sp. NPA1, NPA2, และ NPA3) ในการชักนำไฟโตอเล็กซินเปรียบเทียบกับสารเคมี ($CuSO_4$ และ $AgNO_3$) ผลการตรวจสอบด้วยเทคนิค Thin layer chromatography (TLC) พบการสังเคราะห์ไฟโตอเล็กซินในผักสลัดภายหลังการกระตุ้นด้วยสารเคมีที่ R_f 0.45-0.48 ภายใต้แสงยูวีที่ความยาวคลื่น 365 นาโนเมตร ในขณะที่ไม่พบไฟโตอเล็กซินเมื่อกระตุ้นด้วยเชื้อ *Pythium* sp. ที่ไม่ก่อโรค ผลการทดสอบคุณสมบัติของไฟโตอเล็กซิน พบว่าสามารถยับยั้งการเจริญของเชื้อรา *Aspergillus niger* โดยเกิด clear zone ในบริเวณดังกล่าวบนแผ่น TLC นอกจากนี้สารสกัดหยาบจากผักสลัดที่ถูกกระตุ้นด้วยสารเคมียังสามารถชะลอการงอกของสปอร์เชื้อราสาเหตุโรคพืช *Colletotrichum gloeosporioides*, *Curvularia lunata*, *Fusarium oxysporum* และ *Pythium aphanidermatum* ได้ จากนั้นคัดเลือกเชื้อ *Pythium* ไม่ก่อโรคที่มีคุณสมบัติชักนำความต้านทานต่อโรครากเน่าจากเชื้อที่เจริญอยู่ในระบบไฮโดรโปรนิกส์ โดยเก็บรวบรวม แยกเชื้อและจัดจำแนกเชื้อด้วยลักษณะทางสัณฐานและโมเลกุล จากตัวอย่างทั้งหมดที่แยกได้ 38 ไอโซเลท มี 27 ไอโซเลท ได้มาจากรากที่ไม่แสดงอาการโรค 9 ไอโซเลท ได้มาจากรากที่แสดงอาการโรค และ 2 ไอโซเลท ได้มากจากดินที่ติดเชื้อโรครากเน่า ผล

การจำแนกด้วยลักษณะทางสัณฐานและโมเลกุลพบว่าเป็น *Pythium aphanidermatum* จำนวน 23 ไอโซเลท *P. myriotylum* 11 ไอโซเลท *P. deliense* 1 ไอโซเลท และ *Pythium* sp. 3 ไอโซเลท จากนั้นคัดเลือกเชื้อ *Pythium* sp. ที่ไม่ก่อโรค โดยทดสอบการเกิดโรคและการสร้างเอนไซม์ย่อยสลายผนังเซลล์ พบว่าเชื้อส่วนใหญ่ที่แยกได้จากรากที่ไม่แสดงอาการโรคก่อให้เกิดอาการเมล็ดเน่ารากเน่ากับต้นกล้าผักสลัด แต่ความรุนแรงน้อยกว่าไอโซเลทที่แยกจากรากผักสลัดที่แสดงอาการโรค ซึ่งผลการทดสอบความสามารถสังเคราะห์เอนไซม์เซลลูเลส เพคติเนส และไคติเนส พบว่าเชื้อ *Pythium* ไม่ก่อโรคสามารถสร้างเอนไซม์เซลลูเลสและเพคติเนสได้ แต่สร้างในปริมาณที่น้อย เช่นเดียวกับเอนไซม์ไคติเนส สามารถคัดเลือกเชื้อ *Pythium* sp. ที่ไม่ก่อโรคจำนวน 2 ไอโซเลท ได้แก่ *P. aphanidermatum* ASR23 และ *Pythium* sp. SR36 นำเชื้อดังกล่าวไปทดสอบการชักนำให้ผักสลัดเกิดความต้านทานต่อโรครากเน่า และติดตามการแสดงออกของยีนที่เกี่ยวข้องกับความต้านทาน รวมทั้งกิจกรรมของ reactive oxygen species (ROS) พบว่ายีน *LsPR1b_like*, *LsLTC1*, และ *LsLTC2* มีการแสดงออกในใบผักสลัดที่ชักนำด้วยเชื้อ *Pythium* ASR23 มากกว่าที่ราก สำหรับการแสดงออกของยีน *LsLTC1*, *LsLTC2* ซึ่งเป็นยีนที่เกี่ยวข้องกับการสังเคราะห์สาร sesquiterpene ในผักสลัด มีการแสดงออกในรากหลังจากชักนำด้วย β-aminobutyric acid (BABA) และ เชื้อ *Pythium* ทั้ง 3 ไอโซเลท (ASR23, SR36, และ SR31) และแสดงออกมากขึ้นหลังจากปลูกเชื้อสายพันธุ์ก่อโรค *Pythium* SR31 แสดงว่าผักสลัดเกิดความต้านทานมากขึ้นเมื่อมีการเข้าทำลายของเชื้อ สาเหตุ การวิเคราะห์ histological พบว่าผักสลัดที่ชักนำด้วย BABA และ เชื้อ *Pythium* ASR23 และ SR36 ซึ่งเป็นเชื้อไม่ก่อโรค มีการตอบสนองต่อ PAMPs (flg22) บนใบและรากผักสลัดทั้งสามส่วน (รากส่วนบน กลาง และปลาย) สอดคล้องกับการสะสมของ ROS ที่พบบริเวณกลางรากและปลายราก ผลการทดลองนี้ได้เสนอวิธีตรวจความต้านทานของผักสลัดในระบบไฮโดรโปรนิกส์เพื่อต้านทานโรครากเน่า และคัดเลือกเชื้อไม่ก่อโรค *Pythium* ASR23 จากระบบไฮโดรโปรนิกส์ที่สามารถชักนำให้ผักสลัดต้านทานต่อโรครากเน่าที่เกิดจากเชื้อ *Pythium* ได้ ผลการวิจัยนี้สามารถใช้เป็นข้อมูลและแนวทางในการพัฒนาผักสลัดให้มีความต้านทานต่อโรครากเน่า *Pythium* ได้ เพื่อเป็นประโยชน์สำหรับเกษตรกรผู้ปลูกผักสลัดในระบบไฮโดรโปรนิกส์ต่อไปในอนาคต

คำสำคัญ: โรครากเน่า *Pythium*, ผักสลัด, ไฮโดรโปรนิกส์, Internal transcribed spacer, ไฟโตอเล็กซิน, การแสดงออกของยีน, Reactive oxygen species

INDEPENDENT STUDY TITLE	INDUCTION OF PHYTOALEXIN BIOSYNTHESIS AND ITS ROLE AGAINST *PYTHIUM* ROOT ROT OF LETTUCE (*LACTUCA SATIVA* L.) GROWN IN HYDROPONICS
STUDENT NAME	MS. CHULALAK TALUBNAK
STUDENT ID	53600101
DEGREE	DOCTOR OF PHILOSOPHY
PROGRAM	BIOTECHNOLOGY IN PLANT PATHOLOGY
ADVISOR	ASST. PROF. DR. NONGLAK PARINTHAWONG
CO-ADVISOR	ASSOC. PROF. DR. TANIMNUN JAENAKSORN

ABSTRACT

Lettuce root rot, caused by *Pythium*, is one of the most important diseases on hydroponically-grown lettuce resulting in the loss of crop yields. This disease is more severe in summer. The management of root rot pathogen commonly employs the chemical control. However, using fungicide can cause so many detrimental effects on environment and consumers. Therefore, the best way to achieve the suitable alternative methods to control root rot pathogen, is induction of lettuce resistance to *Pythium* root rot pathogen. Since phytoalexin biosynthesis is the index for disease resistance in plant, 3 non-pathogenic *Pythium* sp. (NPA1, NPA2, and NPA3) and 2 chemicals ($CuSO_4$ and $AgNO_3$) were determined to induce phytoalexin biosynthesis in lettuce. The phytoalexin of lettuce was detected on thin layer chromatography (TLC) after elicitation with chemicals under 365 nm UV light with R_f values of 0.45-0.48, whereas 3 non-pathogenic *Pythium* sp. was unable to induce phytoalexin. The antifungal activity of phytoalexin inhibited *Aspergillus niger* and showed clear zone on the TLC plates. Moreover, crude extract of lettuce elicited with chemicals could retard spore germination of *Colletotrichum gloeosporioides*, *Curvularia lunata*, *Fusarium oxysporum* and *Pythium aphanidermatum*. Hence, non-pathogenic *Pythium* sp. were selected from hydroponics for inducing the resistance to *Pythium* root rot in lettuce. Non-pathogenic *Pythium* sp. were isolated and identified by morphology and molecular characteristics. Of 38 obtained-isolates, 27

isolates were recovered from asymptomatic roots, 9 isolates from symptomatic roots, and 2 isolates from infested soil. The identification results showed that 23 isolates belonged to *Pythium aphanidermatum*, 11 isolates were *P. myriotylum*, 1 isolate was *P. deliense*, and 3 isolates were *Pythium* sp. Furthermore, the pathogenicity test and cell wall degrading enzymes test were employed for screening the real non-pathogenic *Pythium* on lettuce seeds. Most isolates from asymptomatic plants unexpectedly caused seed rot and increased disease incidence in seedlings, but disease severity was less than isolates obtained from symptomatic plants. *Pythium* was tested for their abilities to producing cellulase, pectinase, and chitinase. Most of *Pythium* species could produce a small amount of cellulase and pectinase on agar medium. There was chitinase enzyme at low to medium activity, and could only be detected in *Pythium* species from the non to weakly virulent group. From the results, 2 isolates of the non-pathogenic *P. aphanidermatum* ASR23 and *Pythium* sp. SR36 were selected to induce resistance in lettuce against *Pythium* root rot. The resistance gene expressions and reactive oxygen species (ROS) activity were examined. The results showed that the *LsPR1b_like*, *LsLTC1*, and *LsLTC2* were expressed in lettuce treated with non-pathogenic *Pythium* ASR23 than in roots. Regards to sesquiterpene synthase genes (*LsLTC1* and *LsLTC2*) in lettuce, the roots showed expression after induction with β-aminobutyric acid (BABA) and 3 isolates of *Pythium* (ASR23, SR36, and SR31) and more expression was detected when challenged with pathogenic *Pythium* SR31. Suggesting that strong resistance was induced when lettuce root was infected by the pathogenic *Pythium*. For histological analysis, treatment with BABA and non-pathogenic *Pythium* sp. ASR23 and SR36 showed PAMPs (flg22) responses in leaves and roots (top, middle, bottom). Similarly, ROS accumulation presented strong staining of the middle and bottom parts of the roots. Concludingly, the results presented the methods for inducing resistance to *Pythium* root rot, and the derived *Pythium* ASR23 that could be used to activate resistance. This research is useful for lettuce growers and those who are interested in monitoring the induction of resistance to hydroponically grown-lettuce.

Keywords: *Pythium* root rot, Lettuce, Hydroponics, Internal transcribed spacer, Phytoalexin, Gene expression, Reactive oxygen species

ACKNOWLEDGEMENTS

First of all, I would like to glorify our Lord, Jesus Christ, who has given me the strength and wisdom to complete all the important tasks with his grace, mercy, and love.

I would like to express my deepest and most sincere gratitude to Assoc. Prof. Dr. Tanimnun Jaenaksorn, from the Department of Plant Production Technology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang. She provided me with helpful suggestions and constant encouragement during the preparation of this thesis.

My sincerest gratitude also goes to Asst. Prof. Dr. Nonglak Parinthawong, from the Department of Plant Production Technology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, who provided very helpful supervision through valuable suggestions and constructive feedback. Her guidance helped me through the entire process of conducting research and writing this thesis.

I would like to extend my heartfelt gratitude and appreciation to Dr. Henk-jan Schoonbeek at John Innes Centre, Norwich Research Park, Norwich, UK, who gave me the opportunity to take part in conducting the induced resistance study, and gave me good guidance while experimental procedures were being carried out. He encourages me to work hard in order to become a good scientist.

I would like to express my gratitude to Dr. Chris Ridout at John Innes Centre, Norwich Research Park, Norwich, UK for his help, friendship and accepting me as his student worker. He gave me valuable advice in my research.

I would like to express my gratefulness to all members of the John Innes Centre, Norwich Research Park, Norwich, UK, especially Francessca, Lionel, Sam, Catherine, Sujit, and Kotai, for their help and friendship.

I would like to acknowledge the John Innes Centre, Norwich Research Park, Norwich, UK for providing laboratory instrumental support and all other facilities during conducting my part of the research in induced resistance study.

I would also like to express my genuine thanks to Worrawoot, Chaivarakun, Malatee, Siriporn, Rungarun, and Pennapa, members of the Agricultural

Biotechnology Laboratory, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, for their help, encouragement, friendship and shared knowledge in molecular biology. I would also like to thank Jarongsak, and the members of Plant Pathology Laboratory, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, who helped me both directly and indirectly in accomplishing this thesis.

I would like to acknowledge Dr. Veeranee Tongsri from Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkhaen campus, who provided 2 pathogens from durian root rot.

I would like to acknowledge the Agricultural Biotechnology Laboratory and Plant Pathology Laboratory for providing necessary facilities for conducting the thesis. Thanks also to Faculty of Agricultural Technology and International College at King Mongkut's Institute of Technology Ladkrabang.

I would like to gratefully acknowledge the Thailand Research Fund (TRF), which provided financial assistance for this project through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0286/2551).

Finally, I would like to express my sincere and deepest gratitude to my beloved family, my brother, my family in Christ, and Mr. Tanawut Tantisopharak for their endless love, acceptance, support, and warm encouragement, all of which kept me motivated while pursuing my doctoral study.

Chulalak Talubnak

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Background and Problems

Lettuce (*Lactuca sativa* L.) is an annual plant that belongs in the *Asteraceae* family, and is commercially cultivated at a very large scale in many countries throughout the world (Ryder, 1999). It is commonly incorporated into the human diet due to its good taste, nutritive quality, and low caloric intake (vit A and K, potassium) (Davey *et al.*, 2007; Dan *et al.*, 2014). The main areas where production and consumption of lettuce are abundant are within the United States and Europe (i.e., Italy, Spain, France, the Netherlands, and the United Kingdom). Furthermore, lettuce is also one of the most important greenhouse and field leafy vegetables cultivated in Thailand. It has been reported by the Food and Agriculture Organization of the United Nations (FAO) that in 2013, lettuce was the crop with the highest net import value in Thailand, adding up to approximately 3,335 one-thousand US dollars (import quantity 8,108 tonnes) higher than previous years' statistics, compared to other cultivated edible vegetables, such as roots, tubers, and chicory (FAO, 2016). It is no surprise that lettuce production in Thailand has increased tremendously over the past five years.

The global demand for safe and chemical-free food is rising, along with increasing awareness of healthier food options. The phenomenon gave rise to many new environmentally friendly agricultural techniques and increased manufacturing of organic agricultural products. The concept of food safety covers the entire scope of an agricultural food product, from cultivation to delivery, and ultimately consumption by the consumer. Lettuce, described as the "queen of the salad plants" (Martin and Ruberte, 1975), can be cultivated either with or without the presence of soil. The fact that the vegetable is easily grown and nutritious contributes to it being the one of the most common salad vegetables in the world. In recent years, overall demand for high quality lettuce with minimal or no pesticide residues has risen sharply due to increased local consumption and export demands.

Pythium root rot is one of the most serious diseases of lettuce grown in hydroponic culture and soil. *Pythium* produces flagellate zoospores that can move in nutrient solutions, allowing it to adapt well to the aquatic environment of hydroponic culture systems (Herrero *et al.*, 2003; Vallance *et al.*, 2009). Also, Buysens *et al.* (1995) reported that *Pythium* spp. are pathogens that contribute to the disturbance of the nutrient film technique (NFT) system and other agricultural systems around the world, including those in Thailand (Koohakan *et al.*, 2008). Nowadays, better agricultural practices for disease management should be considered, in order to establish a more sustainable and healthier crop ecosystem. The development of alternative strategies to improve plant disease resistance and control of pathogens should be promoted. Phytoalexin production has received immense attention, especially in the field of plant defense (Ahuja *et al.*, 2012). Accumulation and production of phytoalexin may occur in healthy plant cells, around damaged sites, or in infected plant cells. It is stimulated by harm-signaling (alarm) substances, which are produced and released by a infected plant cell. Abiotic elicitors are usually capable of inducing phytoalexin production in many crops (Angelova *et al.*, 2006; Yean *et al.*, 2009) while biotic elicitors such as microorganisms are also reported to elicit phytoalexins. (Liu *et al.*, 1995).

Lettucenin A was the first to be discovered and reported as the principal phytoalexin in soil-grown lettuce after being elicited by abiotic elicitors (Takasugi *et al.*, 1985). The isolation of Lettucenin A by Takasugi *et al.* (1985) opened up the path for further research on this compound in lettuce, with aims to improve or provide an alternative to plant disease management. Lettucenin A is suggested to contribute to the resistance of lettuce against microbial growth, *Xanthomonas campestris*. The resistance depends on a sufficient concentration of compound and the right time of exposure. It has been shown the bacterial growth rate continues to increase after two hours of incubation time (Yean *et al.*, 2009). However, most researches were conducted to examine the response of lettuce in the production of Lettucenin A only when cultivated in soil. In the present day, there is still no evidence regarding whether or not Lettucenin A can be produced in hydroponically cultivated lettuce. Therefore, I chose to study phytoalexin and its associations with induced resistance to *Pythium* root rot in hydroponic lettuce grown using abiotic and biotic elicitors.

1.2 Objectives

The overall objective of this research was to study *Pythium* resistance in lettuce grown in hydroponic systems. This objective could be further specified into the following aspect.

1) To collect and isolate *Pythium* spp. from hydroponically grown asymptomatic lettuce root.

2) To identify *Pythium* spp. using morphological and molecular characteristics.

3) To test the pathogenicity of *Pythium* spp. on lettuce.

4) To study the *in vitro* screening chitinolytic, cellulolytic, and pectinolytic activity of *Pythium* isolates using solid media.

5) To determine the possible occurrence of phytoalexin in hydroponically-grown lettuce after elicitation with abiotic elicitors at different plant ages.

6) To assess the *in vitro* antifungal activity of crude extract of elicited lettuce against conidial germination of four plant pathogenic fungi.

7) To study gene expression and resistance induction in lettuce after treatment with elicitors and exposure to pathogenic *Pythium*.

1.3 Scope of the Study

In this thesis, the collection, isolation, and identification of the root rot-inducing pathogen, *Pythium* species, from lettuce grown in hydroponics have been proposed. Afterwards, the pathogenicity test of *Pythium* root rot and phytoalexin in hydroponically grown lettuce after elicitation with abiotic and biotic elicitors have been conducted. Finally, the gene expression and resistance induction in lettuce have been studied.

Therefore, this thesis presents the methods of collection, isolation and identification of *Pythium* root rot in lettuce grown in hydroponic farms. The *Pythium* species has been identified through morphological and molecular characteristics. All the isolates of the pathogen were identified and tested for genetic variation by using

the internal transcribed spacer (ITS) region and the test of pathogenicity through seed inoculation and petri-dish assays. Some isolates of non-pathogenic *Pythium* have been used as biotic elicitors in hydroponically grown lettuce. The gene expressions of phytoalexin in lettuce (*LTC1, LTC2*), and pathogenesis-related (PR) proteins (*PR1*) were executed. Moreover, the reactive oxygen species (ROS) have been studied through peroxide and superoxide activity for further examination of induced resistance in lettuce seedlings after treatment with elicitors and exposure to pathogenic *Pythium*.

1.4 Research Setting

The identification and pathogenicity studies were conducted at the Plant Pathology Laboratory and Agricultural Molecular Laboratory, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. The study of gene expression and induced resistance in lettuce was carried out at John Innes Centre, Norwich Research Park, Norwich, UK. The research portion of the thesis began in June 2010, up until May 2016.

CHAPTER 2

LITERATURE REVIEW

This chapter reviews scientific literature on the collection and identification of *Pythium* root rot in hydroponic systems, defense mechanisms in lettuce and other plants, and phytoalexin in lettuce. In addition, specific enzymes that function in degrading the cell wall of *Pythium* species and other pathogenic fungi are identified.

2.1 Lettuce

Lettuce (*Lactuca sativa* L.) is an annual plant, belonging to the Asteraceae (Compositae) family (Dan *et al.*, 2014). Compositae is one of the largest and most widespread angiosperm or flowering families. This family contains over 25,000 species including dandelions, chicory, and endive. Lettuce is grown commercially in many countries around the world, particularly in North America, Western Europe, the Mediterranean basin, Australia, and some parts of Asia. Although lettuce is most easily cultivated under relatively cool and mild temperature conditions, it is commonly grown in some of the most temperate and subtropical areas of the world.

The lettuce plant can be characterized by height ranging from 15 to 30 cm, with colors ranging from bright green to red and yellow. It also takes on a wide range of shapes and textures. Lettuce leaves are spirally arranged on a shortened stem, forming a rosette of leaves. When the vegetative plant growth reaches a mature stage, stem elongation occurs and reproductive development begins. Different types of lettuce are classified according to their physical characteristics, or more specifically, the morphology of its fleshy leaves. This method of classification was able to yield seven types of lettuce by the international code of nomenclature for cultivated plants, namely the following: 1) crisp head, iceberg or cabbage, 2) butter head, 3) cos, 4) leaf or cutting, 5) latin, 6) stem or asparagus, and 7) the oilseed group (Ryder, 1999).

Lettuce is densely packed with primary vitamins, minerals, essential fibres crucial to the human diet, and also a great deal of water. The nutritional content varies in different areas of lettuce depending on the degree of leaf colour, where the green outer part contains higher nutritional value than the white inner part of the

leaves. Additional nutritive and health benefits may come from various biologically active compounds, such as chlorogenic acid, which may contain anticarcinogenic properties (Ryder, 1999).

2.1.1 Major Diseases of Lettuce

Hydroponic cultivation is an agricultural practice often used as a strategy to avoid root diseases and to overcome mono-cropping problems (Stanghellini and Rasmussen, 1994). However, lettuce diseases are still prominent in such methods of cultivation and production, continuously lowering the quality and yield of lettuce and giving rise to many other negative agricultural impacts. Various organisms, such as fungi, bacteria, viruses and virus-like agents, nematodes and certain abiotic factors, are the main causes of disease in lettuce plant. They may occur during various stages of plant life, such as the seedling stage, successive stages of growth and development, or the postharvest stage. Infections may occur at different parts of the plant, most commonly roots, crown, stem, leaves, flowers, fruit or seeds. For example, *Lactuca sativa* Sclerotinia is a common disease-inflicting pathogen in lettuce, which causes rotting of the basal part of stems and leaves (Chitrampalam *et al.*, 2010).

Downy mildew: It is one of the most serious and most studied diseases of lettuce caused by the oomycete *Bremia lactucae*. It can occur in almost all kinds of lettuce regardless of the location of cultivation, and can be particularly troublesome in relatively cool, moist environments. A diseased plant would contain one to many pale yellowish lesions on the upper side of its outer leaves. The lesions are usually defined by veins, giving them an angular appearance. Symptoms may appear on the cap leaves of heading lettuce. Several days after the lesions appear, sporulation takes place, usually on the underside of the leaf. The lesions later turn brown and may kill portions of the leaves or whole leaves. Lesions on butterhead lettuces are very likely to overlap the veins, thus subsequent damage is often more serious than on the other types of lettuce (Cohen *et al.*, 2010; Simko *et al.*, 2015).

Damping off disease: The most damaging and destructive pathogens are those that result in 'damping off', which can cause serious damage or loss of seeds, seedlings and young plants. Damping off disease promotes the death of small seedlings, which occur as a result of attacks by certain fungi, primarily *Pythium ultimum*, *P. aphanidermatum*, *P. myriotylum*, *Phytophthora* spp., and *Rhizoctonia*

solani. The *Pythium* and *Phytopthora* produce spores that are mobile in water. Damping off can occur during various stages of seed germination and subsequent growth of the seed. The optimum temperatures for the growth of *Pythium* and *Rhizoctonia* ranges between 20 and 30 °C, where temperatures above or below results in decreased growth activity. The moisture content of the germination medium is of one of the most important factors in determining the risks of damping-off occurrence (Agrios, 1997; Blancard *et al.*, 2006).

Collar black rot: This disease can be found among plants grown in some hydroponic farms. The plants undergo rotting, also known as "black rot", at the collar region near hydroponic substrates. Although the causal agent of this disease is unidentified, many references have reported that *Rhizoctonia* sp. is one of the possible causes (Koohakan *et al.*, 2008).

Leaf spot: This disease usually occurs during the rainy season. The symptom would first appear among the lower leaves, and then eventually would spread out or extend to the upper leaves. The infected plant would undergo weight loss due to removal of damaged leaves. The causal agent is identified as *Cercospora* sp. (Koohakan *et al.*, 2008).

Bacterial soft rot: This can occur to all types of lettuce as a secondary effect on tissue that has been previously invaded by fungi, is physically damaged, or is dead. It is one of the two most serious diseases of harvested lettuce. Soft rot can be caused by one or more of the following bacterium: *Pseudomonas marginalis*, *Pseudomonas cichorii*, and *Erwinia carotovora* (Cho, 1983; Aysan *et al.*, 2003). Infections usually develop from small soaked lesions at the succulent tissue, such as the petiole. Consequently, the lesions expand to a larger size and spread out to the leaf veins, resulting in leaf sheath rotting (Koohakan *et al.*, 2008).

Bacterial leaf spot: This disease is one of the major problems occurring in lettuce plantation fields in southern Turkey, resulting in great economic loss. Symptoms of the disease can be identified by the formation of water soaked, brown lesions, which later turns black. In some cases, lesions may also be translucent and V-shaped. The formation of these wounds causes the plant to collapse (Yigit, 2011).

Corky root rot: This disease is the second most abundant and problematic disease among lettuce plants. It is defined as the infection of lettuce by gram-

negative bacterium, *Rhizomonas suberifaciens*. The pathogen infects the taproot and main lateral roots, and may cover the entire root surface, resulting in its corky texture. Consequently, plants may wilt, become stunted, and produce small lettuce heads, which are one of the unwanted characteristics of lettuce in the selling market (Moreno-Vázquez *et al.*, 2003).

Moreover, the conditions of lettuce can also be affected by a number of physiological disorders, i.e. disorders in which no organism or virus-like entity has been identified as a cause. Rather, the disorders have been ascribed to environmental causes, such as temperature, moisture, storage conditions and other types of stress (Blancard *et al.*, 2006).

Tip burn: Tip burn is a disorder can appear on all forms of lettuce, and usually occurs at the time of harvest. Thus, damage due to tip burn may result in massive loss of entire fields of lettuce, ultimately rendering all the expenses of field cultivation and plantation operation to go to waste. Symptom expression begins with the appearance of small brown spots located near margins of a leaf. These spots may also be accompanied by necrosis in small veins around the infected area.

Rib disorders: Rib blight is the occurrence of yellow, brown, or black streaks along the midrib and secondary ribs of cap leaves and leaves located below them.

Atmospheric gas effects: A number of atmospheric gases have been shown to have toxic effects on lettuce. This includes ozone, sulphur dioxide, nitrogen dioxide, and peroxyacetyl nitrate (PAN). Plant damage occurs in the form of visible discoloration, pitting, or necrosis of leaves. When such damages can be clearly observed on lettuce, it may render the crop impossible to sell.

Postharvest disorders: Several problems often appear after harvest, during handling, storage, transport, or marketing. The most common occurrences are russet spotting, brown stain, bacterial soft rot, and grey mould.

2.1.2 The Impacts of *Pythium* Root Rot on Hydroponically-Grown Lettuce and Other Plants

Pythium is a soil-borne organism with very similar characteristics to fungi, belonging to the oomycete family, which also includes *Phytophthora* and downy

mildew inflicting organisms. *Pythium* affects seeds, germinating seedlings and the roots of all major grain crops, pastures, and horticulture crops grown in temperate and tropical environments worldwide (van der Plaats Niterink, 1981). *Pythium* species are ubiquitous in all environments, and are commonly recognized as the major cause of damping-off disease in seedlings, resulting in poor germination of seeds and poor emergence of the seedlings. They generally can be found inhabiting surface water, soil, and on dead plant and animal debris as saprophytes or as parasites of fibrous plant roots (Ho *et al.*, 2012). Ho *et al.* (2012) reported that the occurrence and distribution of *Pythium* species constitute up to 2,348 isolates upon isolation of the plant parasite from soil and water of Hainan Island, China. The collected *Pythium* can be classified into 29 different species. *Pythium* can survive in soil as oospores, which are thick walled structures resistant to adverse soil temperature and moisture conditions. These spores stay dormant in the soil until it rains, or when a crop is irrigated. Germinating seeds and growing roots release chemicals that act as growth stimulants and attractants for *Pythium* hyphae and sporangia, enabling the pathogen to grow rapidly, and the rate of root infection to be enhanced (de Boer *et al.*, 2013).

Pythium contains a white, rapidly growing mycelium. The mycelium gives rise to sporangia, which undergo direct germination through formation of one to several germ tubers, or through the production of a short hypha from which a secondary sporangium is formed. Zoospores are produced in the sporangium. The germ tuber penetrates the host tissue and initiates a new infection. In some cases, the germ tube forms another vesicle, allowing several secondary zoospores to be developed and undergo repetition of the process (van der Plaats Niterink, 1981).

Pythium causes a wide range of crops to undergo pre-emergence and post-emergence damping-off. The *Pythium* and *Phytopthora* produce spores that are mobile in water mediums. Damping off may occur at various stages during seed germination and subsequent seedling growth. The optimum temperature for maximal growth lies between 20 and 30 °C, with a decrease in growth activity at temperature conditions above or below the range. The moisture content of the germination medium is one of the most important factors in determining the incidence of damping-off. According to Paulitz (1997); Chatterton *et al.* (2004); and Liu *et al.* (2007) the main symptoms include zones of root-tip browning, stubbiness, proliferation of roots, expansive root browning or yellowing, rotting seedlings, stem

rotting, wilting at mid-day and collapsing of the plant upon pathogen invasion at the crown region. *Pythium* root rot destroys the productivity of numerous kinds of crops in soilless cultures around the world, including cucumber, tomato, sweet pepper, spinach, lettuce, nasturtium, arugula, rose, and chrysanthemum. *Pythium aphanidermatum*, *P. dissotocum*, members of *Pythium* group F, and *P. ultimum* var. *ultimum* are the causal agents responsible for such damage (Sutton *et al.*, 2006). In addition, Teymoori *et al.* (2012) reported, upon detection of the *Pythium* species, that it is responsible for root and stem rot on cantaloupes in Khorasan, Iran. The pathogenic species are identified as *Pythium aphanidermatum*, *P. ultimum* var. *ultimum*, and *P. deliense*. *P. aphanidermatum* is the most prevalent species of *Pythium* discovered within all regions. Later, Bahramisharif *et al.* (2014) published the first report identifying *P. mamillatum, P. pyrilobum*, and *P. myriotylum* as pathogens of lupin, and *P. irregulare* and *P. pyrilobum* as pathogens of oat. However, they also found some non-pathogenic *Pythium*, which can be used against the pathogenic species. The three non-pathogenic *Pythium* spp. are able to significantly reduce the impacts of disease caused by pathogenic species in lupins and oats less susceptible to infections, however, the effect cannot be observed in rooibos. In lupin, the non-pathogenic species enhance the virulence of *Phytophthora cinnamomi.* Recently, Binagwa *et al.* (2016) reported that *Pythium aphanidermatum* and *P. splendens* are widely distributed in the entire surveyed area, constituting 31.25% and 28.13% of the total *Pythium* population, respectively.

Pythium is present in almost all hydroponic systems. The fungus-like organism only infects plants through sites of damage, such as root injuries caused by transplanting, mineral toxicities, nutrient stagnation, or extreme temperature conditions. Accumulation of this disease promotes *Pythium* spore formation as the number of spores in the infected site increases. An indicator of damage by *Pythium* is the initial browning of the root tips, which would eventually blacken and undergo rotting (Figure 2.1). In addition, pathogens with fungal properties produce flagellated asexual swimming spores, also known as zoospores, which can readily move in water and attach themselves to the root, thus infecting it. Zoospores are formed and released from sporangium located on the surface of the roots. Released zoospores mobilize within the hydroponic solution or in water films surrounding solid substrates in a chemotactic manner, or more specifically, directed movement toward root

exudates. Once they encounter a root, they attach, loosen their flagella, undergo encystment through cell wall formation, and penetrate the root with the germ tube (Figure 2.2) (Agrios, 1997; Paulitz, 1997).

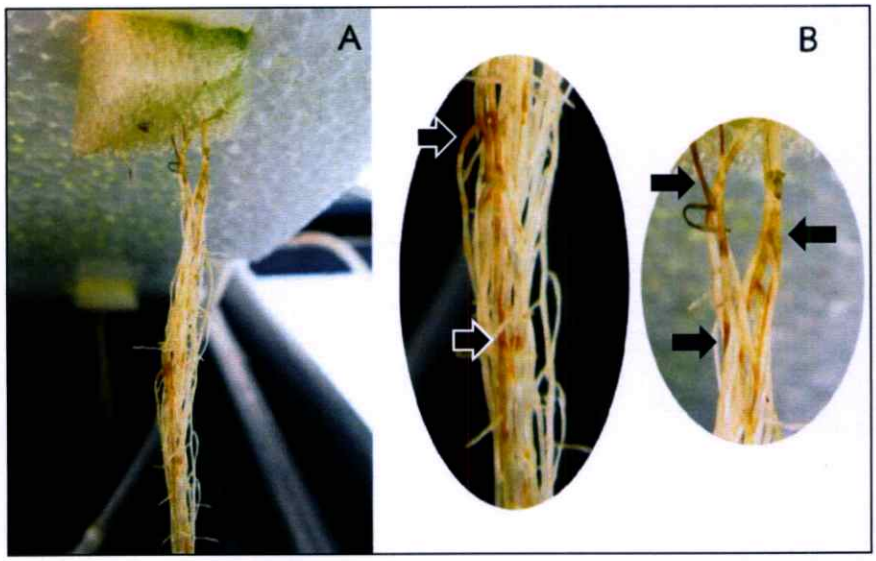

Figure 2.1 Hydroponically-grown lettuce root shows root rot from *Pythium aphanidermatum*. Root or root tips are brown and dead (Black arrow).

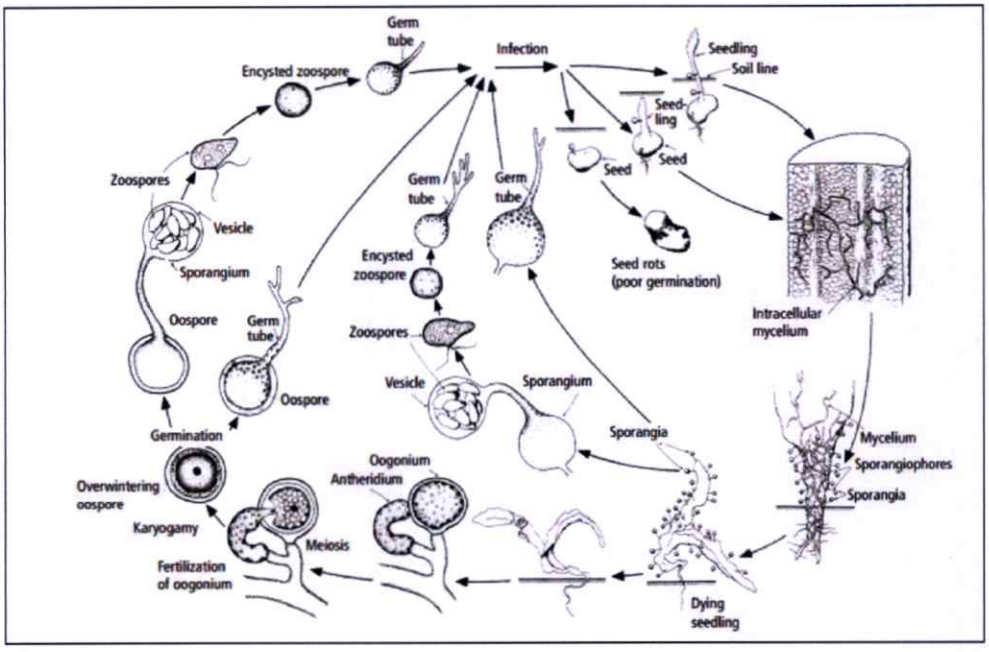

Figure 2.2 Disease cycle of damping-off and seed decay caused by *Pythium* sp. (Source : Agrios, 1997)

Hydroponic and soilless systems are applied to many agricultural lands all over the world to cultivate flowering plants, fruits, and vegetable crops. Nutrient solutions, with or without solid substrates, are added to stimulate root and plant growth. Hydroponic systems without substrates incorporate the nutrient film technique (NFT), the deep flow technique, trough culture, and ebb and flow (Paulitz, 1997). Hydroponic systems have gained increasing popularity over the last 20 years in Northern Europe, the United States, and Canada, and are responsible for the growth of high-value crops in glasshouses. In Thailand, hydroponic cultures of vegetables, especially lettuce, covers more than 300 rai (1 rai = 3.95 acres) of the country's total surface area (Tongaram, 2008). Nutrient solutions added to the hydroponics system constitutes of vitamins and minerals that can be absorbed instantly by the plant itself. This system helps to mitigate the risks of plants from getting root diseases, and avoid the emergence and accumulation of soil-borne pathogens. In order to avoid pathogen contamination in greenhouse plantation systems, agriculturists would mix soil and agricultural substrates together, and then proceed to decontaminate the mixture through adopted sterilization and pasteurization techniques. The following processes eradicate impurities within the soil and substrates prior to usage during cultivation. Hydroponic agriculture is used in practice as a strategy to avoid root diseases and to overcome mono-cropping problems (Stanghellini and Rasmussen, 1994). A nutrient solution is introduced at one end of the structure and flows through troughs within a thin film (3-6 mm) along the gravitational force. The solution then gets pumped into the head of each tray, flows down the gutters, and undergoes recirculation (Ryder, 1999).

Lettuce has been cultured under the hydroponics system for many years. Hydroponics is a disease-free form of cultivation. However, there have been many occurrences of disease in hydroponic lettuce during cultivation and production. Plant diseases seriously affect the quality and yield of lettuce. Various soil-borne pathogens, especially *Pythium* and *Phytophthora* can be commonly found in many hydroponic systems and one introduced, often cause greater crop yield losses compared to soil plantation systems. In particular, *Pythium* is highly destructive effects to the hydroponics system because of its strong pathogenicity and rapid reproduction; it also disperses and spread readily in water as a semi-aquatic fungus. Most of the devastating root diseases that can be found widespread across 800 hectares of hydroponically

grown crops in South Africa, especially in re-circulating systems, are caused by *Pythium* and *Phytophthora* species (Boshoff, 2005). In hydroponic systems, *Pythium* commonly exists in water mediums due to its nature of adaptability and preference for aquatic environments, which can be explained by its mechanism of flagellate zoospore formation, enabling them to swim in various nutrient solutions (Herrero *et al.*, 2003; Vallance *et al.*, 2009). *Pythium* can be introduced into hydroponic systems through contaminated water sources, contaminated soil, dirty farming tools, infected plant materials, or naturally infested peat-based propagation media. *Pythium* can infect roots of lettuce produced in hydroponic systems, and significantly reduce plant growth and yield without the occurrence of any identifiable symptoms (Utkhede *et al.*, 2000). Zhang and Tu (2000) reported that the dispersal of *Pythium* root pathogen is a major concern in closed hydroponic cultivation systems. A small amount of contamination can lead to substantial infection and loss of crop yields from the disease. For example, as few as 20 zoospores of *P. aphanidermatum* introduced into 100 L of nutrient solution in an NFT system resulted in significant yield losses in cucumber plantations (Menzies *et al.*, 1996).

According to Stanghellini and Rasmussen (1994), the fungal genera *Pythium*, *Phytophthora*, and *Olpidium* are capable of infecting seedlings as well as mature, fruit-bearing plants. The most common root pathogens are *P. aphanidermatum*, *P. myriotylum*, *Ph. cryptogea*, and *Ph. nicotianae*. Jenkins and Averre (1983) found that fifty percent of the *Pythium* isolates from hydroponics culture system were identified as *P. aphanidermatum,* which is highly virulent towards tomato seedlings in hydroponic culture systems, resulting in one-hundred percent, or complete damage. Likewise, Gold and Stanghellini (1985) reported that the temperature of the nutrient solution highly affects the cyclic occurrence of *Pythium aphanidermatum* and *P. dissotocum*. It was found that after inoculation of *P. aphanidermatum* at temperature conditions of 21 and 27 °C, plants undergo severe root rot or die off within 3-4 days, whereas following the inoculation of *P. dissotocum,* plants showed symptoms of severe root rot after 7 days of incubation. Bates and Stanghellini (1984) reported that *Pythium aphanidermatum* and *P. dissotocum* caused root rot in hydroponic spinach, which subsequently resulted in the infected plants to be either severely stunted or dead. During warm summer months where the

temperature of nutrient solutions exceed 23 °C, *Pythium aphanidermatum* predominates as the primary causal agent of root rot.

Rafin and Tirilly (1995) reported that necrotic lesions and root rot examined in tomato grown under soilless culture is caused by the *Pythium* species. Samples of *Pythium*, along with its filamentous non-inflated sporangia, were observed in abundant amounts during both latent infections and necrotic phases, constituting seventy-five percent of the total isolates.

Jee *et al.* (2001) reported that the *Phytophthora* root rot in Korea can be observed during all seasons and occurs most severely in summer, from June to August, with an infection rate of over ninety percent in some farms. The fungus, *Phytophthora drechsleri,* responsible for root rot, is highly pathogenic to lettuce and Chinese cabbage. However, it showed no signs of pathogenicity towards other leafy vegetables such as chicory, kale, endive, garland chrysanthemum, spinach beet, and perilla. Garibaldi *et al.* (2014) published the first report of bell pepper root rot in Italy, caused by *Pythium aphanidermatum*. Similarly, in Buenos Aires, Argentina, hydroponically grown poinsettia plants reportedly suffered from root and stem rotting symptoms of *P. aphanidermatum* (Palmucci and Grijalba, 2007). Additional reports also claimed that *Pythium aphanidermatum* was held responsible for the root rotting of hydroponically grown cucumber plants in Japan (Zhao *et al.*, 2000)

In Thailand, *Pythium* is among one of the plant parasites extremely pathogenic to lettuce (*Lactuca sativa* L.), as it is the sole cause of the damping-off disease in lettuce seedlings, and root rot disease in lettuce. Pythium root rot has been a serious and persistent problem in the process of lettuce cultivation in tropical areas i.e., Thailand (Koohakan *et al.*, 2008). *Pythium* can cause damage to lettuce at all stages of its life cycle, as it takes the form of a seed, seedling and upon harvest. The oomycete genus *Pythium* has over 140 species and includes many important soil-borne pathogens, such as *Pythium dissotocum*, *P. aphanidermatum*, *P. irregular*, *P. diclinum*, *P. sylvaticum*, and *P. ultimum*. These organisms were recovered from infected roots of *Kummerowia stipulacea*, *Lactuca sativa* and *Capsicum annuum* L. (Mihail *et al.*, 2002; Van Beneden *et al.*, 2009; Garibaldi *et al.*, 2014). As of the present day, organisms in this genus have been fully recognized as water-borne pathogens. This supports the fact that *P. aphanidermatum*, and

P. dissotocum were always recovered from infected roots of chrysanthemum grown in small-scale hydroponic units. In addition, *Pythium aphanidermatum*, *P. myriotylum*, *P. oligandrum*, *Phytophthora cryptogea*, *P. ultimum*, *P. irregular*, and *Pythium* group F were extracted from various commercial plant roots grown in soilless agricultural systems, such as pea, tomato, cucumber, and lettuce (Lin *et al.*, 2002; Herrero *et al.*, 2003; Calvo-Bado *et al.*, 2006; Liu *et al.*, 2007; Vallance *et al.*, 2009; Koohakan *et al.*, 2008; Schuerger and Hammer, 2009).

2.1.3 Disease Control in Hydroponic Systems

Chemical pesticides, such as fungicides, are usually a main component of disease control programs in agriculture and horticulture. However, according to Paulitz (1997), no fungicides are currently registered for use against root diseases in hydroponic systems that yield food or horticultural crops in the United States or Canada. One of the main threats of fungicide use is the possibility of it inducing phytotoxic effects when applied to hydroponic systems. Another potential problem that could arise is the development of resistance to fungicides by the pathogens. For example, there has been past reports of the development of *Phytophthora*, downy mildews, and *Pythium* resistance to metalaxyl (Staub, 1991). Thus, other methods of disease control are more popular alternatives used to disinfest the nutrient solution, such as UV radiation, filtration, and ozonation, heat treatments, and other agricultural techniques (Ehret *et al.*, 2001). Stanghellini *et al.* (1996) demonstrated that nonionic surfactants (20 µg a.i./ml) in nutrient solutions exhibit complete control of the spread of the pathogen, *Pythium aphanidermatum*. Biological methods incorporating the use of antagonistic microorganisms are applicable to the process of disease suppression within the system (Stanghellini and Rasmussen, 1994). Additionally, the greenhouse environment can also be modified to make conditions less favorable for the pathogen. Since most soilless systems are essentially sterile at the beginning, it is easy to establish high populations of biocontrol agents before an increase in the population of its competitors. Biocontrol agents can easily be added and disperse into the nutrient solution of a hydroponic system. The use of biocontrol could be a marketing advantage, due to the current consumer trend favoring pesticide-free crops (Paulitz, 1997). For example, Chairat and Pasura (2013) reported that all isolates of rhizobacteria, gathered from hydroponic lettuce, could inhibit pathogenic *Pythium* root rot. Moreover, according to an experimental study by Haritha *et al.*

(2010), combination treatments are the most effective in mitigating the occurrence of damping off in tobacco plant nurseries, with the lowest percentage of disease prevalence, at 5.54%.

Jee *et al.* (2002) reported that the direct supplementation of 100-ppm phosphonate into the nutrient solution produced satisfactory results in controlling and mitigating *Phytophthora* root rot in hydroponic systems. This method efficiently controlled the disease, as it did not develop until 28 days after transplanting, and resulted in only less than two percent infection rate at the end of cultivation. On the other hand, the control data (without the presence of 100 ppm phosphonate) suggested rapid development of the disease, numerically represented by an infection rate of over seventy percent within 28 days prior to transplantation. Moreover, Lin *et al.* (2002) developed pesticide-free methods to control *Pythium* root rot of pea seedlings in soilless culture. Used trays were sanitized by leaving to dry under sunlight for a couple of months, or by immersing the trays in calcium hypochlorite solution (2,000 ppm) for 24 hours before seed plantation. There is a significant reduction in the occurrence of root rot disease upon using trays pre-treated with calcium hypochlorite solution, as opposed to trays that have no undergone any treatment. Consequently, Yun (2003) reported that the use of small pore-sized filters is an effective and eco-friendly way in reducing the amount of *Pythium* in recycled water. Even after persistent ozone and chlorine treatments, some traces of the pathogen may still be found in water, thus, the use of filters is crucial. The application of 5-μm pore sized filters into the system allows for the most successive control of the disease.

2.2 The Taxonomy of the Genus *Pythium*

The most recent tools available for identifying species in the *Pythium* genus are proposed in a monograph written by van der Plaats Niterink (1981). Lévesque and de Cock (2004) divided the *Pythium* genus into 11 clades by molecular systematic analysis. These clades are generally segmented based on morphological characteristics, which help to identify different *Pythium* species, i.e. sporangia, oogonia, antheridia, and oospores (van der Plaats-Niterink, 1981). However, there has been many cases of frequent overlapping of morphological characteristics, which makes the process of identification extremely difficult and time consuming. For

example, *Pythium aphanidermatum* and *P. deliense* are physically indistinguishable, but clearly portrays different characteristics under the RAPD analysis (Herrero and Klemsdal, 1998). Recently, further examination of molecular characteristics has contributed to the advancement of the morphological study of *Pythium* species. Many molecular approaches were held, which include incorporating the use of simple sequence repeat (SSR) markers (Lee and Moorman, 2008), PCR-restriction fragment length polymorphism (RFLP) (Watanabe *et al.*, 2007; Gómez-Alpízar *et al.*, 2011), amplified fragment length polymorphism (AFLP) fingerprinting (Garzón *et al.*, 2005), random amplified polymorphic DNA (RAPD) (Herrero and Klemsdal, 1998), DNA oligonucleotide macroarrays (Tambong *et al.*, 2006), the species-specific primers (Lévesque *et al.*, 1994; Godfrey *et al.*, 2003; Wang *et al.*, 2003), real-time polymerase chain reaction (RT-PCR) (Schroeder *et al.*, 2006), and PCR analysis of internal transcribed spacers (ITS) regions (Matsumoto *et al.*, 1999; Paul, 2000, 2001; Mathew *et al.*, 2003; Singh *et al.*, 2003; Bala *et al.*, 2006; Al-Sheikh and Abdelzaher, 2012).

A few DNA regions have been used in DNA sequence-based phylogenetic studies of Oomycetes. The nuclear rDNA is the main region used in the phylogenetic studies of genera and species within the Saprolegniomycetidae, including some species of the *Pythium* genus. Moreover, the internal transcribed spacer (ITS) has been used extensively in systematic studies of mycology, primarily because the PCR primers, developed by White *et al.* (1990), universally amplified a highly variable region throughout all taxa, including Oomycetes. Lévesque and de Cock (2004) reported the phylogeny of 116 species, and the variety of the characteristics of *Pythium* using parsimony and phonetic analysis of the ITS region of nuclear ribosomal DNA. Matsumoto *et al.* (1999) also performed a phylogenetic analysis of 30 *Pythium* species using the ITS region. Likewise, Schurko *et al.* (2003) used the ITS region to study the genetic variation and phylogeny of *P. insidiosum* and species closely related to it. Lévesque and de Cock (2004) presented the phylogeny of *Pythium* species based on ITS1, 2 and the 5.8S gene of nuclear rDNA. They stated that *Pythium deliense* and *P. aphanidermatum* are both grouped in clade A, and in the same cluster due to their strikingly similar characteristics. Matsumoto *et al.* (1999) suggested that the ITS regions served as great templates for designing species-specific primers that function in identification and detection of *Pythium* species. Some other reports claim that the ITS region is also used in the process of molecular analysis.

Alaei and Rostami (2013) revealed that twenty-three isolates of *Pythium aphanidermatum* were identified according to their molecular characteristics through the use of PCR primers, which are essentially synthesized from ITS regions of rDNA.

Matsuura *et al.* (2010) studied the identification method of *Pythium* species based on morphological characteristics and the rDNA-ITS sequences. *Pythium uncinulatum* was identified as the *Pythium* species responsible for wilting of lettuce in Japanese plantation farms. According to Nzungize *et al.* (2011), 96 typical *Pythium* colonies were isolated from the collected samples, which were then classified into 16 *Pythium* species according to their respective molecular sequences derived from ribosomal ITS fragments. In addition, Kageyama *et al.* (2003) stated that the *Pythium* P group isolates and *P. helicoides* had the exact same morphological characteristics and size of sporangia, and took on the same sporangial germination mode. Hence, the molecular technique was carried out by restriction fragment length polymorphism analysis of the rDNA ITS region. The results visually demonstrated and compared the banding patterns of enzymes in group P and *P. helicoides*, where five out of six extracted enzymes are identical and the only difference being the *Hha*I. Tambong *et al.* (2006) reported that the oligonucleotides complementary to specific diagnostic regions of internal transcribed spacers (ITS) of more than 100 species were developed and used for identification and detection of *Pythium* species. Likewise, Pornsuriya *et al.* (2008) was able to identify *Pythium graminicola*, an organism responsible for pineapple root rot, through amplification and sequencing techniques, using the ITS region of the ribosomal nuclear DNA, and by building a phylogenetic tree to confirm the species. Lévesque *et al.* (1994) developed a species-specific probe to accurately identify and differentiate *Pythium ultimum* using restriction sites to map the ITS region of the organism's nuclear ribosomal DNA (rDNA).

Garibaldi *et al.* (2014) was the first to publish a report of the occurrence of root rot caused by *Pythium aphanidermatum* in bell pepper. In this scientific paper, specific kinds of *Pythium* were characterized by their morphology and the different ITS regions along the rDNA sequence. The microorganism's morphological characters identified it as *Pythium* sp. The specific species of the *Pythium* was determined by BLAST analysis on the collected sample rDNA with ITS sequence of the microorganism, which showed 100% homology with the ITS sequence from an isolate sample *P. aphanidermatum* in GenBank (AY598622.2). Zitnick-Anderson and Nelson

Jr. (2015) also identified the *Pythium* species through examining their morphological features and employing molecular techniques. DNA sequences have been used to identify all isolates of the species. The internal transcribed spacer (ITS) sequence is a described as a special DNA region that contains good resolution, and is commonly used by the mycology community for species identification. They recovered twenty-six known and three unknown species of *Pythium* spp. from soybean seedling roots. In a report by Santoso *et al.* (2015), durian pathogens were identified by their molecular and morphological characteristics. Based on ITS-nrDNA sequences, six Pythiaceae species were identified, namely *Pythium cucurbitacearum*, *P. vexans*, *Pythium* sp. D37, *P. deliense*, *Phytophthora cinnamomi* var. *parvispora*, and *Ph. Palmivora*.

2.3 Mechanisms of Defense in Plants

Generally, higher plants have a broad spectrum of defensive mechanisms against various types of pathogens and other physical, chemical and biological stresses. Plants can induce biochemical and physiological changes in response to invading pathogens through the release of phytoalexins, which are known as low molecular mass secondary metabolites containing antimicrobial activity, lytic enzymes, oxidizing agents, suberization, deposition of callose, formation of reactive oxygen species (ROS), reinforcement of the localized cell-wall, and the synthesis of pathogenesis-related (PR) proteins (Ebrahim *et al.*, 2011; Ahuja *et al.*, 2012). Plants respond to the pathogenic penetration of microorganisms through the accumulation and release of reactive oxygen species (ROS), which function as crucial signaling molecule. ROS are produced by many different enzymatic systems, and besides exhibiting direct antimicrobial activity, the molecules also function in triggering programmed cell death and initiating hypersensitive response (HR) at the site of plant infection. It is known that in response to pathogen invasion, plants often produce excessive amounts reactive oxygen species (ROS), which contain toxic properties, including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals. These toxic molecules are generated by oxidative burst, a process that occurs via a membrane-bound NADPH oxidase (Nox), carried out to limit the spread of pathogens in plant structures (Greenberg and Yao, 2004; Marino *et al.*, 2012). Park and Paek (2007) defined hypersensitive response (HR) as distinctive

localized, rapid death of host cells and restriction of growth of biotrophic pathogens by incompatible interactions resulting from the production of antimicrobial compounds and limited the nutrient uptake. Regulation of the HR involves oxidative burst, ion channel activity, and NO, as well as various interactions among some of these signals. Other signaling molecules involved in the HR include salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). Lehotai *et al.* (2011) has studied about the physiological responses of pea roots to long-term copper exposure, and further examined growth parameters, metal uptake, levels of different reactive oxygen species (hydrogenperoxide, H_2O_2 and superoxide radical, $O_2^{\cdot}$) and reactive nitrogen species (nitric oxide, NO and peroxynitrite, $ONOO^{\cdot}$), and cell death in the meristem cells of pea roots.

Złotek and Gawlik-Dziki (2015) studied the biochemical properties of polyphenol oxidase (PPO), which were sampled from lettuce leaves elicited by DL-β-amino-n-butyric acid (BABA). PPO is an induced defense enzyme commonly found in plants, containing many important defensive properties. BABA is a chemical elicitor used to protect and preserve the wellbeing of crops, due to it being highly stable. The non-protein amino acid DL-β-amino-n-butyric acid is one of the most well known chemical inducers of systemic resistance. Likewise, Cohen *et al.* (2010) have studied the mechanisms of local and systemic resistance against *Bremia lactucae* induced by DL-β-amino-butyric acid (BABA) in lettuce (*Lactuca sativa*). Although BABA did not affect the rate of spore germination, appressorium formation, or penetration of *Bremia lactucae* into the host, it induced rapid encasement of structures within the pathogen, contributing to primary infection, with callose. Hydrogen peroxide, a reactive oxygen species, can be detected in infected cells of *Lactuca* spp. that has been previously challenged with *Bremia lactucae* (race NL16) (Sedlárová *et al.*, 2007). In addition, Perrone *et al.* (2003) demonstrated that in some plants, H_2O_2 mediates the elicitor-induced accumulation of secondary metabolites. However in other plants, O_2 is the mediator for the elicitor-induced production of secondary plant metabolites, such as phytoalexin accumulation in tobacco.

Primary plant immune response is referred to as PAMP-triggered immunity (PTI). PTI is initiated upon recognition of conserved microbial structures (PAMPs) by plant surface receptors. More importantly, PAMP-induced immune responses have recently been shown to contribute to basal resistance of host plants against virulent

pathogens, and to be crucial in stabilizing non-host resistance (Zipfel and Felix, 2005). During the first stage of plant defense triggered by pathogenic bacteria, PAMPs, or general elicitors, the plant would rapidly activate a number of defense mechanisms, such as cell-wall reinforcement by callose deposition, production of reactive oxygen species (ROS), and induction of numerous defense-related genes (Zipfel *et al.*, 2006; Mishra *et al.*, 2012). For example, the bacterial flagellins, flg22, can activate flg22-FLS2 interaction, which leads to the production of reactive oxygen species (ROS) (Navarro *et al.*, 2004). In a similar case, Sano *et al.* (2014) demonstrated that activation of the SA biosynthesis pathway by flg22 requires the presence of light. Moreover, the Nep1-like proteins (NLPs) and flagellin (Flg22) trigger plant defense and cell death as a result of pathogenic exposure. The NLPs can activate defense-associated responses, by the synthesis of phytoalexins and ethylene, the accumulation of defense-related transcripts, and cell death. These proteins can contribute to the virulence of necrotrophic fungal and bacterial pathogens (Veit *et al.*, 2001). The bacterial PAMP, flg22, can trigger PTI responses in *Arabidopsis* by binding to its cognate pattern recognition receptor, FLS2. FLS2 contains a receptor site, which binds the bacterial PAMP, flagellin. Flagellin activity is required for full immunity against bacteria, due to the fact that plants deficient in FLS2 are more susceptible to adapted and non-adapted bacterial pathogens (Zipfel, 2009). Upon application of flg22 to intact seedlings of *Arabidopsis*, the process of callose formation is triggered, along with the accumulation of the defense protein PR1 and strong inhibition of seedlings growth (Mishra *et al.*, 2012).

Simultaneous or subsequently invasion of plants by multiple aggressors can affect the effectiveness of its principal induced response (Zhang *et al.*, 2010). Plants accumulate several kinds of pathogenesis-related (PR) proteins in response to infection by pathogens, such as fungi, bacteria or viruses. The PR proteins are downstream components of systemic acquired resistance (SAR) in plants. They have been used routinely in the defense mechanism of plants with positive antimicrobial activity. PR-proteins are induced in response to pathogenic attacks. Plants are able to coordinate the expression of specific defense-related genes in response to attack by relevant pathogens at the molecular level. Various signaling molecules are responsible for mediating the induction of PR gene expression during pathogenic infection (Kitajima and Sato, 1999; Zhang *et al.*, 2010). The PR proteins have been

divided and classified, initially, into 5 families based on molecular mass, isoelectric point, localization, and biological activity. PR-proteins are currently categorized into 17 families based on their properties and functions. This includes β-1, 3-glucanse, chitinase, thaumatin-like proteins, peroxidases, ribosome-inactivating proteins, defense, thionins, nonspecific lipid transfer proteins, oxalate oxidase, and oxalate-oxidase-like proteins (Ebrahim *et al.*, 2011). Among the PR-proteins families, PR-1 proteins accumulate in most abundant amounts after pathogen infection, with concomitant accumulation of the transcript during pathogenic attack (Kitajima and Sato, 1999). It was supported by Hammerschmidt (2007) that plants must have all genes necessary to mount effective defense, and that the induced treatment primes or sensitizes that plant in such a way that allows rapid expression of a broad set of defenses upon infection by a pathogen. For example, Zhang *et al.* (2010) studied the expression of *PR* genes in the tissue of *Malus hupehensis* (*MhPR1*, *MhPR5*, *MhPR8*) seedlings following treatment with salicylic acid (SA), methyl jasmonate (MeJA), and ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) in leaves, stems, and roots. The expressions of *MhPR1*, *MhPR5*, and *MhPR8* are enhanced during the first 48 h post-induced treatment with SA, MeJA, and ACC. Later, Kim *et al.* (2015) reported the activation of pathogenesis-directing genes by the rhizobacterium, *Bacillus* sp. strain JS, which induces systemic resistance in tobacco plants. The experimental results show that tobacco leaves pre-treated with volatiles of *Bacillus* sp. strain JS developed smaller lesions prior to damage by *Rhizoctonia solani* and *Phytophthora nicotianae* compared to those found on control plant leaves (untreated). In PR gene expression analysis, volatiles of *Bacillus* sp. strain JS caused the up-regulation of PR-2 encoding β-1, 3-glucanase and acidic PR-3 encoding chitinase. However, PR-1 genes, markers of salicylic acid (SA) induced resistance, are not expressed.

Moreover, the kinetics of allene oxide synthase (AOS), which occurs in response to wounding and elicitors, is stimulated in a manner similar to that of previously studied wounds that are capable of inducing the gene expression of PIN II (proteinase inhibitor II). AOS stimulation occurs in the leaves through wounding, or in the presence of systemin, 12-oxophytodienoic acid, and methyl jasmonate. Lab results show that levels of AOS mRNA started to decline 4 hours after induction, whereas the levels of *PIN II* mRNA continued to increase up to 20 hours after induction (Sivasankar *et al.*, 2000).

2.4 Phytoalexin

Plant resistance to pathogens and pests can either be active or passive, while induced resistance is classified as a type of active resistance. According to reports by many researchers, phytoalexin is defined as "antibiotics produced as a result of the interaction between two different metabolic systems of the host and parasite, which subsequently inhibit the growth of microorganisms pathogenic to plants" (Kuć, 1972). According to Yoshikawa (1978), phytoalexins are inducibly-formed higher plant metabolites that can be used as antibiotics to eradicate certain potential plant pathogens. At least 75 plant species from 20 families have been shown to produce high amounts of phytoalexins in response to infection. Phytoalexins also accumulate in plants in response to various agents, also known as elicitors, such as substances that originate or arise from the pathogen (biotic elicitors) and abiotic elicitors such as heavy metal salts and detergents. In 1995, Kuć redefined phytoalexin as a low-molecular-weight antimicrobial compound that can be synthesized and can accumulate in plants after infection or introduced stress by biotic or abiotic elicitors. The occurrence of rapid phytoalexin accumulation occurs as a plant defense mechanism to resist contracting diseases caused by fungi and bacteria, despite the fact that genetic information for phytoalexin synthesis can be found in both susceptible and resistant plants. This suggests that phytoalexins serve as only one of the many components of the complex mechanisms of disease resistance in plants.

Phytoalexins are mainly produced by healthy cells located adjacent to localized damaged or necrotic cells. On the other hand, PR proteins not only accumulate within the infected areas and surrounding tissues, but also in remote uninfected tissues (Ebrahim *et al.*, 2011). Phytoalexins accumulate as a result of compatible plant-pathogen interactions induced by biotic elicitors, such as pathogenic or non-pathogenic fungi. For example, Bednarek *et al.* (2005) showed that a pathogenic oomycete, *Pythium sylvaticum,* induced the synthesis of camalexin in roots. Hall *et al.* (2011) reported the accumulation of terpenoid phytoalexins in infected xylem vessel lumens and in adjacent parenchyma cells three days after inoculation with *Fusarium oxysporum* f. sp. *vasinfectum*. In another report by Monde *et al.* (1990), a sesquiterpenoid phytoalexin, cichoralexin, was extracted from a chicory plant (*Cichorium intybus*) infected by bacterial pathogens. On the first day of the experiment, traces of cichoralexin were detected, which subsequently increased

to a maximum population number on the third day after inoculation, and underwent a population decline afterwards. Likewise, Liu *et al.* (1995) detected phytoalexin, kievitone, phaseollinisoflavan, and phaseollin in the roots of bean seedlings cultured under the hydroponics system. These phytoalexins are stimulated by microorganisms in the soil responsible for root rot disease in beans, mainly the *Pythium* spp. species identified as *P. ultimum* and *P. sylvaticum*. Moreover, glyphosate application did not significantly affect the accumulation or exudation of phytoalexins in the roots of hydroponic beans. Sarig *et al.* (1997) reported the secretion of phytoalexins, resveratrol and pterostilbene, from berries of the *Vitis vinifera* species infected with *Rhizopus stolonifer.* Both phytoalexins existed in highest concentration 24 hours after inoculation, followed by gradual reduction. Also, Ongena *et al.* (2000) demonstrated that the PGPR, *Pseudomonas putida* isolate BTP1, can elicit phytoalexins systemically in cucumber to defend itself against *Pythium* root rot. The overall defense response is not based on a single type of phytoalexin, as it is otherwise chemically complex and organ-specific. The accumulation of stilbenoids, a phytoalexin, in peanuts (*Arachis hypogaea* L.) is stimulated by infections caused by microbial pathogen. Upon further investigation of compound activity in a broad spectrum of biological assays, it appears that compounds all play important roles in the plant defense mechanisms (Sobolev *et al.*, 2011). Gutierrez *et al.* (1995) revealed that coumarin phytoalexins, scopoletin and ayapin, were derived from sunflower plants (*Helianthus annuus*) treated with abiotic elicitors, such as $CuCl_2$ or sucrose. The effectiveness of coumarin elicitation is dependent on the type of elicitor used and the age of the plant tissues.

2.4.1 Phytoalexin in Lettuce

Takasugi *et al.* (1985) published the first report on accumulation of phytoalexin in lettuce leaves inoculated with pathogenic bacterium, *Pseudomonas cichorii,* upon exposure to two antifungal sesquiterpenes, costunolide and lettucenin A. Bennett *et al.* (1994) developed a hypothesis that sesquiterpenoid phytoalexins are involved in the defense mechanisms of lettuce, however, none of any specific structures could be observed. In 1995, Bestwick *et al.* (1995) reported that the sesquiterpenoid phytoalexin extracted from lettuce seedlings with physiological disorder in the form of visible red spots is "lettucenin A". The formation of phytoalexin can be observed by increasing activity of 3-hydroxy-3-methylglutaryl

coenzyme A reductase. It was observed that lettucenin A accumulation is associated with the occurrence of discoloration in affected cotyledons, which were examined 7 days after sowing.

Hanawa *et al.* (1995) found that the leaves of *Taraxacum hondoense*, *Lactuca dentata*, *L. scariola*, *Sonchus oleraceus*, *S. asper*, and *Ixexis repens* synthesized lettucenin A upon challenge with cupric chloride and *Cladosporium herbarum*. The fungal elicitor and cupric chloride were able to promote the synthesis of lettucenin A within 2 hours, while 12 hours of UV light irradiation was required for the same process to completely occur.

Bennett *et al.* (2002) reported that the seedlings with red spot disorder, accumulating high amounts of phytoalexins, were used as cloning tools of sesquiterpene synthase genes from lettuce (*LTC1* and *LTC2*). The results show constitutive expressions of *LTC1* and *LTC2* in roots, hypocotyls and true leaves, but not in cotyledons. Ong and Chong (2009) mentioned the phytoalexin, lettucenin A, were synthesized in lettuce leaves after being challenged with different elicitors and processes, such as $AgNO_3$, $CuSO_4$, ultraviolet irradiation and freeze-thawing. The maximum concentration of lettucenin A accumulated in lettuce leaves after being sprayed with $AgNO_3$ and $CuSO_4$. The elicitations of lettucenin A were dependent on the type of elicitor and plant age. Experimental results revealed that the lettucenin A accumulation increased significantly from week nine, reached a maximum at week twelve, and then decreased by the eighteenth week. Likewise, Yean *et al.* (2009) reported that lettucenin A was found in leaves lettuce introduced to 5% $CuSO_4$ and 1% $AgNO_3$, and as a result, *Aspergillus niger* and *Xanthomonas campestris* developed effective antifungal activity. Recently, The research of lettucenin A has carried out with the main purpose of studying the relationship between discoloration of iceberg lettuce and enzymatic browning. It was discovered that sesquiterpenes, lettucenin A and B, started to accumulate within 40 hours after cutting the lettuce, and thus, lettucenin A can be used as an identity marker within the genus of *Lactuca* (Mai and Glomb. 2014).

2.5 Enzyme Production of Oomycetes and Plant Pathogens

Plant cell wall degrading enzymes play significant roles in plant pathogenesis. They are produced and secreted by many plant pathogenic fungi, and are considered to be of major importance in the process of intercellular invasion and maceration of host tissue. During fungal infection, a range of hydrolytic enzymes is secreted to promote and initiate host colonization. Enzymes are important towards plants, as they help in cell wall expansion and fruit ripening, while some enzymes, such as pectinase, cellulose, and ligninase function in protecting the plant cell. A plant's biomass constitutes of abundant amounts of lignin, cellulose and hemicellulose. The enzymes are synthesized by plants, insects, and microorganisms, such as bacteria and fungi (Priya and Sashi. 2014). These enzymes play an importable role in pathogenesis and determine the virulence of many pathogens, as they are responsible for degradation of the host cell wall and colonization of the host tissue. (Neethu *et al.*, 2012). Generally, the ability of a pathogenic fungus to produce a wide range of enzymes is directly associated with higher levels of pathogenicity. Differences in cell wall degrading enzyme levels and mycelial growth rates of pathogen isolates are positively correlated with their levels of virulence (Chen *et al.* 1998; Owen-Going *et al.*, 2004; Onuh and Ohazurike. 2008).

2.5.1 Pectinase

Pectins are high molecular weight polysaccharides found in higher plants. They compose the primary cell wall, and are main components of the middle lamella. The hydrolysis of pectin backbone is initiated by the synergistic action of several enzymes. Among the enzymes secreted, polygalacturanase and pectinase are responsible for cell maceration and death of plant tissues (Priya and Sashi, 2014). Enzymes that attack pectic substances in the plant cell wall contribute immensely to an organism's pathogenicity. Pectin degrading enzymes directly cause cell wall degradation, and the ability to synthesize those molecules is what determines the virulence of many pathogens (Lionetti *et al.*, 2012). In phytopathogenic fungi, pectinases are involved in plant cell wall-degradation and are thought to be the important determinants of pathogenicity and virulence. Chen *et al.* (1998) reported that pectin lyase (PL) can be synthesized by *Pythium splendens*, a pathogenic plant fungus, which causes damping-off of seedlings and fruit rot in cucumber plants. Also

the softening of cucumber fruit as a symptom of the cottony leak disease, caused by *P. aphanidermatum*, is shown to associate with the activities of endo-polygalacturonase and cellulase (Zamski and Peretz, 1996). Synthesis of pectolytic and cellulolytic enzymes has been identified in several plant pathogenic *Pythium* species, including the *P. aphanidermatum* (Janardhanan and Husain, 1974; Sutton *et al.*, 2006). Additional support for enzyme secretion by pathogens has been reported by Boudjeko *et al.* (2006), claiming that when *Pythium myriotylum* infects a plant host, the fungus contributes to the breakdown of significant amounts of pectin, possibly via degradation by hydrolytic enzymes. Moreover, a sugar analysis that was held demonstrated a significant decrease in galacturonic acid content in infected root cell walls, which served as evidence for pectin degradation. Maccheroni, Jr. *et al.* (2004) demonstrated that secretion of specific pectinolytic activities by phytopathogenic fungi is dependent on the pH of the culture medium. Thus, pectin or pectate lyases (PLs) usually have base optimum pH for activity. Tested enzymes have been secreted out from all isolates in a pH-dependent manner, and were then later examined. It was found that endophytes and pathogens showed distinct patterns of protease secretion, under respective alkaline and acidic optimum pH growth conditions. For example, pectate lyase (PL) secretion has been detected when the pH conditions reached 5.8, and the level of secretion increased as the pH 6.5. However, PL secretion could be detected when the pH of the inducing medium is lower than 5.8, or when the *Colletotrichum gloeospoioides* hypha has been transferred from PL-secreting conditions at pH 6.5 to pH 3.8 (Yakoby *et al.*, 2000).

Mccombs (1961) reported that pectin methylesterase (PME) could be detected in both healthy and diseased cucumber fruits infected by *Pythium aphanidermatum,* however, the enzyme could not be observed in fungus culture. Janardhanan and Husain (1974) demonstrated that *Pythium butleri*, *P. debaryanum*, *P. aphanidermatum*, *P. irregular*, and *P. ultimum*, the pathogens causing soft rot diseases, produced significant amounts of endo-polygalacturonase, endo-polymethylgalacturonase, exo-polygalacturonase, polygalacturonate trans-eliminase and pectin methyl-trans-eliminase in media containing glucose and pectin as carbon sources. Likewise, Koleosho *et al.* (1987) reported the concentrations of oxalic acid and polygalacturonase (PG) found in cowpea tissue, which plays an important role in the pathogenesis of susceptible varieties of cowpea caused by *Pythium*

aphanidermatum infection. In comparison to resistant species, cowpea plants more susceptible to contracting diseases contain higher levels of oxalic acid and PG. Moreover, maximum accumulation occurs during the infective stage of disease and declines during the serious stage. Therefore, early accumulation of oxalic acid in cowpea tissue during pathogenesis may be a useful tool for monitoring disease severity. The synthesis of cell-wall degrading enzyme of *Pythium violae*, *P. sulcatum*, and *P. ultimum* in response to the cavity spot disease has lead to a conclusion that upon *P. violae* infection, pectate lyases and cellulase are produced in very minimal amounts at very slow rate, which results in slow and restrictive expression of its symptoms upon disease contraction (Campion *et al.*, 1997). Moreover, Fernando *et al.* (2001) reported that *Colletotrichum acutatum*, the pathogen responsible for leaf fall disease in rubber was examined to secrete polygalacturonase (PG), pectin lyase (PL) and the cellulolytic enzymes, β-glucosidase, and cellobiase in culture. The results showed that PG and PL were secreted in both healthy and infected plant leaves, and the process is associated with lesion expansion. β-glucosidase is present in both healthy and infected leaves while cellobiase has been detected only in infected leaves.

In addition, an extracellular enzyme in *Fusarium oxysporum* and *F. proliferatum,* pectinase, exhibited strong enzymatic activity in solid medium (Kwon *et al.*, 2007). Hubballi *et al.* (2011) reported that the infection of noni (*Morinda citrifolia*) by *Alternaria alternata* has yielded more cellulolytic (C1 and Cx) and pectinolytic (macerating enzymes, pectin methyl esterase and endo-polygalacturonase) enzymes than the avirulent isolates. The activity of cellulolytic enzymes increased with increase in age of the culture, whereas the activity of pextinolytic enzymes decreased with the increase in the age of the culture and were found to be highly active in 10-day-old cultures. Saleem *et al.* (2012) found that eight phytopathogenic fungi are capable of secreting pectinase, especially *Alternaria citri* and *A. raphanin,* which exhibited exceptionally high pectinase activity. They have been examined over a span of 8 days under 30 °C and pH 6 conditions in liquid medium supplemented with citrus pectin and ammonium sulphate as carbon and nitrogen sources, respectively. Additionally, Deep *et al.* (2014) found that the pathogenic isolate of *Alternaria brassicicola* infected crucifers by synthesizing enzymes, specifically exo-β-1,4-glucanase and endo-β-1,4-glucanase. Also, a report by Hernández-Silva *et al.*

(2007) revealed that pathogenic races of *Colletotrichum lindemuthianum* treated with different sources of carbon and nitrogen in liquid culture was able to yield higher extracellular pectin lyase activity compared to the non-pathogenic races.

2.5.2 Cellulase

Cellulase enzymes are produced by plants, insects and microorganisms, such as bacteria and fungi (Priya and Sashi., 2014). These enzymes play a significant role in pathogenesis, through processes such as host cell wall degradation and colonization of the host tissue. A non-pathogenic microorganism capable of producing useful enzymes, the filamentous fungi, is particularly gaining scientific interest due to their ability to produce vast amounts of extracellular enzymes. Florencio *et al.* (2012) has reported that the *Trichoderma* strain CG104NH yielded the highest rate of cellulase production with EGase activity (25.39 UI/g). Moreover, three pathogenic *Pythium* species, namely *P. violae*, *P. sulcatum* and *P. ultimum,* are primarily responsible for cavity spots on carrot roots, and can secrete pectate lyase, cellulase, polygalacturonase and β-1, 4-glucanase in various amounts at different times (Campion *et al.*, 1997). Likewise, Okunowo *et al.* (2010) reported that submerged cultures of phytopathogenic *Myrothecium roridum* is capable of producing cellulases and xylanase enzyme, but to a lesser degree compared to non-pathogenic *Fusarium solani* and *Curvularia pallescence* Boedjin. Reanprayoon and Pathomsiriwong (2012) revealed that tropical soil fungi, *Aspergillus niger*, *Aspergillus* sp., *Chaetomium murorum*, and *Trichoderma* sp., yield the highest concentrations of cellulose, while a single isolate of *Pythium ultimum* shows weak cellulase-secreting activity.

2.5.3 Chitinase

Chitinase enzymes are receiving attention in the field of scientific research with regards to their potential as microbial biocontrol agents. In this sense, biological control of some soil-borne fungal diseases is directly correlated with chitinase production (Herrera-Estrella and Chet, 1999). Chitinases have many important biophysiological functions, and can be scientifically applied in many ways, especially in the control of phytopathogens and production of chito-oligosaccharides, which have numerous uses, especially in treatment and degradation of chitinous biowaste. Chitinases are also produced as competition or part of a defense mechanism against

other fungi, or for the colonization of other arthropods, including insects or nematodes (Karthik *et al.*, 2014). Chitinase may exist in abundant amounts, as the molecule plays the main role in plant defense against fungal pathogen by degrading the organism's cell wall, which is composed primarily of chitin. Chitin is known as a major structural component in the cell walls of many pathogenic fungi (Ebrahim *et al.*, 2011). Colloidal chitin is the best inducer of chitinase enzyme in comparison to other sources of chitin. All organisms that have chitin in their cell wall composition can synthesize chitinases, including microorganisms such as fungi (Seidl, 2008) and bacteria (Kielak *et al.*, 2013). Some microorganisms produce chitinases to degrade polymers and use them as nutrient source. Other organisms produce these enzymes to protect themselves against microorganisms with chitin in their composition. Some plants also produce chitinases, probably in response to infections by chitin-containing microorganisms (Karthik *et al.*, 2014).

Souza *et al.* (2003) reported that the phytopathogenic fungus, *Colletotrichum gloeosporioides*, secreted endochitinase and exochitinase when grown in the presence of chitin as the sole carbon source.

CHAPTER 3

MATERIALS AND METHODS

3.1 Collection, Identification, and Study of *Pythium* Species in Asymptomatic and Symptomatic Lettuce

3.1.1 Collection and Isolation of *Pythium*

Healthy (asymptomatic) and diseased roots (symptomatic) of hydroponically grown lettuce were used to extract and isolate *Pythium* spp. The samples were collected from 1) a hydroponic farm in Pak Chong, Nakorn Ratchasima province, 2) a greenhouse at KMITL, Bangkok and 3) a hydroponic farm in Bangkok. Root samples were placed on 2% water agar (WA), and the infested soil of lettuce root rot was used to isolate *Pythium* spp. using baiting technique with cucumber seeds (Dhingra and Sinclair, 1994). In addition, one *Pythium* sp. isolate and one *Phytophthora* sp. isolate were provided by the Department of Plant Pathology, Faculty of Agriculture, Kasetsart University Bang Khaen. They were recovered from the infested soil of durian plant in Trad province.

3.1.2 Morphological Characteristics

In order to study morphological characteristics, the purified isolates of *Pythium* were grown on Potato-carrot agar (PCA) and Potato dextrose agar (PDA). PCA and PDA media preparation were carried out by following a standardized procedure, proposed by Dhingra and Sinclair (1994). Formation of the sporangia, oogonia, antheridia, and oospore were triggered by grass-leaf culture technique (Abad *et al.*, 1994). Five agar plugs (Ø 0.5 mm) from 3-day-old *Pythium* sp. cultures were transferred to Petri-dishes containing 10 ml of sterilized water and 3 pieces of boiled grass-leaf. The plates were incubated at room temperature (25-30 °C) under fluorescent light for 2-3 days or until sporangia, oogonia, and oospores are produced. Measurements of various reproductive structures laid out on a glass slide are conducted by submerging the samples in either water or lactophenol blue. Thirty measurements were taken for each morphological characteristic, such as the sporangia, oogonia, oospores, and colony morphology. *Pythium* species and its varieties were identified based on monographs or an identification key developed by van der Plaats-Niterink (1981).

Images were captured with a high-resolution digital camera (DXM 1200, Nikon), and examinations under standardized and calibrated magnification were held using a computer-based software system (NIS-Elements version D, Nikon Canada Inc.).

3.1.3 Molecular Characteristics

3.1.3.1 DNA Extraction

Three agar plugs of each *Pythium* isolate were cultured in 50 ml of 20% V8 juice broth in a 250 ml flask. After five days of incubation at room temperature, mycelial mats were collected on filter paper, washed three times with sterile distilled water, and left to freeze at -80 °C. The process of total genomic DNA extraction was carried out according to a procedure proposed by Matsumoto *et al.* (1999). The mycelia were ground into fine powder using a mortar and pestle under the addition of liquid nitrogen. The mycelial powder was then suspended in 600 µl of lysis buffer (50 mM Tris-HCl; pH 7.5, 50 mM EDTA, 3% SDS, 1% 2-mercaptoethanol) and heated at 65 °C for 1 hour. After centrifugation at the speed of 14000 rpm for 5 minutes, the supernatant was transferred to a new tube. DNA was extracted two times with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v), and centrifuged at 14000 rpm for 20 minutes. The supernatant was then again transferred to a new microtube, and the fungal DNA was precipitated out by the addition of 2 volumes of absolute ethanol (99.9%) in the presence of 3 M sodium acetate (0.1 vol) and centrifuged at 14000 rpm for 3 min. The precipitated DNA was rinsed with 70% ethanol, centrifuged at 14000 rpm for 2 minutes, dried up, and dissolved in 50 µl of TE buffer (10 mM Tris-HCl; pH 7.5, 0.1 mM EDTA). 2 µl of RNase (Fermentas, China) were added to the extracted DNA sample and incubated at 37 °C for 1 hour for the complete removal of RNA from genomic DNA. Quantification of purified DNA was conducted by using a spectrophotometer at OD 280 nm (Eppendorf, Model 6132, Germany).

3.1.3.2 PCR amplification

The ITS rDNA region was amplified using forward primer ITS1 (5´ TCCGTAGGTGAACCTGCGG 3´) and reverse primer ITS4 (5´ TCCTCCGCTTATTGATATGC 3´), for product sizes at around 750 bps (White *et al.*, 1990). The primers were synthesized at Integrated DNA Technologies, Singapore. A total volume of 50 µl of the reaction mixture contained 0.2 µM each of primer, 1.25 units of *Taq* polymerase

(Thermo Scientific, China), 0.2 mM dNTP mix, 1X PCR buffer (10 mM Tris-HCl; pH 8.8, 50 mM KCl), 1 mM $MgCl_2$, and 10-20 ng/µl of the DNA template. The reaction was carried out using a DNA Thermal Cycler (Model Tpersonal Combi-block, Biometra GmbH, Germany). The temperature cycling parameters were programmed for one 3 minute cycle at 94 °C, followed by 30 rounds of the cycle (45 s at 94 °C, 45 s at 55 °C, 50 s at 72 °C), and one 10 minute cycle at 72 °C. The resulting PCR products were separated by electrophoresis involving 0.8% (w/v) agarose gels (Vivantis, Malaysia) in 0.5X TAE buffer at 100 V for 40 minutes at room temperature, stained with ethidium bromide, observed under UV light, and photographed using a gel documentation system (GeneSnap Ver. 7.02, England). The size of the amplified product was estimated by comparison with the size of a 1 kb DNA ladder (Fermentas, China). The target bands were cut out and purified with GenepHlow™ Gel Extraction Kit (Geneaid, Taiwan), and sent out to Macrogen, Korea, for sequence analysis.

3.1.3.3 Phylogenetic Analysis

Contiguous sequencing was conducted using BioEdit version 7.2.2 (Hall, 1999). The sequenced data of *Pythium* species was aligned using a multiple sequence alignment program, also known as the CLUSTAL OMEGA (Clustal Omega, 2015). Alignment gaps were treated as missing data, and ambiguous positions were excluded from the analysis. All the ITS sequences were analyzed by UPGMA (Unweighted Pair Group Method with Arithmetic mean), while phylogenetic analysis was performed using MEGA version 6.06 (Tamura *et al.*, 2013). The bootstrap analysis was achieved using 1000 replicates of heuristic searches to determine the confidence levels of inferred phylogenies. The two isolates from Trat province were used as outgroup of phylogenetic analysis. The sequence of each isolate was conducted with BLAST alignment to identify the isolates and analyse the homology of these sequences in comparison to sequences of the same species available in the GenBank database at NCBI (National Center for Biotechnology Information, 2015) (Zhang *et al.*, 2000).

3.2 *In vitro* Screening Pathogenicity of *Pythium* Isolates from Asymptomatic and Symptomatic Lettuce and Selection of Non Pathogenic Strains

3.2.1 Pathogenicity Test

3.2.1.1 Preparation of Plant Material

Seeds of butterhead and green oak lettuce being tested for pathogenicity were surface sterilized by immersion in 6% sodium hypochlorite for 5 minutes, thorough washing for three times with sterilized water, and then drying on sterilized tissue paper.

3.2.1.2 Petri-Dish Assay

The inoculum was prepared from the culture of each isolate of *Pythium* grown on 20% carrot agar over a span of three days. An agar plug (Ø 0.5 mm) of *Pythium* was placed on the center of a Petri-dish (9 cm) containing 2% water agar. Afterwards, ten surface-sterilized seeds were planted surrounding the agar plug and incubated at 26 °C (Zhang and Yang, 2000). An agar plug of 2% water agar without mycelium was used as the control sample. The numbers of germinated seeds and non-germinated seeds (rotten seeds) were counted two days after inoculation (DAI). Disease incidence (DI) and disease severity (DS) of the seedlings were recorded 4 DAI. Five replicates for each isolate were experimented upon, and all experiments were repeated twice.

3.2.1.3 Seed Inoculation Method

The inoculum was prepared using the grass-leaf culture technique (Abad *et al.*, 1994). Ten agar plugs of *Pythium* (Ø 0.5 mm) previously grown on 20% carrot agar for 3 days were placed in a petri dish containing 10 ml of sterilized water and 3 pieces of boiled grass leaves (each 2 cm long). The plates were incubated at room temperature (25-30 °C) under fluorescent light for 2-3 days, or until reproductive structures, sporangia or oospores, were produced. Afterwards, ten seeds of surface-sterilized seeds were immersed in inoculum for 10 minutes, and the infected seeds were placed on a Petri-dish containing sterilized towel paper moistened with 3 ml of sterilized water (Dhingra and Sinclair, 1994). All Petri-dishes were sealed with plastic wrap to prevent water loss and incubated at 26 °C. The numbers of germinated seeds and non-germinated seeds (rotten seeds) were

counted 2 DAI. Disease incidence and disease severity of the seedlings were recorded 4 DAI. The experimental set up contained five replicates of each isolate, and all the experiments were repeated twice.

3.2.1.4 Evaluation of DI and DS

Percentage of DI and DS was calculated by the formula:

$$DI\ (\%) = \frac{\text{(The number of infected germinated seeds)} \times 100}{\text{The total number of germinated seeds}}$$

$$DS\ (\%) = \frac{\Sigma\text{(Disease severity index} \times \text{The number of infected seeds)} \times 100}{\text{(Maximum index} \times \text{The total number of germinated seeds)}}$$

Disease severity index was scored based on a scale from 0 to 5, where the value of 0 represents an asymptomatic root, 1 = 1-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-100%, and 5 = complete root rot, or dead seedling and no occurrence of germination (Figure 3.1) (Rey *et al.*, 1998).

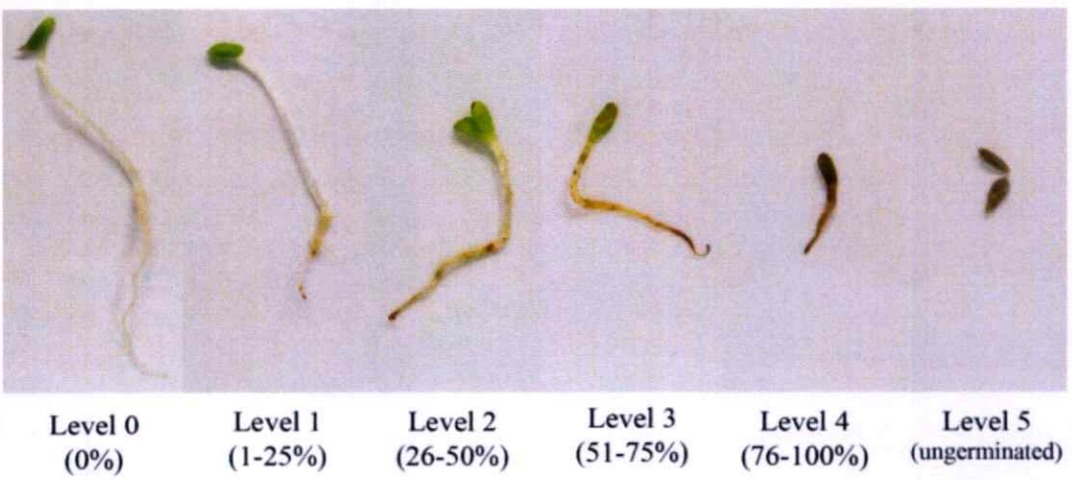

Figure 3.1 Level of disease severity of lettuce seedlings after immersion in spore suspension of *Pythium* species for 4 days.

3.2.1.5 Statistical Analysis

All data were collected and processed from un-germinated seeds (rotten seeds), disease incidence and disease severity of seedlings, as well as root length. The results were analyzed by examination of variance. Treatment means were separated using Duncan's multiple range tests.

3.2.2 Enzymatic Assay

3.2.2.1 *Pythium* Culture

Thirty isolates of *Pythium* from asymptomatic and symptomatic lettuce roots were examined for pathogenicity (Talubnak *et al.*, 2014). Pathogenic and non-pathogenic *Pythium* were selected from pathogenicity test by seed inoculation method. Five isolates of non-aggressive *Pythium* (ASR23, SR33, SR36, IS39, IS40) and three isolates of aggressive *Pythium* (ASR9, ASR26, SR31) were tested using qualitative enzymatic assay on solid media. *Pythium* isolates were cultured on PDA (25 °C, 3 days) and used for enzymatic assay.

3.2.2.2 Enzymatic Production

Cellulase Enzyme

Screening for cellulolytic activity of *Pythium* was conducted on carboxymethylcellulose (CMC) agar medium (0.023 M $NaNO_3$, 0.0057 M K_2HPO_4, 0.00327 M $MgSO_4$, 0.0067 M KCl, carboxymethylcellulose (CMC) sodium salt 0.2% (w/v), peptone 0.02% (w/v), agar 1.7% (w/v), and water 1 L) and the sample was then qualitatively determined using the iodine plate assay (Kasana *et al.*, 2008). A three-day-old agar plug of *Pythium* isolate was placed on CMC medium and incubated at 25 °C for 2-3 days. The plates were flooded with 10 ml of iodine solution (containing 0.04 M KI and 0.013 M iodine in 300 ml distilled water) for 3-5 minutes, and draining off the excess stain for clearer visual observation of the plate zones surrounded by the colonies. Ten microliters of enzymatic solution (10 mg/mL) of standard cellulase extracted from *Aspergillus niger* (Sigma-Aldrich, USA) was used as positive control.

Pectinase Enzyme

The pectinolytic activity of *Pythium* on Czapek agar medium (0.0235 M $NaNO_3$, 0.0057 M K_2HPO_4, 0.002 M $MgSO_4 \cdot 7.H_2O$, 0.5 g; 0.0067 M

KCl, 1 ml of 0.062 M $ZnSO_4$, 1 ml of 0.014 M $CuSO_4$, sucrose 3 % (w/v), agar 2 % (w/v), water 1 L) containing 1% of pectin was determined qualitatively by iodine plate assay (Priya and Sashi, 2014). The pH value was adjusted to 6 before autoclaving at 121 °C for 15 minutes. A three-day-old agar plug containing *Pythium* isolates was placed on pectin medium and incubated at 25 °C for 2-3 days. The plates were flooded with 10 ml of iodine solution (containing 0.04 M KI and 0.013 M iodine in 300 ml distilled water) for 3-5 minutes, draining off the excess stain for better visual observation of the zone (of clearance) around the colony. Ten µl of enzymatic solution (10 mg/mL) of standard pectinase (Sigma-Aldrich, USA) was used as the positive control sample.

Chitinase Enzyme

Screening for chitinolytic activity of *Pythium* was held in a colloidal chitin medium (0.001 M $MgSO_4 \cdot 7H_2O$, 0.023 M $(NH_4)_2SO_4$, 0.011 M KH_2PO_4, 0.005 M citric acid monohydrate, agar 2 % (w/v), 200 µl of Tween-80; colloidal chitin 0.45 % (w/v), bromocresol purple 0.015 % (w/v), and 1 L of water) (Agrawal and Kotasthane, 2012) with adjusted pH value of 4.7. The agar was autoclaved at 121 °C for 15 minutes. A three-day-old agar plug of *Pythium* isolates was placed a medium containing colloidal chitin, and incubated at 25 °C for 5-7 days. The chitinase activity was observed by purple colored zone formation. The positive control sample of *Trichoderma* sp. was obtained from Plant Pathology Laboratory, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL).

3.2.2.3 Calculation of Enzymatic Activity Assay

Cellulase, pectinase and chitinase producing *Pythium* species was examined based on their potential to degrade cellulose, pectin and chitin, which can be derived from the hydrolysis capacity (HC) value. The HC-value is calculated by the following equation (Taechapoempol *et al.*, 2011):

$$\text{Hydrolysis capacity value} = \frac{\text{Diameter of clear zone (cm)}}{\text{Diameter of fungi colony (cm)}}$$

Hydrolysis capacity value is divided into 5 levels, where - = absence; + = HC value < 1.00; ++ = 1.01-2.00; +++ = 2.01-3.00 and ++++ = > 3.00.

3.3 Phytoalexin Production in Lettuce Grown Hydroponically with Elicitors

3.3.1 Phytoalexin Production

3.3.1.1 Plant Materials

Five varieties of lettuce seeds such as butterhead, green oak, red oak, red coral, and cos were germinated on moist sponges in seedling trays at room temperature. After 7 days, seedlings were transferred to the nutrient film technique (NFT) system containing a nutrient solution (EC = 1.8 mS/cm, pH=5.8) (modified after Benoit, 1992), in order to prepare for treatment of 4-11 week old plants with elicitors.

3.3.1.2 Preparation of Elicitors

Copper sulphate ($CuSO_4$) and silver nitrate ($AgNO_3$) were used to stimulate phytoalexin production. $CuSO_4$ was prepared in 2.5% (0.072 M) and 5% (0.144 M) concentrations. $AgNO_3$ was prepared in 0.5% (0.029 M), 1% (0.059 M) concentrations, dissolving readily in sterilized water (modified after Ong and Chong, 2009).

Three isolates *Pythium* (NPA1, NPA2, NPA3) from asymptomatic lettuce were used to induce phytoalexin production by either pouring the suspension on perlite near the plant stem, or spraying it onto the leaf surface until it runs out. The growth of sporangia, zoospores, and oospores of each isolate was stimulated by application of the grass leaf technique (Abad *et al.*, 1994). For sporulation, five agar plugs (Ø 0.5 mm) were placed on a plastic plate (Ø 90 mm) at 28 °C for 5 days under white fluorescent light. The zoospores were released by sporangia under 4 °C temperature for 15 minutes. To induce the encystment of zoospores, the zoospore suspension was agitated in a test tube for 1 minute by a Vortex mixer (Ho and Ko, 1997). The concentration of inoculum was adjusted to 10^4 spore/ml.

3.3.1.3 Elicitation of Phytoalexin

First experiment, phytoalexin production of lettuce was conducted as described by Ong and Chong (2009) using abiotic inducers (1% $AgNO_3$ and 5% $CuSO_4$). The sterilized water was used on the control group.

Second experiment, lettuce leaves were elicited upon getting sprayed with different concentration of abiotic elicitors (0.5% $AgNO_3$, 1% $AgNO_3$, 2.5%

$CuSO_4$ and 5% $CuSO_4$) while sterilized water was used on the control group. In addition, oospores suspensions of non-pathogenic *Pythium* used as biotic elicitor were applied on lettuce root. Treated lettuce was left to grow under normal conditions for 3 days prior to extraction.

3.3.1.4 Extraction of Phytoalexin

The treated leaves of lettuce were washed in running tap water, and homogenized using mortar and pestle. Then, ethanol 60 % (v/v) was added to the sample with a ratio of 10 ml of solvent per gram of tissue, and was left overnight in complete darkness at room temperature (25-30 °C). The homogenate was filtered using Whatman No.1 filter paper, and the residue was again re-extracted under the same method as described before. The extracts were evaporated at 46 °C using a rotary evaporator. The extract was left to evaporate until ~30 % of the total original volume remained. Subsequently, the concentrate was extracted three times with chloroform ($CHCl_3$) and then filtered through Whatman No.1 filter paper before getting concentrated down to a volume of 2 ml. From the extract, phytoalexin and antifungal activity were detected by using thin layer chromatography (TLC) bioassays (Ong and Chong, 2009).

3.3.1.5 Detection of Phytoalexin

Twenty µl of lettuce extract in each week-tested was added to TLC plates (Merck Kieselgel 60 F254 silica gel). The plates were placed in a mixture of hexane:ethyl acetate (1:1 v/v). When the solvent reached 7 cm from the starting point, the plates were taken out, dried, and examined under UV light at 365 nm wavelength. Lettucenin A emitted a greenish yellow florescence (R_f 0.45) (Ong and Chong, 2009).

3.3.1.6 Antifungal Activity of Phytoalexin

The developed TLC plates were sprayed with spores of *Aspergillus niger* (4×10^6 spore/ml), suspended in potato dextrose broth (PDB). The inoculated TLC plates were kept in a moist chamber made from plastic box, and incubated at 25 °C in complete darkness for 3 days. Inhibition zones and the retardation factor (R_f) value were measured as following equation.

$$R_f = \frac{\text{The migration distance of the compound from the origin}}{\text{The migration distance of the solvent from the origin}}$$

3.3.2 Effect of Lettuce Leaf Extracts on Spores of Plant Pathogenic Fungi and Their Pathogenicity Test

To determine the effect of lettuce extracts on spores of all tested plant pathogenic fungi (*Curvularia lunata*, *Fusarium oxysporum*, *Colletotrichum gloeosporioides* and *Pythium aphanidermatum*), 50 µl of the spore suspension (1 x 10^6 spore/ml) were added into welled slides, containing 50 µl of the extract. The slides were incubated in moist plastic box chamber at room temperature (25-32 °C). The spore germination was observed after 30 minutes, 12 hours, 24 hours, and 72 hours after incubation. Abnormal spores and spore germination were randomly counted on each slide under the light microscope for ten times. The abnormal spores and spore germination were calculated by the following equation:

$$\text{Abnormal spore or spore germination (\%)} = \frac{\text{(Number of spore in control – Number of spore in treatment) x 100}}{\text{Number of spore in control}}$$

Thereafter, the treated spores of *Curvularia lunata*, *Fusarium oxysporum*, *Colletotrichum gloeosporioides* and *Pythium aphanidermatum* where inoculated onto chili leaves and lettuce roots. The experiment comprised of 5 treatments and 3 replications (1 plant/replication). The disease symptoms were observed after 5 days after inoculation. The disease was examined by rating disease severity (- = no symptom, + = a weak symptom, ++ = a moderate symptom, +++ = a severe symptom, and ++++ = seedling died).

3.4 Induced Resistance to *Pythium* Root Rot in Hydroponically Grown Lettuce after Stimulation with Elicitors

The experiment was conducted in a hydroponic system and designed in CRD with 7 different kinds of treatments and four experimental replications (60 plants per replication):

T1 = Healthy control (non-treated, non-inoculated)

T2 = Inoculated control with pathogenic *Pythium* SR31

T3 = Inoculated control with *Botrytis cinerea* strain B05.10

T3 = $CuSO_4$ 0.05 mM (Lehotai *et al.*, 2011)

T4 = $AgNO_3$ 0.01 mM

T5 = BABA 0.1 mM (Parinthawong and Koohakan, 2009)

T6 = Non-pathogenic *Pythium* SR36

T7 = Non-pathogenic *Pythium* sp. ASR23

3.4.1 Plant Materials

The butterhead lettuce seeds (*Lactuca sativa* L.; from HI-Q Agricultural Co., LTD., Bangkok, Thailand) were used in this study. Seeds were surface-sterilized (2.5 % (v/v) sodium hypochlorite, 5 min), rinsed with 100 ml of sterilized water, and incubated at 4 °C for one day. Seeds were planted in seedling trays with autoclaved perlite and placed in the growth chamber at 28 °C, either under 12 hours of light and 12 hours of darkness. Seven days after seeding, the seedlings were fertilized with nutrient solution (Benoit, 1992).

3.4.2 Preparation of Inducer

3.4.2.1 Chemical Preparation

A stock solution containing 1 M $CuSO_4$ $5H_2O$ and 1 M DL-3-amino-*n*-butanoic acid (BABA) was used in this study. All chemicals were dissolved in sterilized water before use.

3.4.2.2 Fungal Preparation

Three isolates of *Pythium*, ASR23, SR31 and SR36, were individually cultured on PDA (Potato dextrose agar) at 28 °C for 2 days. The growth of Sporangia, zoospores, and oospores of each isolate was stimulated by application of the grass leaf technique (Abad *et al.*, 1994). For sporulation, five agar plugs (Ø 0.5 mm) were placed on a plastic plate (Ø 90 mm) at 28 °C for 5 days under white fluorescent light. The zoospores were released by sporangia under 4 °C temperature for 15 minutes. To induce the encystment of zoospores, the zoospore suspension

was agitated in a test tube for 1 minute by a Vortex mixer (Ho and Ko, 1997). The concentration of inoculum was adjusted to 10^4 spore/ml.

The spore suspension of *Botrytis cinerea* strain B05.10 was derived by Dr. Schoonbeek, H.J. (John Innes Centre, UK). Fresh PDA plates of *B. cinerea* were cultured on PDA. When a matt of aerial hyphae became clearly visible, 10 ml of sterilized water mixed with 0.05% tween 20 was added, then scraped mycelium on PDA. The spores and hyphae were filtered by cotton using the flat side of a pipette tip. The spore suspension was collected, and spore density was counted with a haemocytometer. Suspensions were usually diluted to approximately 1 x 10^8 spores/ml for storage at 4 °C. The spore suspensions were stored at 4 °C, and collected over a span of 3 days (Lloyd *et al.*, 2014).

3.4.3 Fungal and Chemical Treatments

Pythium and *B. cinerea* suspensions were added to 3-week-old seedlings of lettuce plants by either pouring the suspension on perlite near the plant stem, or spraying it onto the leaf surface until it runs out (about 2 ml/plant). Chemicals were individually mixed in the nutrient solution, with a final concentration of 25 mM $CuSO_4.5H_2O$ and 0.1 mM BABA. Plants were incubated in the growth chamber at 28 °C under 12 hours of light and 12 hours of darkness. After one day of incubation, plants were inoculated with pathogenic *Pythium* SR31 by pouring the suspension on perlite. Sterilized water was applied as mock treatment.

3.4.4 Analysis of Induced Resistance

3.4.4.1 Gene Expression Assays

In the study of gene expressions, the treated leaves and roots were collected at 0, 24, and 48 hours after inoculation. Refer to Table 3.1 For the list of gene expressions used in this research.

Extraction of Total RNA from Tissue

The leaves and roots of the butterhead lettuce were collected at different time intervals (0, 24, and 48 hours post induction (hpi)). The samples were immediately left to freeze in liquid nitrogen, and were stored at -80 °C until extraction. For RNA extraction, TRIzol reagent (Sigma) was used. A total of 100-200 mg of leaves and 50-100 mg of roots were grounded with a 2010 Geno/Grinder®

(United States). 1.5 ml of TRIzol reagent was added to all samples and shaken vigorously. After shaking, samples were incubated (5 min) and centrifuged (10 min; 12000 g) at room temperature, and the lysate was transferred to a new 2 ml Eppendorf tube. 150 µl of BCP (1-bromo 3-chloropropane) was added to the supernatant and shaken vigorously for 15 seconds to isolate RNA from DNA and proteins. After incubation (10 min, room temperature), samples were centrifuged (12000 g, 10 min, 4 °C) to separate the layers. The upper aqueous layer was transferred to a new 2 ml Eppendorf tube. Afterwards, RNA precipitates were collected upon addition of 800 µl of isopropanol and flipping of the Eppendorf tube upside down. The RNA precipitate was incubated for 5 minutes at room temperature, and centrifuged for 8 minutes at 4 °C (12000 g). After discarding the supernatant, 1000 µl of 75% ethanol was added, and then the pellet was washed gently by flipping the Eppendorf tube. After centrifugation (12000 g; 5 min; 4 °C), the supernatant was discarded and the RNA pellet was left to dry at room temperature until all the ethanol evaporates before the resuspension of the RNA pellet in 60 µl DEPC water. The RNA samples were stored at -20 °C.

Treatment with DNase and Synthesis of cDNA

All RNA samples were treated with Ambion Turbo DNase™ to eliminate gDNA contamination. 30 µl of RNA sample was added and left to react with 4 µl of Turbo mixtures (3.5 µl of 10x Turbo DNase™ buffer and 0.5 µl of Turbo DNase™ enzyme) in a 1.5 ml Eppendorf tube. After incubation for 20 min at 37°C, to a 0.5 µl sample of Turbo DNase™ enzyme was added and incubated for 20 min, then 3.45 µl of Turbo DNase™ inactivating substance was added to each sample. The PCR plate was left for 2 min at room temperature and then centrifuged (10000 g for 1.5 min). The RNA was transferred to a new 1.5 ml Eppendorf tube and quantified by a spectrophotometer (Thermo NanoDrop™, United States). For the observation of the 260/280 ratios, wavelengths of 260 nm and 280 nm were used. Samples with values that ranged from 1.8 to 2.1 were considered to be of high quality. The synthesis of cDNA was performed using SuperScript® IV Reverse Transcriptase (Invitrogen). The first strand of cDNA was synthesized using a mixture of 10 µl of 1000 ng of total RNA in water, 1 µl of Oligo $(dT)_{20}$ primers (50 µM), 1 µl of dNTP mix (10 mM), and 1 ul of random hexamers (50 µM) (Invitrogen), which was then briefly centrifuged. The RNA-primers were incubated at 65 °C for 10 min in order for it be annealed to template

RNA, and then incubated in ice for at least 1 min. Thereafter, the RT reaction mix was prepared by mixing 4 µl of 5x SSIV buffer, 1 µl of 100 mM DTT and 1 µl of SuperScript® IV Reverse Transcriptase (200 U/µl) together, 6 µl of RT reaction mix was then added to each RNA sample. The combined mixture of annealed RNA and RT reaction mix was then briefly centrifuged. After centrifugation, the combined reaction mixture was placed in a G-Strom (GS1 Thermal Cyclers, United Kingdom) and incubated at 50 °C for 15 min, and then 80 °C for 10 min. The cDNA samples were stored in a freezer at -20 °C.

Quantitative Reverse Transcription PCR (qRT-PCR)

Synthetic cDNAs were used as templates in qRT-PCR, along with specific primers for each gene expression (Table 3.1) and SYBR® Green (Sigma). All qRT-PCR processes were carried out using LightCycler® 480 (Roche, Germany). Cycling parameters for all qRT-PCR were 96°C for 4 minutes, followed by 45 cycles at 94 °C for 20 seconds, 60 °C for 20 seconds and 72 °C for 20 seconds. Ten µl reaction mixtures are composed of 2.5 µl of 20x cDNA (1 µg), 0.2 µl of each primer (10 µM), 5 µl of SYBR® green and 2.1 µl of sterilized water. The qRT-PCR was performed over three repetitions for each sample.

3.4.4.2 PAMP Response Assay

Oxidative Burst

Seven day-old butterhead lettuce seedlings grown on MS medium were pre-treated with various elicitors such as 0.1 mM of BABA, 0.25 mM of $CuSO_4.5H_2O$, non-pathogenic *Pythium* ASR23, SR36 and pathogenic *Pythium* SR31 one day prior to ROS measurement. For the mock treatment, half of the MS broth was used. The ROS measurement was performed using a luminol/peroxidase-based assay (Lloyd *et al.*, 2014). Leaf discs (d = 4 mm) were cut with a cork borer. Seedling roots (7 day after pre-treatment) were divided into 3 parts - top, middle and bottom root (Figure 3.2). Each sample segment was left to suspend in an individual well of a 96-well microtiter plate, each containing 200 µl of sterilized water, and then incubated for 24 hours at room temperature (18-22 °C) in complete darkness. The water was drained and replaced by a solution containing 34 mg/L (0.2 nM) luminol, 20 mg/l horseradish peroxidase (HRP) and PAMPs (flg22, HaNLP, chitinase, and xylanse). Luminescence was recorded by counting photons in 120 reads over a 60-minute

period, using a Varioskan Flash plate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Table 3.1 The primer name and sequences for the expression analysis using a qRT-PCR.

Primer name	Oligonucleotide sequence (5'-3')
LsDEF	F: AGCAACCGTGAAGGGAGAAT
	R: ACTGGTCGTCGCAGTGTCTT
LsLTC1	F: GATGCTTTGGCCTGGTATGA
	R: GGCTGATTGACCCCTTTCTC
LsLTC2	F: AGAAGCCGAGTGGACAAACA
	R: TGTCGCCCATACCCACTAAA
LsEIF2α	F: TAGGCGAGTGGAGAAGCATT
	R: GTAGAAACAGCAACAGGCAAA
LsTIP4	F: GAGAGATTTGCTGGAGGGAAACTA
	R: CCTTTGACTGATGATGTTTGGA
LsPR1b_like	F: ATGCACTCCCAAAACCGTCC
	R: CTCGCCAACCCACATGTTCA
LsERF1	F: TCGCCGGTGATGTCCAGTTATCAA
	R: TGTTTCCCTCTCTGCTGGTTCACA
LsAOS	F: TCTTCCAACTCCAAAGTCGTCGCT
	R: TTGAGTGCATGGTGGGTAGGTTCA
PyITS1	F: ATGTCTAGGCTCGCACATCG
	R: GGCATACTTCCAGGCATAACC
PyITS2	F: ATGTCTAGGCTCGCACATCG
	R: AGTGCAATGTGCGTTCAAAAT

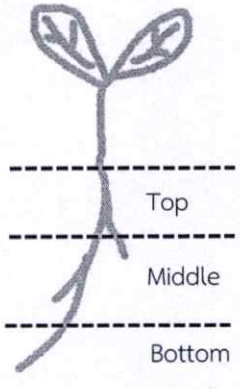

Figure 3.2 The schematic of lettuce seedling plant presenting the position where lettuce root was cut.

Histological Analysis

Superoxide (O_2^-) and peroxidase (H_2O_2) were detected by staining methods, which involved nitroblue tetrazolium (NBT) and 3, 3'-diaminobenzidine tetrachloride (DAB), respectively (Sigma-Aldrich).

CHAPTER 4

RESULTS

4.1 Collection, Identification and Studies of *Pythium* Species from Asymptomatic and Symptomatic Lettuce

4.1.1 Collection and Isolation of *Pythium*

Asymptomatic and symptomatic lettuce roots were collected from the following hydroponic farms in Thailand: 1) commercial hydroponic farm at Pak Chong, Nakorn Ratchasima province, 2) Greenhouse at KMITL, Bangkok, and 3) commercial hydroponic farm at Bangkok. The samples were randomly collected depending on disease severity and lettuce varieties. 36 isoates of asymptomatic and symptomatic *Pythium* spp. were extracted from hydroponic grown lettuce roots, while 2 isoates of symptomatic *Pythium* spp. were isolated from infested soil grown lettuce roots. Finally, 1 isolate of *Pythium* sp. and 1 isolate of *Phytophthora* sp. were provided by Dr. Veeranee Tongsri, from the Department of Plant Pathology, Faculty of Agriculture, Kasetsart University Bang Khaen. It was recovered from infested soil of durian plant at Trad province. The details of origin and morphological descriptions of each isolate of *Pythium* are presented in Table 4.1.

4.1.2 Morphological Characterization

Pythium species were identified according to their morphological characteristics. The 30 each of morphological features were measured and taken photo such as sporangia, oogonia, oospores and colony morphology. Identification of specific species or variety was based on monographs or the identification key of van der Plaats-Niterink (1981). The morphological characteristics of 40 isolates were divided into 4 groups.

1) This group developed a white colony in PDA after 4 days of inubation, showing aerial cottony mycelium with no special pattern, while the colony in PCA medium showed some little loose aerial mycelium (Figure 4.1A-B). Main hyphae were up to 7 µm wide, hyaline, coenocytic. Sporangium may be terminal or intercalary, filamentous inflated, simple or branched (Figure 4.1C). Oogonia may be

Table 4.1 Origin and morphological descriptions of *Pythium* species isolated from hydroponic farms and cultivated soil.

No.	Isolates	Origin				Morphological Descriptions						Species[5/]
										Oospores		
		Source[1/]	Host[2/]	Origin (Thailand)[3/]	Colony	Hyphal Width (µm)[4/]	Sporangia (µm)[4/]	Oogonia Diameter (µm)[4/]	Antheridia No. per Oogonium[4/]	Diameter (µm)[4/]	Wall (µm)[4/]	
1	ASR1	H	RO	Bangkok	No pattern	5.66±0.76	8.27±1.35	23.14±2.85	1	17.59±2.12	1.81±0.34	Pa
2	ASR2	H	RO	Bangkok	No pattern	6.29±0.63	8.13±2.23	24.51±2.20	1	15.85±1.35	2.36±0.39	Pa
3	ASR3	H	RO	Bangkok	No pattern	6.14±0.95	10.49±2.22	22.53±1.40	1	15.54±1.04	1.78±0.25	Pa
4	ASR4	H	RO	Bangkok	No pattern	6.03±1.16	7.45±1.40	20.55±1.77	1	15.05±1.64	1.61±0.39	Pa
5	ASR5	H	BH	Bangkok	No pattern	5.53±0.78	6.15±1.60	22.77±1.19	1	17.49±1.79	1.54±0.24	Pa
6	ASR6	H	BH	Bangkok	No pattern	6.06±0.75	8.76±1.58	22.02±1.99	1	16.52±2.00	1.57±0.35	Pa
7	ASR7	H	GO	Bangkok	No pattern	6.18±1.05	7.89±2.62	21.91±1.46	1	15.97±1.51	1.43±0.30	Pa
8	ASR8	H	GO	Bangkok	No pattern	6.90±1.15	7.50±2.13	20.95±1.34	1	14.74±1.33	1.43±0.34	Pa
9	ASR9	H	RC	Bangkok	No pattern	4.95±0.70	9.03±3.16	22.22±1.63	1	16.26±2.09	1.58±0.30	Pa
10	ASR10	H	RC	Bangkok	No pattern	6.55±1.41	7.98±2.72	33.15±6.93	4-10	23.88±3.19	1.69±0.28	Pm
11	ASR11	H	RO	Bangkok	No pattern	5.83±1.17	7.81±2.53	23.28±2.92	1	16.26±0.98	1.76±0.25	Pa
12	ASR12	H	RO	Bangkok	No pattern	5.01±1.11	6.83±3.18	25.67±4.76	1	17.23±1.93	1.41±0.19	Pa

Table 4.1 Continued.

No.	Isolates	Origin				Morphological Descriptions						Species[5/]
										Oospores		
		Source[1/]	Host[2/]	Origin (Thailand)[3/]	Colony	Hyphal Width (µm)[4/]	Sporangia (µm)[4/]	Oogonia Diameter (µm)[4/]	Antheridia No. per Oogonium[4/]	Diameter (µm)[4/]	Wall (µm)[4/]	
13	ASR13	H	RO	Bangkok	No pattern	5.26±0.75	7.82±3.15	23.08±2.68	1	17.78±2.10	1.19±0.38	Pa
14	ASR14	H	BH	Bangkok	No pattern	5.27±0.62	8.81±2.42	22.83±1.26	1	16.44±1.95	1.69±0.23	Pa
15	ASR15	H	BH	Bangkok	No pattern	6.35±1.01	7.16±1.42	24.21±2.24	1	17.21±1.58	1.73±0.34	Pa
16	ASR16	H	BH	Bangkok	No pattern	5.42±0.91	9.38±1.14	24.76±2.71	1	18.15±1.70	1.16±0.36	Pa
17	ASR17	H	BH	Bangkok	Rosette pattern	No Data	No Data	No Data	No Data	No Data	No Data	-
18	ASR18	H	GO	Bangkok	No pattern	5.88±1.06	7.89±2.26	19.88±1.51	1	16.01±1.28	1.28±0.23	Pa
19	ASR19	H	GO	Bangkok	No pattern	5.34±1.07	8.30±1.78	22.56±3.59	1	16.25±7.54	1.44±0.21	Pa
20	ASR20	H	COS	Bangkok	No pattern	4.75±0.87	8.87±2.12	34.27±5.97	4-10	23.85±2.56	1.52±0.28	Pm
21	ASR21	H	COS	Bangkok	No pattern	6.84±1.41	8.54±3.33	35.20±4.14	4-10	21.84±4.55	1.12±0.20	Pm
22	ASR22	H	COS	Bangkok	No pattern	6.40±1.44	7.87±2.27	40.32±3.31	4-10	25.68±4.30	1.35±0.50	Pm
23	ASR23	H	RC	NRC	No pattern	4.81±0.69	6.99±2.14	21.64±1.97	1	17.42±1.47	1.40±0.20	Pa
24	ASR24	H	BH	NRC	No pattern	5.07±0.56	7.56±2.14	23.38±2.54	1	16.51±1.38	1.36±0.39	Pa
25	ASR25	H	BH	NRC	No pattern	5.46±0.78	7.93±2.14	25.14±2.65	1	18.33±1.73	1.29±0.32	Pa

Table 4.1 Continued.

No.	Isolates	Origin				Morphological Descriptions						Species[5/]
										Oospores		
		Source[1/]	Host[2/]	Origin (Thailand)[3/]	Colony	Hyphal Width (µm)[4/]	Sporangia (µm)[4/]	Oogonia Diameter (µm)[4/]	Antheridia No. per Oogonium[4/]	Diameter (µm)[4/]	Wall (µm)[4/]	
26	ASR26	H	BH	NRC	No pattern	5.52±0.89	8.14±2.18	21.62±1.21	1	15.38±1.36	1.52±0.29	Pa
27	ASR27	H	GO	NRC	No pattern	5.39±0.82	8.04±1.65	24.47±2.01	1	17.58±1.85	1.35±0.33	Pa
28	SR28	H	RC	Bangkok	No pattern	6.36±1.47	6.48±1.92	33.78±3.13	4-10	21.21±2.42	1.26±0.30	Pm
29	SR29	H	RO	Bangkok	No pattern	5.24±1.41	7.15±1.19	39.19±4.89	4-10	22.99±3.79	1.68±0.37	Pm
30	SR30	H	RO	Bangkok	No pattern	6.21±0.99	8.83±1.61	33.54±2.15	4-10	23.21±2.39	1.54±0.25	Pm
31	SR31	H	RO	Bangkok	No pattern	6.62±1.73	9.28±2.52	24.86±3.65	4-10	21.78±2.60	1.49±0.20	Pm
32	SR32	H	RO	Bangkok	No pattern	5.74±0.84	7.68±1.66	29.94±3.97	4-10	22.77±2.93	1.33±0.20	Pm
33	SR33	H	RO	Bangkok	Rosette pattern	No Data	No Data	7.50±0.91	No Data	No Data	No Data	-
34	SR34	H	RO	Bangkok	No pattern	4.69±0.74	7.00±2.37	36.31±3.93	4-10	24.58±4.59	1.37±0.27	Pm
35	SR35	H	RO	Bangkok	No pattern	5.74±1.22	6.32±2.08	34.54±4.77	4-10	19.52±3.31	1.56±0.36	Pm
36	SR36	H	RO	Bangkok	Rosette pattern	No Data	No Data	No Data	No Data	No Data	No Data	-
37	IS37	S	GO	Bangkok	No pattern	5.37±0.79	10.85±1.77	26.77±2.19	1	19.03±1.35	1.37±0.22	Pa

Table 4.1 Continued.

No.	Isolates	Origin				Morphological Descriptions						Species[5/]
										Oospores		
		Source[1/]	Host[2/]	Origin (Thailand)[3/]	Colony	Hyphal Width (µm)[4/]	Sporangia (µm)[4/]	Oogonia Diameter (µm)[4/]	Antheridia No. per Oogonium[4/]	Diameter (µm)[4/]	Wall (µm)[4/]	
38	IS38	S	GO	Bangkok	No pattern	5.94±0.79	9.56±2.07	19.01±1.81	1	12.64±1.87	0.93±0.24	Pd
39	IS39	S	Durian	TRD	Chrysanthemum pattern	3.00±0.58	No Data	18.84±1.41	No Data	13.36±1.04	1.11±0.26	-
40	IS40	S	Durian	TRD	Distinct pattern	6.08±1.01	29.54±5.05 x 40.46±8.34			23.40±4.41	1.57±0.30	*Phytophthora*

[1/]H=Hydroponics, S=Soil; [2/]RO=Red Oak, BH=Butterhead, GO=Green Oak, RC=Red Coral; [3/]NRC=NakornRatchasima province, TRD=Trad province; [4/]Mean ± Standard deviation; [5/]Species determined according to morphological descriptions of type strains by (Plaats-Niterink, 1981) PA=*Pythium aphanidermatum*, PM=*Pythium myriotylum*, Pd=*Pythium deliense*.

terminal or intercalary, is spherical, 23.14±2.85 µm in diameter, and smooth walled (Figure 4.1D-E). Antheridia mostly intercalary, sometimes terminal, antheridial branches monoclinous, 1 per oogonium (Figure 4.1D-E). Oospores aplerotic, spherical, 17.59±2.12 µm in diameter, wall 1.81±0.34 µm thick, smooth walled (Figure 4.1F-H). These characteristics were used to identify *Pythium aphanidermatum*. It was found in 24 isolates (ASR1-ASR9, ASR11-ASR16, ASR18-ASR19, IS37, and IS38) (Table 4.1).

2) This group was presented by a 4 day old white colony in PDA, showing aerial cottony mycelium with no special pattern, while the colony formed in PCA medium contains aerial cottony mycelium at the edge of colony (Figure 4.2A-B). Main hyphae were up to 7 µm in width, hyaline, coenocytic. Sporangium filamentous inflated in a simple or branched manner, terminal or intercalary (Figure 4.2C). Oogonia either intercalary or terminal, spherical, 34.27±5.97 µm in diameter, smooth walled (Figure 4.2D-F). Antheridia present, antheridial stalk branched, mostly diclinous, 4-10 per oogonium (Figure 4.2D-F). Oospores aplerotic, spherical, 23.85±2.56 µm in diameter, wall 1.52±0.28 µm thick, smooth walled (Figure 4.2G-H). 11 isolates were classified in this group, identified as *Pythium myriotylum*. This included the following isolates: ASR10, ASR20-ASR27, SR28-SR32, SR34, and SR35 (Table 4.1).

3) This group developed a white rosette patterned colony on PDA after 4 days, showing flat cottony mycelium, while colony on PCA medium showed small traces of loose flat mycelium (Figure 4.3A-B). Hyphae hyaline, coenocytic. Swelling hypha was seen as filamentous and sporangium-like, mostly terminal (Figure 4.3C). Small oogonia and antheridia-like structures were mostly terminal or intercalary, globose, smooth walled (Figure 4.3D-E). Four isolates were classified in this group, identified as unknown *Pythium* sp. There were isolates ASR17, SR33, SR36, and IS39 (Table 4.1).

4) This group presented a distinct colony pattern on PDA after 8 days of incubation, showing loose aerial white mycelium, while colony on PCA medium showed loose flat aerial mycelium (Figure 4.4A-B). Hyphae hyaline, coenocytic, 6.08±1.01 µm in width. Sporangium limoniform or ovoid single or branched, papillae, terminal or intercalary, 29.54±5.05 µm in width, 40.46±8.34 µm in length (Figure 4.4D-E). Oogonia terminal or intercalary, globose, smooth walled (Figure 4.4C). Antheridia single,

monoclinous, 1 per oogonium (Figure 4.4C). Oospores aplerotic, globose, 23.4±4.41 µm in diameter, wall 1.57±0.30 µm thick, smooth walled (Figure 4.4F). Only 1 isolate, IS40, was classified in this group, and identified as *Phytophthora* sp. (Table 4.1).

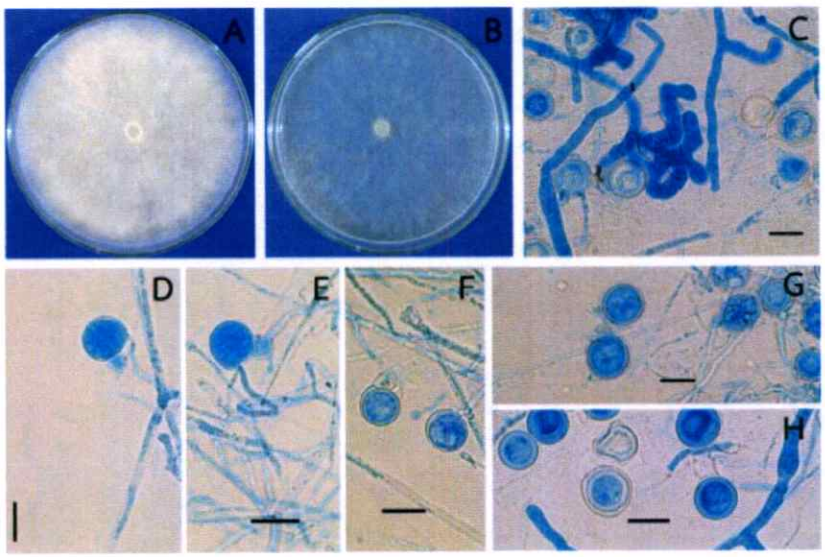

Figure 4.1 *Pythium aphanidermatum* strain ASR1, representation of group 1. A-B. colony on PDA and PCA incubated for 4 days at 26 °C, C. filamentous and lobulate sporangium, D-E. oogonium with terminal antheridium, F-H. aplerotic oospore. Scale Bars = 20 µm.

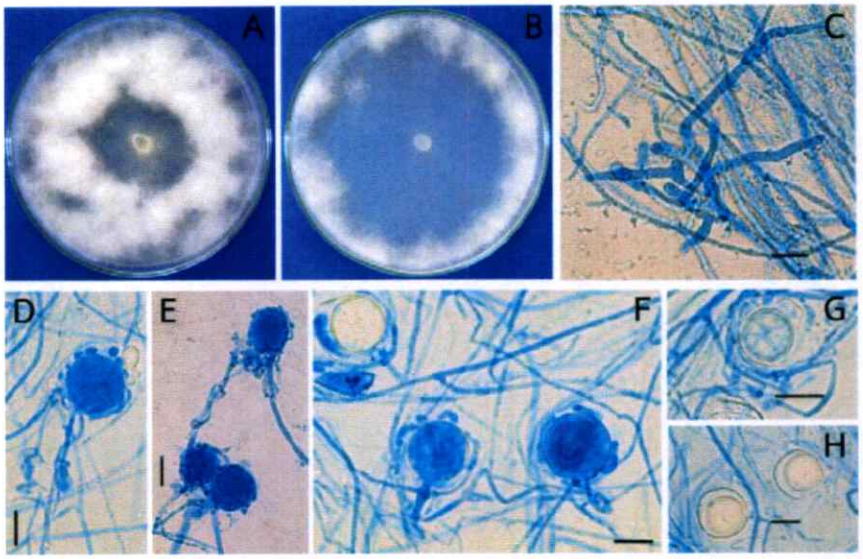

Figure 4.2 *Pythium myriotylum* strain SR31, representation of group 2. A-B. colony on PDA and PCA incubated for 4 days at 26 °C, C. filamentous inflate sporangium, D-F. oogonia surrounded by curve-shaped, mostly diclinous antheridia, G-H. aplerotic oospore. Scale Bars = 20 µm.

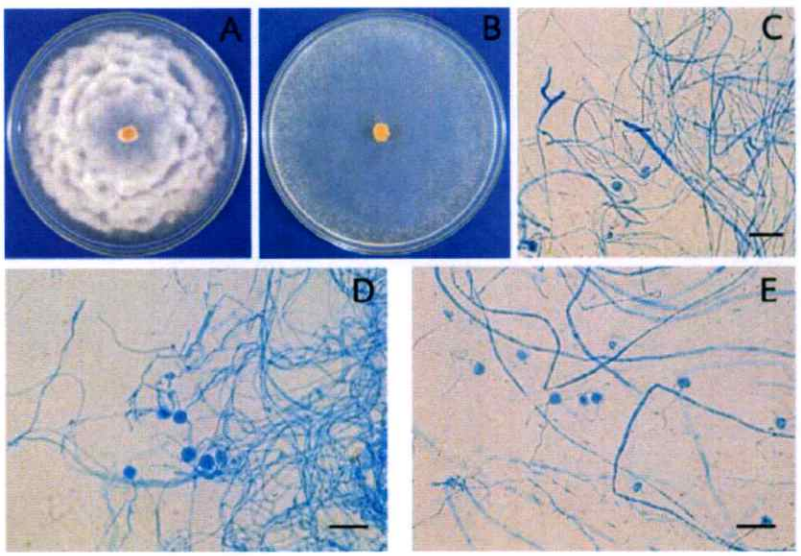

Figure 4.3 Unknown *Pythium* sp. strain SR33, representation of group 3. A-B. colony on PDA and PCA incubated for 4 days at 26 °C, C. filamentous sporangium-like, D-E. small oogonia and antheridia-like. Scale Bars = 20 µm.

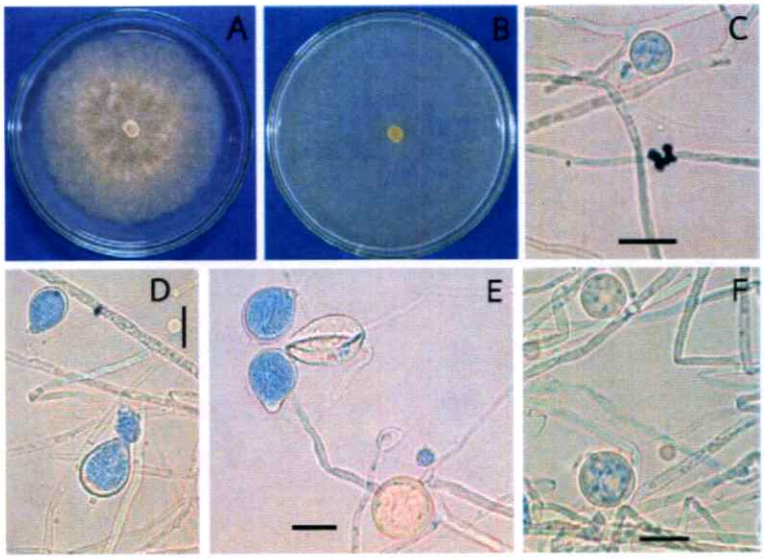

Figure 4.4 *Phytophthora* sp. strain IS40, representation of group 4. A-B. colony on PDA and PCA incubated for 8 days at 26 °C, C. oogonium with antheridium, D-E. Sporangium limoniform or ovoid single or branched, papillae, terminal or intercalary lemon sporangium, F. plerotic oospore. Scale Bars = 20 µm.

4.1.3 Molecular Characteristics

The results of the *Pythium* species identification using DNA sequence analysis yielded multiple identities of a single isolate according to the NCBI database. Therefore, species identification in these cases was based primarily on morphological features and comparison of the unknowns to various species identified by sequence analysis. A total of 40 isolates was identified based on morphological features, which included the *Pythium aphanidermatum* group, *P. myriotylum* group, unknown *Pythium* sp. group, and *Phytophthora* sp. group. All isolates were re-identified through a molecular method using the internal transcribed spacer (ITS) region of rDNA with specific primers. The amplified rDNA-ITS region of all *Pythium* isolates was between 750-800 base pairs in length, and BLAST search yielded the highest similarity to oomycetes. Results from the obtained UPGMA phylogenetic tree showed that the grouping of isolates based on rDNA-ITS analyses was in agreement with the grouping obtained from morphological characteristics. The 38 isolates of *Pythium* isolated from lettuce were grouped in the same cluster, while 2 isolates from durian

were a seperated group (Figure 4.5A). The 2 isolates IS40 and IS39 showed 100% similarity to *Phytophthora palmivora* and *Pythium cucurbitacearum*, respectively, which were catergorized under the "out" group. Among sequencing data of 38 isolates, data of 23 isolates were 100% identitical to those of *P. aphanidermatum* (KT336808 and KR095341). The 4 isolates (SR34, ASR10, SR29 and SR35) showed 100% similarity to *Pythium myriotylum* (KM434129), while 6 isolates (SR30, ASR20, SR32, ASR21, ASR22 and SR31) showed 99% identity to *P. myriotylum* (KT364295) and 1 isolate, IS38, showed 100% identity to *P. deliense* (AJ233442 and KP183964). The 3 isolates that could not be identified morphologically (SR33, SR36 and ASR17) all clustered together with *Pythium* sp. CLE-2015d (KT247392) (Figure 4.5B).

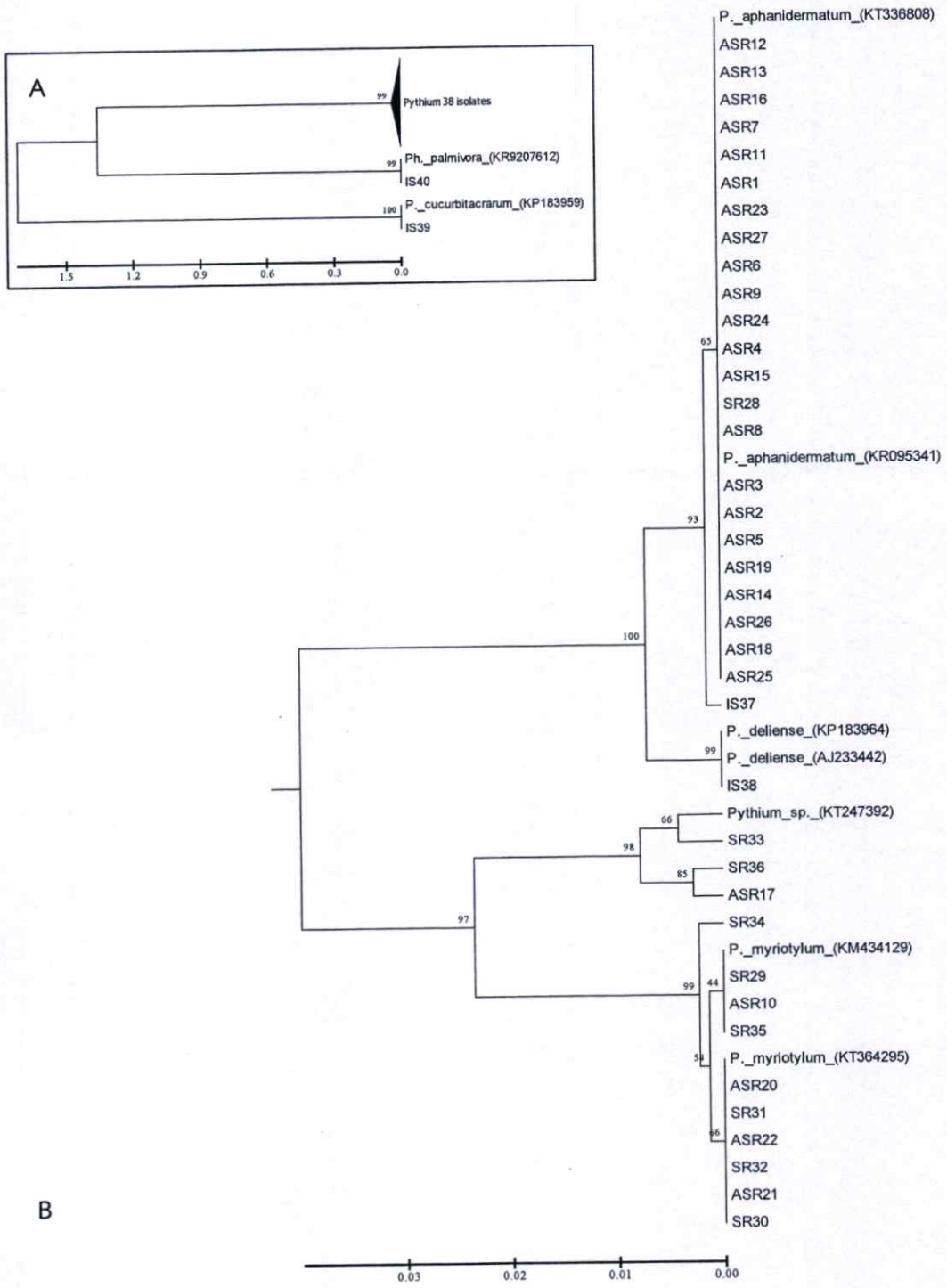

Figure 4.5 Phylogenetic tree of *Pythium* species using the rDNA-ITS region sequence data based on UPGMA analysis. Numbers within the tree represent the bootstrap values (1,000 replications), (bar = 0.02). A = Phylogenetic tree showing all 40 isolates, B = Tree showing only group of *Pythium* 38 isolates from A

4.2 *In vitro* Screening Pathogenicity of *Pythium* Isolates from Asymptomatic and Symptomatic Lettuce and Selection of Non Pathogenic Strains

4.2.1 Pathogenicity Test

A total of 40 isolates of *Pythium* spp. responsible for root rot disease was recovered from asymptomatic and symptomatic roots of lettuce grown in hydroponic systems and infested soil. All collected isolates of *Pythium*, either from asymptomatic or symptomatic plants, were shown to be pathogenic to one or both varieties of lettuce seedlings, but differ based on how severely they infect their hosts (level of disease severity can be categorized in the following manner: 0=no symptom; 1=root rot 1-25%; 2=root rot 26-50%; 3=root rot 51-75%; 4=root rot 76-100% and 5=ungerminated or died seed). The symptoms of root rot disease such as root discoloration, brown necrosis on tap root or stem, and soft rot appeared within 48 to 96 hours after inoculation. Results derived from 2 pathogenicity tests, upon comparison, revealed more than 14% seed germination among 2 lettuce species. Butter head and green oak revealed 4% of fresh un-germinated seeds in Petri-dish assay, while 14 and 0 % were respectively shown in the seed inoculation method. Regarding the Petri-dish assay, *Pythium* isolates from asymptomatic lettuce roots caused 0-20 and 0-32% seed rot, respectively, in butterhead and green oak. These isolates were also responsible for 0-12% percent seed rotting of the butterhead, and 0-16% seed rotting of the green oak. The previously mentioned seed rot percentage was not statistically different compared to the control group. Based on disease occurrence in seedlings, 3 isolates from asymptomatic roots caused DI in butterhead and green oak were categorized according to the following ranges of 0-25% of disease incidence with DS mostly less than 50%. Meanwhile, 4 and 3 isolates from symptomatic roots caused DI in butterhead and green oak, respectively, were categorized according to the following ranges of 0-25% of disease incidence with varied levels of DS. This result indicated that *Pythium* from symptomatic root could infect plant and show more severe than *Pythium* from asymptomatic root. Root lengths varied in groups receiving different treatments, and were strongly correlated with with DI and DS. That is, the higher the value of DI and DS, the shorter of the root length. ASR23 (*Pythium* isolate from asymptomatic root) and SR33 (*Pythium* isolate from symptomatic root) were proven to be non-pathogenic isolates, accordingly (Table 4.2). Seed rot and seedling symptoms in Petri-dish assay are depicted in Figure 4.6A-B.

Table 4.2 The percentage of ungerminated seeds, disease severity, disease incidence and root length of lettuce seedlings using Petri-dish assay.

Isolates[1/]	Host[2/]	Petri-dish Assay							
		BH				GO			
		2 DAI	4 DAI Post-emergence			2 DAI	4 DAI Post-emergence		
		Ungerminated seeds (%)[3/]	DI (%)[5/]	DS (%)[6/]	Root Length (cm)[7/]	Ungerminated seeds (%)	DI (%)	DS (%)	Root Length (cm)
Control	-	4ab[4/]	0	0	2.35±0.37a-d	4a	0	0	2.28±0.47b-d
ASR1	RO	4ab	50	13.5	1.75±0.36e-i	0a	80	20	1.76±0.24e-i
ASR2	RO	16a-c	71.4	19	1.43±0.55f-m	8a	56.5	16.3	1.38±0.35i-k
ASR3	RO	16a-c	66.7	16.7	2.19±0.29a-e	0a	32	8	1.92±0.15c-g
ASR4	RO	16a-c	38.1	9.5	1.68±0.41e-j	0a	48	13	1.92±0.3c-g
ASR5	BH	4ab	75	22.9	1.2±0.42i-q	0a	64	24	1.88±0.59d-h
ASR6	BH	8a-c	69.1	23.9	1.65±0.75e-k	4a	66.7	20.8	1.99±0.2b-g
ASR7	GO	0a	28	7	1.46±0.32f-m	8a	21.7	5.4	1.9±0.19c-g
ASR8	GO	4ab	29.2	7.3	1.97±0.35b-f	0a	40	10	1.44±0.32h-k
ASR9	RC	4ab	66.7	18.8	1.61±0.50f-k	4a	100	28.1	1.72±0.48f-i
ASR10	RC	8a-c	60.9	15.2	1.69±0.41e-j	8a	95.7	28.3	1.66±0.28f-i
ASR11	RO	12a-c	100	37.5	1.39±0.72f-n	4a	54.2	13.5	1.85±0.27d-h
ASR12	RO	4ab	100	40.6	0.76±0.16p-t	12a	100	48.9	0.83±0.14l-o
ASR13	RO	0a	72	24	1.61±0.52f-k	4a	62.5	16.7	1.78±0.27e-i
ASR14	BH	12a-c	100	26.1	0.73±0.15q-t	12a	100	32.9	0.94±0.19l-o
ASR15	BH	24c	100	39.5	1.19±0.57i-r	8a	91.3	27.2	1.87±0.23d-h
ASR16	BH	12a-c	100	38.6	1.84±0.64d-h	32b	88.2	32.4	1.17±0.21kl
ASR17	BH	20bc	25	6.3	1.31±0.29g-p	4a	16.7	4.17	2.25±0.42b-d
ASR18	GO	12a-c	100	53.4	0.98±0.13l-t	8a	60.9	16.3	2.33±0.13bc
ASR19	GO	8a-c	100	48.9	0.84±0.13n-t	12a	54.6	13.6	2.07±0.25b-f
ASR20	COS	8a-c	100	92.4	0.55±0.08st	4a	100	96.9	0.72±0.12m-o
ASR21	COS	0a	100	31	1.78±0.52e-h	12a	100	50	1.02±0.31k-n
ASR22	COS	4ab	100	39.6	1.13±0.24j-r	8a	100	53.3	0.73±0.13m-o
ASR23	RC	4ab	0	0	1.97±0.54b-f	0a	20	5	1.73±0.29f-i
ASR24	BH	16a-c	14.3	26.2	1.72±0.39e-i	4a	33.3	10.4	1.6±0.08g-j
ASR25	BH	12a-c	54.6	21.6	1.52±0.31f-l	0a	48	13	1.87±0.28d-h
ASR26	BH	4ab	100	25	1.1±0.27k-s	8a	91.3	26.1	1.18±0.17j-l
ASR27	GO	8a-c	100	27.2	1.86±0.15c-g	0a	76	21	1.86±0.34d-h
SR28	RC	12a-c	100	42	0.77±0.47o-t	4a	62.5	32.3	1.4±0.65i-k
SR29	RO	8a-c	100	79.4	1.2±0.17i-q	8a	100	89.1	1.12±0.15k-m
SR30	RO	0a	100	84	0.75±0.11p-t	8a	100	98.9	0.68±0.12no
SR31	RO	4ab	100	97.9	0.5±0.09t	4a	100	100	0.54±0.12o
SR32	RO	20bc	100	85	0.9±0.27m-t	12a	100	96.6	0.52±0.07o
SR33	RO	8a-c	0	0	2.42±0.43ab	8a	8.69	2.2	2.4±0.44b
SR34	RO	12a-c	100	88.6	0.62±0.06r-t	8a	100	98.9	0.7±0.29m-o
SR35	RO	4ab	100	84.4	0.73±0.11q-t	16a	100	100	0.65±0.24no
SR36	RO	4ab	16.7	4.2	2.39±0.27a-c	8a	60.9	16.3	2.43±0.14b
IS37	GO	8a-c	69.6	17.4	1.34±0.47g-o	4a	66.7	16.7	1.22±0.28j-l
IS38	GO	12a-c	63.6	22.7	1.26±0.22h-q	8a	73.9	23.9	1.75±0.33f-i

Table 4.2 Continued.

Isolates[1/]	Host[2/]	Petri-dish Assay							
		BH				GO			
		2 DAI	4 DAI Post-emergence			2 DAI	4 DAI Post-emergence		
		Ungerminated seeds (%)[3/]	DI (%)[5/]	DS (%)[6/]	Root Length (cm)[7/]	Ungerminated seeds (%)	DI (%)	DS (%)	Root Length (cm)
IS39	Durian	16a-c	4.8	1.2	2.59±0.36a	0a	0	0	2.86±0.26a
IS40	Durian	4ab	16.7	4.2	2.54±0.38a	12a	18.2	4.6	2.21±0.56b-e

[1/]ASR *Pythium* isolated from asymptomatic root, SR =*Pythium* isolated from symptomatic root, IS = *Pythium* isolated from infected soil (root rot disease)

[2/]RO = Red oak, BH = Butter head, GO = Green oak, RC = Red coral

[3/]Ungerminated seed (%) = (the number of non-germinated seeds x 100)/the number of total seeds

[4/]Mean values within the column followed by the same letter are not significantly different at P=0.05.

[5/]Disease incidence; DI (%) = (the number of infected germinated seeds x 100)/the number of total germinated seeds

[6/]Disease severity; DS (%) = Σ(disease severity index x the number of infected seeds) x 100/(maximum index x the number of total germinated seeds)

[7/]The mean and standard deviation are calculated from 5 replications of seedlings.

With regards to the seed inoculation method (Table 4.3), the percentage of seed rot caused by *Pythium* isolates from both asymptomatic (16-66 and 2-62% for butterhead and green oak) and symptomatic plants (10-82 and 0-28% for butterhead and green oak) seemed to be rather high in both varieties of tested lettuce. The high percentage of seed rot occurrence in this pathogenicity test was attributed to the complete pathogen inoculation method, which involved soaking the seed in spore suspension. On contrary to the occurrence of seed rot, the DI and DS among the seedlings were not so obvious, probably due to the unavailability of nutrients for pathogens from the agar medium (this method was done on tissue paper) during infection. Pathogenicity results from the seed inoculation method still remained in agreement with those of the Petri-dish assay. Root lengths of severely diseased seeds were significantly shorter than those of the control group. Figure 4.6C-D depicts seed rot and seedling symptoms in the seed inoculation method. From compiled results, ASR23 and ASR4 (*Pythium* isolates from asymptomatic root) as well as SR33 (*Pythium* isolate from symptomatic root) were all shown to be non-pathogenic isolates, accordingly.

Table 4.3 The percentage of ungerminated seeds, disease severity, disease incidence and root length of lettuce seedlings using seed inoculation method.

Isolates[1/]	Host[2/]	Seed Inoculation Method							
		BH				GO			
		2 DAI	4 DAI Post-emergence			2 DAI	4 DAI Post-emergence		
		Ungerminated seeds (%)[3/]	DI (%)[5/]	DS (%)[6/]	Root Length (cm)[7/]	Ungerminated seeds (%)	DI (%)	DS (%)	Root Length (cm)
Control	-	14ab[4/]	0	0	2.25±0.22b	0a	0	0	2.42±0.18ab
ASR1	RO	34a-g	51.5	31.8	1.42±0.47de	6a-c	19.1	7.4	2.00±0.37a-g
ASR2	RO	42d-k	100	58.6	0.84±0.18f-n	8a-d	50	19.6	1.86±0.44c-h
ASR3	RO	24a-d	44.7	24.3	1.19±0.41d-i	12a-d	13.6	4.6	2.10±0.32a-e
ASR4	RO	34a-g	51.5	14.4	1.60±0.39cd	4ab	0	0	2.30±0.16a-c
ASR5	BH	54f-m	100	44.6	1.35±0.16d-f	22b-d	53.9	19.2	2.23±0.2a-d
ASR6	BH	36b-h	56.3	33.6	1.51±0.77de	4ab	2.1	0.5	2.04±0.15a-g
ASR7	GO	40c-j	90	27.5	2.02±0.28bc	62g	57.9	14.5	1.52±0.93g-j
ASR8	GO	54f-m	82.6	34.8	0.87±0.28f-m	10a-d	35.6	11.1	1.91±0.24b-h
ASR9	RC	66k-n	100	85.3	0.47±0.11l-n	20a-d	100	64.4	0.69±0.15mn
ASR10	RC	62i-n	100	50	0.41±0.07mn	26c-e	100	61.5	0.69±0.24mn
ASR11	RO	44d-l	92.9	69.6	0.48±0.23l-n	8a-d	58.7	23.9	1.17±0.12j-m
ASR12	RO	40c-j	66.7	19.2	2.12±0.51b	12a-d	2.3	0.6	2.06±0.45a-f
ASR13	RO	50d-l	100	51	1.01±0.3e-l	6a-c	65.9	23.4	2.12±0.47a-e
ASR14	BH	16a-c	47.6	17.9	2.02±0.64bc	16a-d	14.3	5.4	1.71±0.28d-i
ASR15	BH	16a-c	54.1	39	0.79±0.29g-n	8a-d	41.3	15.2	1.56±0.24f-j
ASR16	BH	48d-l	96.2	48.1	1.03±0.22e-j	16a-d	66.7	36.3	1.25±0.43i-k
ASR17	BH	30a-f	88.6	23.6	1.28±0.17d-g	2ab	38.8	9.7	2.29±0.33a-c
ASR18	GO	42d-k	100	43.1	0.86±0.19f-m	6a-c	38.3	10.6	2.28±0.27a-c
ASR19	GO	44d-l	100	40.2	0.74±0.27h-n	12a-d	34.1	10.2	2.11±0.26a-e
ASR20	COS	42d-k	89.7	23.3	1.02±0.41e-k	4ab	100	25	2.04±0.11a-g
ASR21	COS	38b-i	100	63.7	0.69±0.16i-n	42ef	100	73.3	0.72±0.21l-n
ASR22	COS	54f-m	100	67.4	0.47±0.08l-n	16a-d	100	75	0.71±0.19l-n
ASR23	RC	44d-l	7.1	7.1	1.22±0.27d-h	10a-d	2.2	0.6	1.77±0.32c-h
ASR24	BH	44d-l	100	82.1	0.41±0.06mn	28de	100	88.2	0.57±0.07n
ASR25	BH	50d-l	100	70	0.67±0.14i-n	10a-d	62.2	27.8	1.47±0.52h-j
ASR26	BH	60h-n	85	40	0.85±0.39f-m	12a-d	79.6	21	1.51±0.41g-j
ASR27	GO	60h-n	90	31.3	0.99±0.29e-l	20a-d	100	61.3	1.13±0.16j-m
SR28	RC	46d-l	100	73.1	0.54±0.09j-n	22b-d	89.7	55.1	1.22±0.37i-l
SR29	RO	80n	100	82.5	0.47±0.11l-n	14a-d	88.4	74.4	1.26±0.54i-k
SR30	RO	64j-n	100	59.7	0.52±0.11j-n	48fg	100	88.5	0.76±0.19k-n
SR31	RO	52f-m	100	62.5	0.58±0.12j-n	20a-d	100	69.4	0.96±0.22k-n
SR32	RO	82n	100	69.4	0.37±0.05n	22b-d	100	66	0.95±0.21k-n
SR33	RO	26a-e	21.6	9.5	2.10±0.43b	0a	0	0	1.71±0.26d-i
SR34	RO	58g-n	100	59.5	0.48±0.1l-n	60g	100	76.3	0.71±0.28l-n
SR35	RO	68l-n	100	45.3	0.49±0.1k-n	28de	100	50.7	0.77±0.12k-n
SR36	RO	62i-n	73.7	18.4	1.25±0.33d-h	14a-d	2.3	0.6	1.77±0.1c-h
IS37	GO	76mn	91.7	39.6	0.90±0.28f-m	8a-d	30.4	11.4	2.22±0.52a-e
IS38	GO	48d-l	11.5	3.9	1.47±0.37de	8a-d	86.9	24.5	1.70±0.13e-i

Table 4.3 Continued.

Isolates[1/]	Host[2/]	Seed Inoculation Method							
		BH				GO			
		2 DAI	4 DAI Post-emergence			2 DAI	4 DAI Post-emergence		
		Ungerminated seeds (%)[3/]	DI (%)[5/]	DS (%)[6/]	Root Length (cm)[7/]	Ungerminated seeds (%)	DI (%)	DS (%)	Root Length (cm)
IS39	Durian	10a	2.2	2.2	3.52±0.54a	6a-c	0	0	2.45±0.34a
IS40	Durian	44d-l	10.7	2.7	2.44±0.22b	8a-d	17.4	7.6	1.77±0.3c-h

[1/] ASR *Pythium* isolated from asymptomatic root, SR =*Pythium* isolated from symptomatic root, IS = *Pythium* isolated from infected soil (root rot disease)

[2/] RO = Red oak, BH = Butter head, GO = Green oak, RC = Red coral

[3/] Ungerminated seed (%) = (the number of non-germinated seeds x 100)/the number of total seeds

[4/] Mean values within the column followed by the same letter are not significantly different at P=0.05.

[5/] Disease incidence; DI (%) = (the number of infected germinated seeds x 100)/the number of total germinated seeds

[6/] Disease severity; DS (%) = Σ(disease severity index x the number of infected seeds) x 100/(maximum index x the number of total germinated seeds)

[7/] The mean and standard deviation are calculated from 5 replications of seedlings.

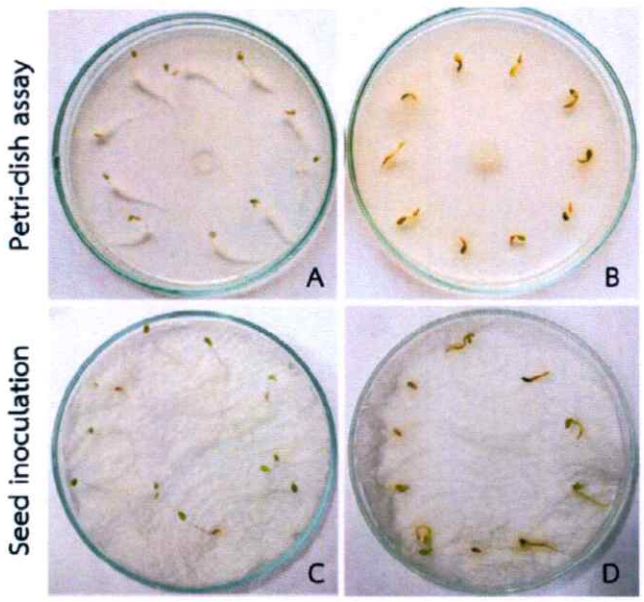

Figure 4.6 Pathogenicity test of lettuce seeds inoculated with *Pythium* spp. causing root rot disease. A = control, B = inoculated seeds of Petri-dish assay; C = control, D = inoculated seeds of seeds inoculation method

The results suggested that both lettuce varieties had different germination rates, probably due to the use of different lots of seeds during the experiment. Unexpectedly, most isolates from asymptomatic plants inhibited the germination of seeds, making them rot 2 DAI, as well as promoting disease incidence of the seedlings of 2 tested varieties of lettuce from both experimental methods 4 DAI with different levels of disease severity. The Petri-dish assay presented 4 isolates, ASR7, 8, 17, and 23, of asymptomatic *Pythium* spp., which did not inflict any disease symptoms on both samples of lettuce. Moreover, 4 isolates (SR33, SR36, IS39, and IS40) from symptomatic lettuce root rot also did not cause any symptom to occur on both lettuce seedlings. In contrast to the assay, seed inoculation method presented that 1 isolate (*P. aphanidermatum* ASR23) from asymptomatic lettuce root and 4 isolates (SR33, SR36, IS39, and IS40) from rotten symptomatic lettuce root did not show induce of any symptoms in both lettuce varieties.

4.2.2 Enzymatic Assay

In vitro production of cell wall degrading enzymes by *Pythium* species isolated from asymptomatic and symptomatic lettuce roots was studied. The samples of *Pythium* spp. were selected via pathogenicity test. 2 groups of *Pythium* were divided depending on disease severity, as non to weakly virulent and moderately virulent groups. Isolates ASR23, SR33, SR36, and IS39 (non to weakly virulent group), and ASR9, ASR26, and SR31 (moderately virulent) were chosen to be tested upon in order to determine their abilities in producing cell wall degrading enzymes on solid media. The results showed that all tested isolates of both groups showed low potential in producing cellulase, with HC value of less than 1.00, while *Phytophthora palmivora* IS40 (a reference for non-virulent oomycete) did not secrete any cellulase, and when compared to the standard enzyme, where the HC value of more than 3 (Table 4.4, Figure 4.7). With regards to pectinase production, standard pectinase has a HC value of more than 3, while all 3 isolates of *Pythium* from the moderately virulent group and IS39 from the non to weakly virulent group could produce a small amount of pectinase, with HC value of less than 1. It is noticeable that the rest of the isolates in the non to weakly virulent group did not produce pectinase. As for chitinase, only 3 isolates (ASR23, SR36, and IS39) from the non to weakly virulent group could produce the enzyme, showing low to medium activity, while high activity could be observed from the standard chitinase enzyme.

Table 4.4 Cellulase, pectinase and chitinase enzyme production on solid media.

Isolates	Cellulase[1/]	Pectinase[1/]	Chitinase[2/]
Standard enzyme (Control)	++++	++++	high
The non to weakly virulent group			
Pythium ASR23	+	-	medium
*Pythium*SR33	+	-	absent
Pythium SR36	+	-	medium
Pythium IS39	+	+	low
The moderately virulent group			
Pythium ASR9	+	+	absent
Pythium ASR26	+	+	absent
Pythium SR31	+	+	absent
Non-virulent oomycete (reference)			
Phytophthora IS40	-	-	absent

[1/]HC values as follows: - = absence; + = HC value is < 1.00; ++ = HC value is 1.01-2; +++ = HC value is 2.01-3 and ++++ = HC value is > 3. [2/]Absent; low, medium, high activity was categorized based on relative increase in diameter as well as intensity of purple color on colloidal chitin medium.

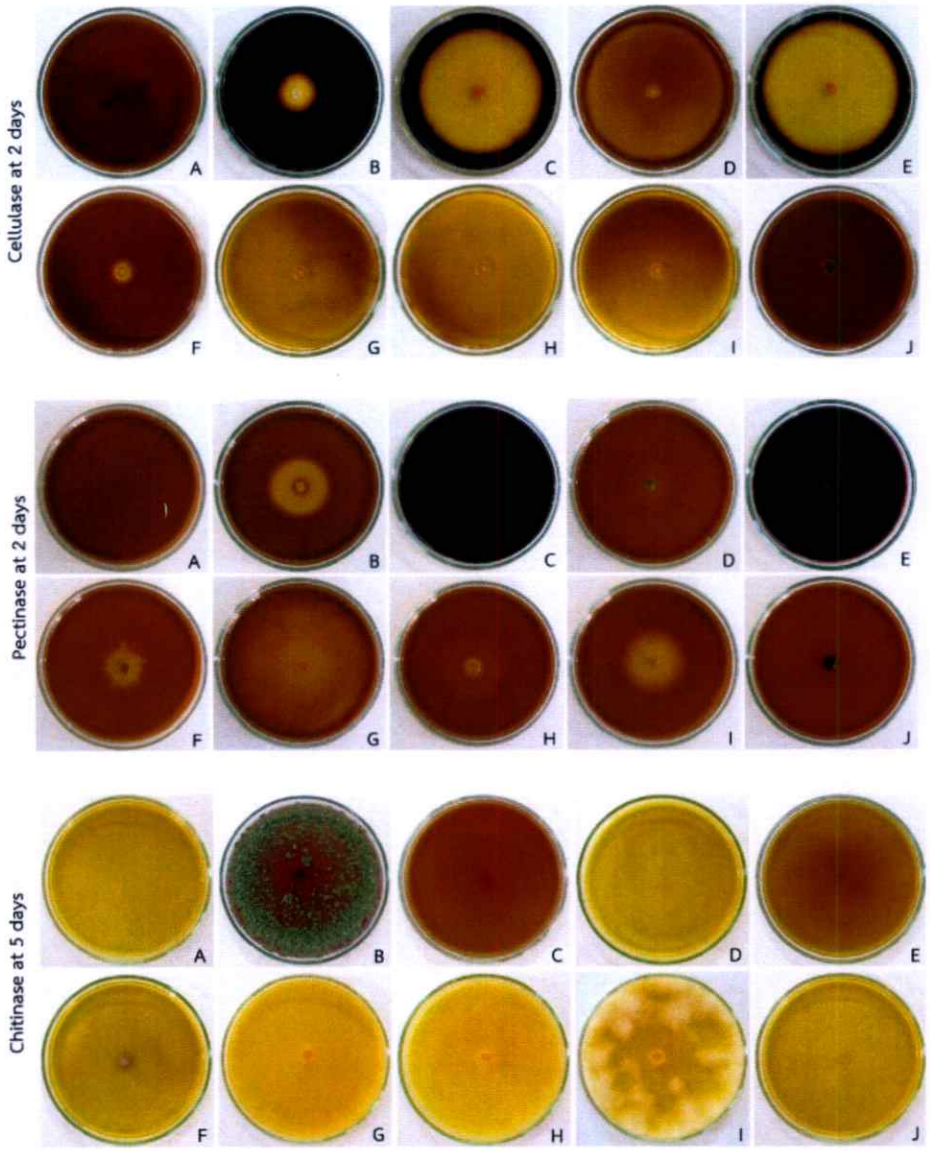

Figure 4.7 Hydrolyzed zones of cellulase, pectinase, and chitinase activity by *Pythium* isolates on supplemented medium. A = uninoculated (negative control), B = inoculated with standard (positive control), C = *Pythium aphanidermatum* ASR23, D = *Pythium* sp. SR33, E = *Pythium* sp. SR36, F =*Pythium cucurbitacearum* IS39, G =*Pythium aphanidermatum* ASR9, H =*Pythium aphanidermatum* ASR26, I = *Pythium myriotylum* SR31, and J = *Phytophthora palmivora* IS40

4.3 Phytoalexin Production in Lettuce Grown in Hydroponic Systems with Elicitors

4.3.1 Phytoalexin Production

Occurrence of phytoalexin in the hydroponically grown butterhead variety after abiotic elicitation was studied. Phytoalexin production being examined at different ages of butterhead lettuce grown in hydroponics system with abiotic elicitors was presented in Table 4.5. The results revealed that phytoalexin could be produced in hydroponically cultivated butterhead lettuce after elicitation with abiotic elicitors (5% $CuSO_4$ and 1% $AgNO_3$) at the plant age of 4 to 11 weeks. In TLC bioassays, phytoalexin exhibited a yellow fluorescent spot under 365 nm UV light with corresponding R_f values of 0.45-0.48 (Figure 4.8). Both abiotic chemical elicitors, $CuSO_4$ and $AgNO_3$, showed the same pattern of phytoalexin production. Clear inhibition zones (about 0.5-1.1 cm) where the fungi, *Aspergillus niger,* failed to develop on TLC plates dipped in hexane:ethyl acetate (1:1, v/v) were observed at the same R_f. Furthermore, the inhibition zones (0.9 and 1.1 cm) were largest and most clearly observed particularly at the plant age of 10 weeks upon use of 5% $CuSO_4$ and 1% $AgNO_3$, respectively (Figure 4.8, Table 4.5). The control (unelicited) plant sample did not develop any yellow fluorescent spots under 365 nm UV light or reveal the inhibition zones of *A. niger* on TLC plates throughout the experiment.

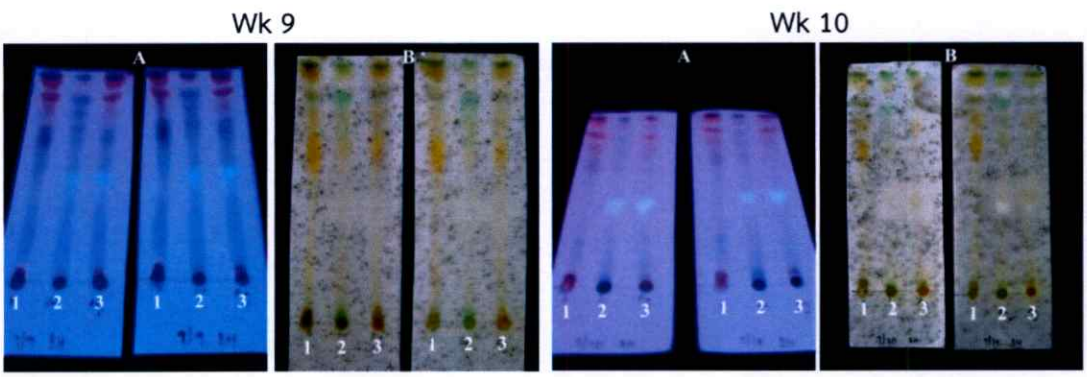

Figure 4.8 TLC plate bioassay. 365 nm UV light (A) and *Aspergillus niger* (B) detects phytoalexins (at R_f 0.45) in the tissue of lettuce leaves elicited with SW (Lane 1); 5% $CuSO_4$ (Lane 2) and 1% $AgNO_3$ (Lane 3) at the age of 9 and 10 weeks.

Table 4.5 Effect of abiotic elicitors on phytoalexin production in the leaves of hydroponically grown butterhead lettuce.

	Plant age at which being treated with elicitors																						
	Wk4			Wk5			Wk6			Wk7			Wk8			Wk9			Wk10			Wk11		
	UV[1/]	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone	
	R_f[2/]	Size	R_f	R_f	Size	R_f	R_f	Size	R_f	R_f	Size	R_f	R_f	Size	R_f	R_f	Size	R_f	R_f	Size	R_f	R_f	Size	R_f
SW	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5% $CuSO_4$	0.45	0.5[3/]	0.45	0.45	0.6	0.45	0.42	0.8	0.42	0.43	0.5	0.43	0.43	0.7	0.43	0.45	0.8	0.45	0.45	0.9	0.45	0.45	0.5	0.45
1% $AgNO_3$	0.48	0.7	0.48	0.46	1.0	0.46	0.43	0.7	0.43	0.45	0.6	0.45	0.45	0.8	0.45	0.45	0.7	0.45	0.45	1.1	0.45	0.45	0.5	0.45

[1/]UV light 365 nm; [2/]R_f value as the ratio of the distance travelled by compound and the distance travelled by solvent front; [3/]Size in centimetre

In addition, the results of the experiment involving phytoalexin production in 5 lettuce varieties after biotic and abiotic elicitation were examined. The result showed that all 5 of the tested varieties (red oak, green oak, red coral, cos, and butterhead) of hydroponically grown lettuce could produce phytoalexin after elicitation with abiotic elicitors, whereas only green oak lettuce demonstrated constant phytoalexin production throughout the plant ages up until the maturity stage (4 to 12 weeks) (Table 4.6). Phytoalexins are depicted as yellow fluorescent spots under 365 nm UV light with R_f value of 0.38-0.6, as well as antifungal activity against *A. niger* on TLC plate, which corresponds with the previous experiment conducted on butterhead lettuce. Among the chemical abiotic elicitors, all lettuces elicited with half concentrations of both chemicals (2.5% $CuSO_4$ and 0.5% $AgNO_3$) could produce phytoalexin throughout the experimental trial, however less constantly compared to those elicited with the reference dose (5% $CuSO_4$ and 1% $AgNO_3$). Surprisingly, elicitation using 3 isolates of *Pythium* sp. from asymptomatic roots as biotic elicitors on all 5 tested varieties of lettuce did not yield any yellow fluorescent spot on the TLC plates under 365 nm UV light, however, the inhibition zones on TLC plates against *A. niger* at R_f=0.9 were detected from 3 varieties of lettuce at the plant age of 8-9 weeks, namely red oak, green oak and red coral. In addition, sizes of inhibition zones were in the range of 0.5-1.5 cm.

Table 4.6 Effect of biotic and abiotic elicitors on phytoalexin production in the leaves of hydroponically grown of 5 lettuce varieties.

	Plant age at which being treated with elicitors																							
	Wk4			Wk5			Wk6			Wk7			Wk8			Wk9			Wk10			Wk11		
	UV 365 nm*	Antifungal activity zone		UV 365 nm	Antifungal activity zone		UV 365 nm	Antifungal activity zone		UV 365 nm	Antifungal activity zone		UV 365 nm	Antifungal activity zone		UV 365 nm	Antifungal activity zone		UV 365 nm	Antifungal activity zone		UV 365 nm	Antifungal activity zone	
	R_f	Size (cm)	R_f	R_f	Size (cm)	R_f	R_f	Size (cm)	R_f	R_f	Size (cm)	R_f	R_f	Size (cm)	R_f	R_f	Size (cm)	R_f	R_f	Size (cm)	R_f	R_f	Size (cm)	R_f
Red oak																								
SW	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2.5% $CuSO_4$	-	-	-	-	-	-	-	-	-	-	-	-	0.38	0.5	0.38	-	-	-	-	-	-	-	-	-
5% $CuSO_4$	-	-	-	-	-	-	0.46	0.7	0.5	-	-	-	0.4	0.8	0.4	0.4	0.9	0.4	-	-	-	-	-	-
0.5% $AgNO_3$	-	-	-	0.42	0.9	0.42	-	-	-	-	-	-	0.4	0.8	0.4	-	-	-	0.47	0.8	0.47	-	-	-
1% $AgNO_3$	-	-	-	0.39	1	0.4	-	-	-	0.45	0.8	0.45	0.4	0.8	0.4	-	-	-	0.45	1	0.5	-	-	-
NPA 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.8	0.4	-	-	-	-	-	-
NPA 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.8	0.4	-	-	-	-	-	-
NPA 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.8	0.4	-	-	-	-	-	-
Green oak																								
SW	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2.5% $CuSO_4$	-	-	-	0.46	0.9	0.46	0.44	0.6	0.44	0.68	0.7	0.68	0.35	0.5	0.35	0.52	0.7	0.52	-	-	-	0.38	0.7	0.38
5% $CuSO_4$	-	-	-	0.43	0.8	0.43	0.58	0.9	0.58	0.67	0.7	0.67	0.33	0.8	0.33	0.5	0.65	0.5	-	-	-	0.45	0.7	0.45
0.5% $AgNO_3$	0.5	0.8	0.5	-	-	-	0.42	0.6	0.42	0.67	0.9	0.67	0.33	0.8	0.33	0.54	0.65	0.54	-	-	-	-	-	-
1% $AgNO_3$	0.42	0.9	0.4	-	-	-	-	-	-	0.67	0.6	0.67	- 0.35	0.8 0.9	0.7 0.35	0.55	0.7	0.55	0.46	0.8	0.46	0.46	0.7	0.46

[1/]UV light 365 nm; [2/]R_f value as the ratio of the distance travelled by compound and the distance travelled by solvent front; [3/]Size in centimeter

Table 4.6 Continued.

	Plant age at which being treated with elicitors																							
	Wk4			Wk5			Wk6			Wk7			Wk8			Wk9			Wk10			Wk11		
	UV 365 nm*	Antifungal activity zone		UV 365 nm	Antifungal activity zone		UV 365 nm	Antifungal activity zone		UV 365 nm	Antifungal activity zone		UV 365 nm	Antifungal activity zone		UV 365 nm	Antifungal activity zone		UV 365 nm	Antifungal activity zone		UV 365 nm	Antifungal activity zone	
	R_f	Size (cm)	R_f	R_f	Size (cm)	R_f	R_f	Size (cm)	R_f	R_f	Size (cm)	R_f	R_f	Size (cm)	R_f	R_f	Size (cm)	R_f	R_f	Size (cm)	R_f	R_f	Size (cm)	R_f
Red coral																								
SW	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2.5% $CuSO_4$	0.34	0.5	0.34	0.36	0.6	0.36	0.35	1.1	0.35	0.4	0.5	0.4	-	-	-	-	-	-	0.85	0.9	0.85	-	-	-
							-	0.6	0.6															
5% $CuSO_4$	0.4	0.7	0.4	0.42	0.7	0.42				0.39	0.5	0.39							0.45	1.0	0.45	0.42	0.6	0.42
0.5% $AgNO_3$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.43	0.8	0.43	-	-	-
1% $AgNO_3$	-	-	-	-	-	-	0.4	0.8	0.4	-	-	-	0.38	0.5	0.38	0.41	0.9	0.41	-	-	-	0.38	0.7	0.38
Cos																								
SW	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2.5% $CuSO_4$	-	-	-	-	-	-	0.4	1	0.4	-	-	-	0.45	0.5	0.45	0.36	0.7	0.36	-	-	-	0.38	0.8	0.38
5% $CuSO_4$	-	-	-	0.38	0.8	0.38	0.39	0.7	0.39	0.45	1.1	0.45	-	-	-	0.48	0.4	0.48	-	-	-	-	-	-
0.5% $AgNO_3$	-	-	-	-	-	-	-	-	-	0.42	0.9	0.42	-	-	-	-	-	-	0.43	0.9	0.43	0.45	0.5	0.45
																			-	1.8	1.8			
1% $AgNO_3$	0.42	0.9	0.42	-	-	-	-	-	-	0.47	0.4	0.47	0.4	0.8	0.4	0.41	0.8	0.41	0.43	0.5	0.43	0.45	0.6	0.45
Butterhead																								
SW	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2.5% $CuSO_4$	-	-	-	0.38	1.2	0.38	0.34	1.3	0.34	-	-	-	0.38	1.1	0.38	-	-	-	-	-	-	-	-	-
							-	1.2	0.6															
5% $CuSO_4$	-	-	-	0.45	1.1	0.45	-	-	-	0.38	0.6	0.38	0.4	0.7	0.4	0.37	0.6	0.37	-	-	-	0.45	0.9	0.45
0.5% $AgNO_3$	0.45	0.8	0.45	0.37	1.3	0.37	0.46	0.7	0.46	0.41	0.5	0.41	-	-	-	-	-	-	0.36	0.8	0.36	-	-	-
				-	1.3	0.7																		
1% $AgNO_3$	0.47	0.9	0.5	0.4	1.2	0.4	0.36	1.1	0.36	0.4	0.9	0.4	0.45	1	0.45	-	-	-	0.39	0.8	0.39	0.43	1	0.43
				-	1.5	0.7	-	1.6	0.6															

[1/]UV light 365 nm; [2/]R_f value as the ratio of the distance travelled by compound and the distance travelled by solvent front; [3/]Size in centimeter

4.3.2 Assessment of the *In Vitro* Antifungal Activity of Crude Extract of Elicited Lettuce against Conidial Germination of 4 Plant Pathogenic Fungi

The conidia germination of 4 plant pathogenic fungi were examined after 30 minutes, 12, 24 and 72 hours of incubation with crude extract from lettuce elicitation with $CuSO_4$ and $AgNO_3$. According to the results, in control treatment, conidia germination of all tested fungi except *Pythium aphanidermatum* started after 30 minutes and increased continuously with increasing incubation times, and reached the highest percentage of germination after 72 hours of incubation (55.63% for *Colletotrichum gloeosporioides*, 58.59% for *C. lunata* and 65.2% for *Fusarium oxysporum*). On the other hand, the tested conidia, except for *P. aphanidermatum*, in both crude extract treatments did not germinate or only slightly germinated throughout the entire process of incubation, yielding results that were not significantly different among the crude extract treatments (Figure 4.9).

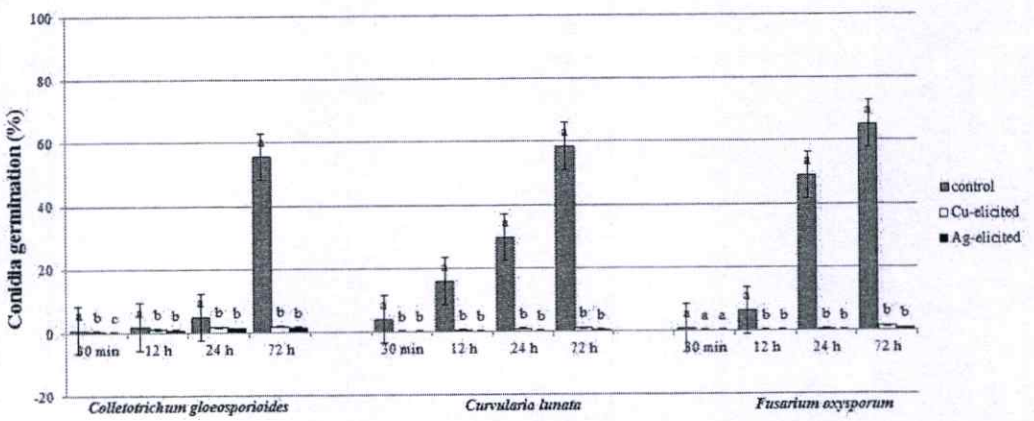

Figure 4.9 Effect of crude extract of elicited lettuce on conidia germination of plant pathogenic fungi. Each bar represents the percentage of spore germination of *C. gloeosporioides*, *C. lunata* and *F. oxysporum* ($P < 0.05$). Different letters indicate statistically significant differences between type of crude extract and time incubation.

In addition, abnormal conidia could only be observed, under the light microscope, in crude extract treatments. Abnormalities of conidia included damaged cell, swelling, lysis, deformation and a granular cytoplasm with intense vacuolization at 72 hours of incubation (Figure 4.10). With regards to *P. aphanidermatum*, observations of the germination process of sporangium, zoospore and oospore were made, but no such data could be obtained either in the control samples or the ones with crude extract treatments due to experimental difficulties. However, changes in the structures of sporangium, oogonium, antheridium and oospore were detected, such as lysis of sporangium and antheridium, granular cytoplasm with an intense vacuolization in oogonium and oospore. Lysis was also detected on hyphae. From the above findings, it is suggested that crude extracts of hydroponically grown lettuce elicited with $CuSO_4$ and $AgNO_3$ possessed *in vitro* antifungal activity against *C. gloeosporioides*, *C. lunata*, *F. oxysporum* and *P. aphanidermatum* probably due to the presence of phytoalexin (lettucenin A).

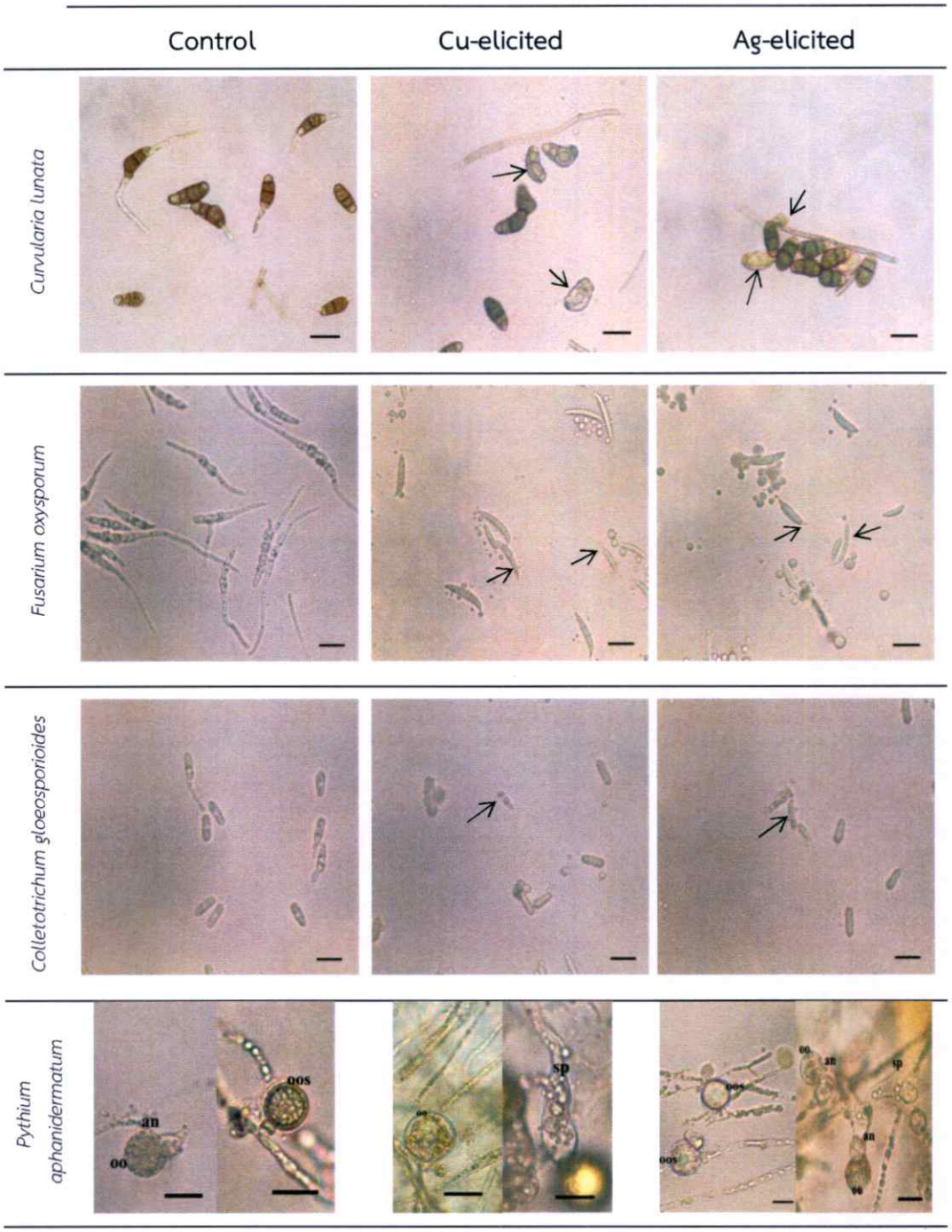

Figure 4.10 Effect of crude extract from lettuce leaves stimulated by abiotic elicitors (5% $CuSO_4$ and 1% $AgNO_3$) on conidia abnormalities and conidia germination upon exposure to plant pathogenic fungi for 72 hours. Vacuolisation, distortion and degradation were shown. Scale bar = 30 µm. *oo = oogonium; an = antheridium; oos = oospore; sp = sporangium

Thereafter, the treated spores of *Curvularia lunata*, *Fusarium oxysporum*, *Colletotrichum gloeosporioides* and *Pythium aphanidermatum* inoculated onto chili leaves and lettuce roots. The disease symptoms were observed after inoculation. The result showed that treated spores of pathogenic fungi on plants could infect chili leaves and lettuce roots as presented in Table 4.7. Inoculated treatment showed more severe symptom than crude extract treatments, while mock treatment did not show any symptom on tested plants. Spores treated with crude extract caused less symptoms on leaves than untreated control spores. Leaves inoculated with spores treated with crude extracts from Cu or Ag elicited lettuce did not show a further reduction in virulence. Lettuce root inoculated with *Pythium* showed symptom in all treatments. Root showed brown necrosis lesion on tip or middle root (Figure 4.11). The symptoms were less after treatment of spores with crude extract from lettuce elicited with $CuSO_4$ than after elicitation with sterilized water and $AgNO_3$. This result suggests that crude extract from elicited lettuce could inhibit spore germination but does not entirely kill the spores.

Table 4.7 Effect of treated spores of pathogenic fungi on plants.

Treatments	*Curvularia*	*Colletotrichum*	*Fusarium*	*Pythium*
Mock	-[1/]	-	-	-
Inoculated	++	+++	++	++
Ext.1[2/]	-	+	+	++
Ext.2[3/]	++	++	++	++
Ext.3[4/]	+	+	++	+

[1/]Level of disease severity as follows: - = no symptom; + = a weak symptom; ++ = a moderate symptom; +++ = a severe symptom and ++++ = seedling died. [2/]Crude extract from elicited lettuce with sterilized water. [3/]Crude extract from elicited lettuce with $AgNO_3$. [4/]Crude extract from elicited lettuce with $CuSO_4$.

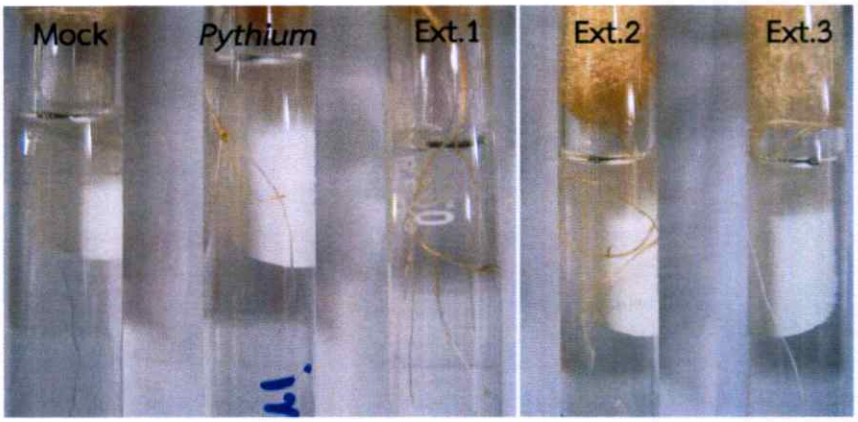

Figure 4.11 Lettuce root showed brown necrosis lesion on tip or middle root after infection with treated-*Pythium* spores. Ext.1 = Crude extract from elicited lettuce with sterilized water, Ext.2 = Crude extract from elicited lettuce with $AgNO_3$, Ext.3 = Crude extract from elicited lettuce with $CuSO_4$.

4.4 Induced Resistance to *Pythium* Root Rot in Hydroponically Grown Lettuce after Induction with Elicitors

4.4.1 Effect of Biotic and Abiotic Elicitors on the *Pythium* Induced Resistance and Gene Expressions

The results from quantitative RT-PCR were used to assess the expression levels of 7 selected genes involved in defense responses, for both controlled and uncontrolled experiments of lettuce leaves and roots. The expression profiles of lettucenin A biosynthesis, PR protein and *Pythium* genes were compared using specific primers. The primers were designed for lettuce and *Pythium* genes from sequences available in the GenBank database. The reference genes, Elongation factor 2-alpha (*LsEIF2a*) and *LsTIP4*, were employed as expression control genes. The *LsLTC1* and *LsLTC2* genes were expressed in lettuce leaves and roots after exposure to *Botrytis cinerea* B05.10, 3 strains of *Pythium* spp. (*P. aphanidermatum* ASR23, *Pythium* sp. SR36, and *P. myriotylum* SR31), or BABA at 24 hpi (Figure 4.12). Then at 48 hpi, the expressions were clearly observed after challenging with *Pythium* SR31, with no expressions of *LsLTC1* and *LsLTC2* genes in leaves pre-treated with $CuSO_4$. On the other hand, *LsLTC1* and *LsLTC2* were expressed in roots exposed to $CuSO_4$ after

Pythium SR31 infection. The expression of *LsPR1b_like*, a gene involved in the SA pathway, was stimulated by *B. cinerea* B05.10, 3 strains of *Pythium* sp., and BABA. However, the accumulation of pathogenesis-related proteins genes was not altered by BABA. It was found that BABA-treated plants only accumulated a small amount of those proteins as compared to other sampled plants. *LsPR1b_like* accumulation was seen in mock-inoculated plants at 4 dpi (days post inoculation). Among all elicitors, the gene induction by *Pythium* sp. ASR23 in the leaf was strongest. There was no expression of *LsPR1b_like* genes in leaves and roots stimulated with $CuSO_4$ (Figure 4.13). The expression of *Pythium* markers in roots, *PyITS1* and *PyITS2*, were strongly triggered by 3 strains of *Pythium* sp. (SR31, SR36, ASR23), and BABA, demonstrating no expression upon application of mock and $CuSO_4$ treatments (Figure 4.14). There was no expression of *PyITS1* and *PyITS2* markers in leaves stimulated with elicitors.

The gene expressions of *LsPR1b_like*, *LsLTC1*, and *LsLTC2* were higher in lettuce leaves treated with *P. aphanidermatum* ASR23 than in roots as determined by qRT-PCR when compare to the mock. In addition, lettuce leaves were treated with *B. cinerea* showed expression of *LsLTC1* and *LsLTC2*. However, the relative expression of *LsLTC1* in roots after treatment with 3 strains of *Pythium* sp. and BABA, and subsequent infection with pathogenic *P. myriotylum* SR31, showed no significant differences compared to the expression of *LsLTC2*. Therefore, the results suggested that changes in these gene expressions as a method of defense response against *Pythium* infection increased after pre-treatment with various elicitors over 24 hours. It can also be observed that the expressions significantly increased when chemically stimulated lettuce was infected by pathogenic *P. myriotylum* SR31. The result suggested that non-pathogenic *P. aphanidermatum* ASR23 could be used as an elicitor in *Pythium* defense mechanisms.

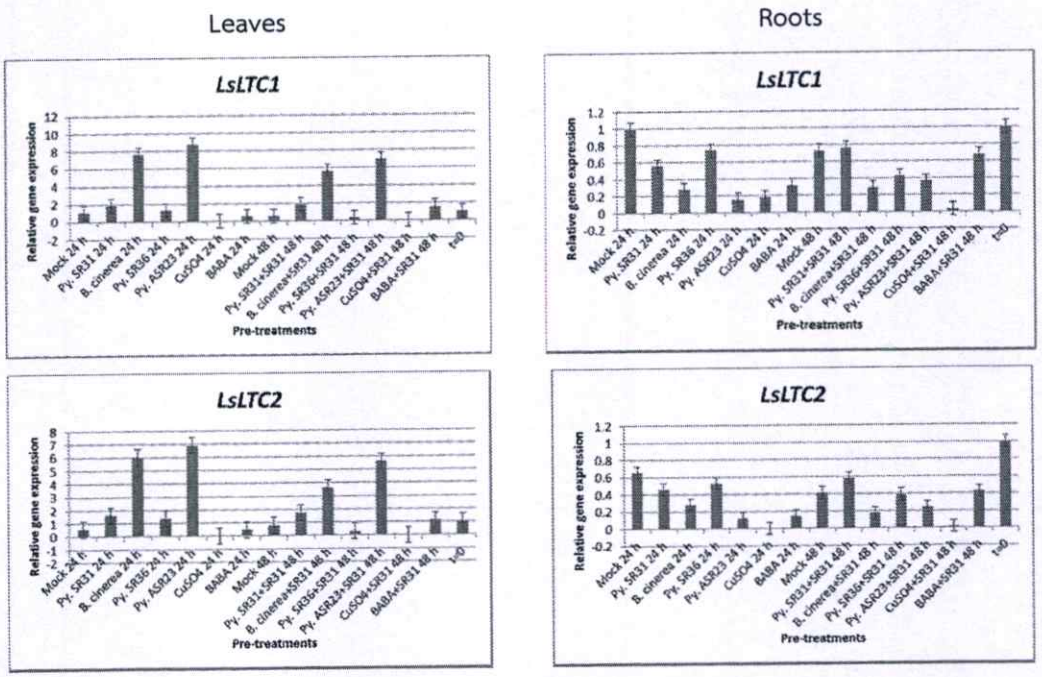

Figure 4.12 Expression profiles of *LsLTC1* and *LsLTC2* in response to induction with elicitors and inoculation with *P. myriotylum* SR31. Expression levels of genes were determined by quantitative real-time PCR. Total RNA was prepared from separate lettuce leaves and roots of 3 week-old seedlings at the indicated times. Expression values are relative to the absolute non-treated control (mock) level. Values represent the mean with standard deviation (SD) of results from triplicate experiments. Lines indicate values of non-treated control (Mock).

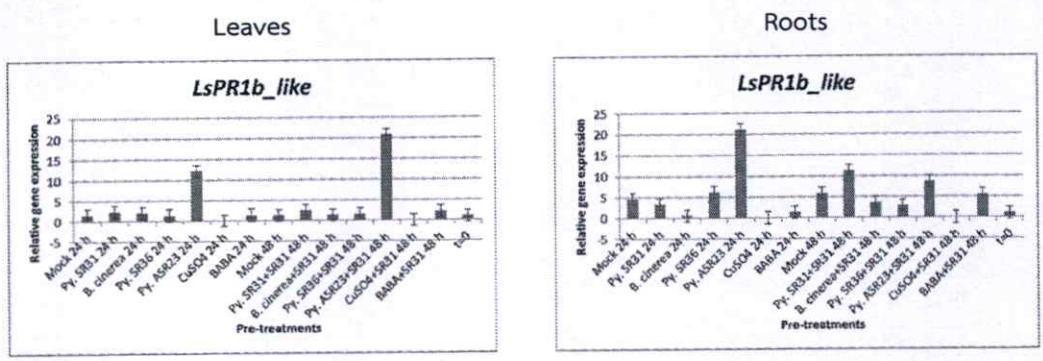

Figure 4.13 Expression profiles of *LsPR1b_like* in response to induction with elicitors and inoculation with *P. myriotylum* SR31. Expression levels of genes were determined by quantitative real-time PCR. Total RNA was prepared from separate lettuce leaves and roots of 3 week-old seedlings at the indicated times. Expression values are relative to the absolute non-treated control (mock) level. Values represent the mean with standard deviation (SD) of results from triplicate experiments. Lines indicate values of non-treated control (Mock).

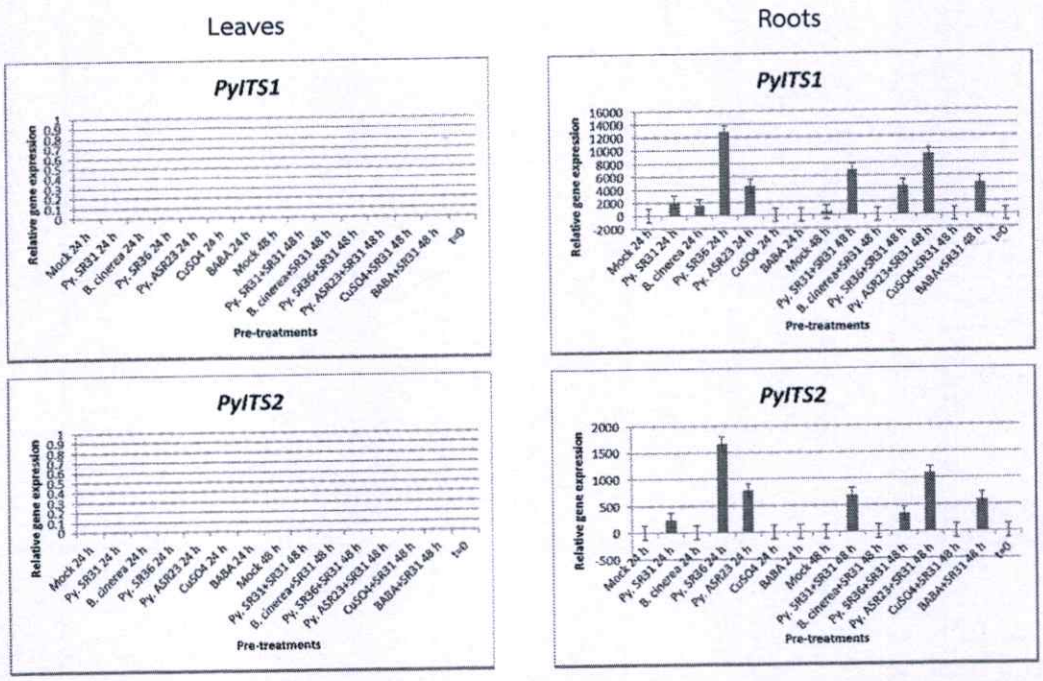

Figure 4.14 Expression profiles of *PyITS1* and *PyITS2* in response to induction with elicitors and inoculation with *P. myriotylum* SR31. Expression levels of genes were determined by quantitative real-time PCR. Total RNA was prepared from separate lettuce leaves and roots of 3 week-old seedlings at the indicated times. Expression values are relative to the absolute non-treated control (mock) level. Values represent the mean with standard deviation (SD) of results from triplicate experiments. Lines indicate values of non-treated control (Mock).

4.4.2 ROS Accumulation and Histological Analysis

The accumulation of reactive oxygen species (H_2O_2 and O_2^-) was analyzed. During the experiment, whole plants were collected at 24 and 48 hours after induction and inoculation (hpi).

Hydrogen peroxide (H_2O_2) production was determined by DAB staining. DAB was applied to treated lettuce, inoculated leaves (*Botrytis cinerea* B05.10) and roots (*Pythium* sp.) at 24 and 48 hpi. They produced extensive brown color on the leaves and roots infected by pathogens (Figure 4.15). Slight stains were observed in mock, *Pythium* sp. SR31, SR36, ASR23, and BABA-treated lettuce at 24 hpi, suggesting no accumulation of H_2O_2 in the host cells of leaves of treated lettuce. DAB staining of leaves inoculated with *B. cinerea* at 24 hpi produced brown color in the

inoculated sites. No staining was observed in $CuSO_4$-treated lettuce at 24 and 48 hpi, suggesting plant death after the treatment.

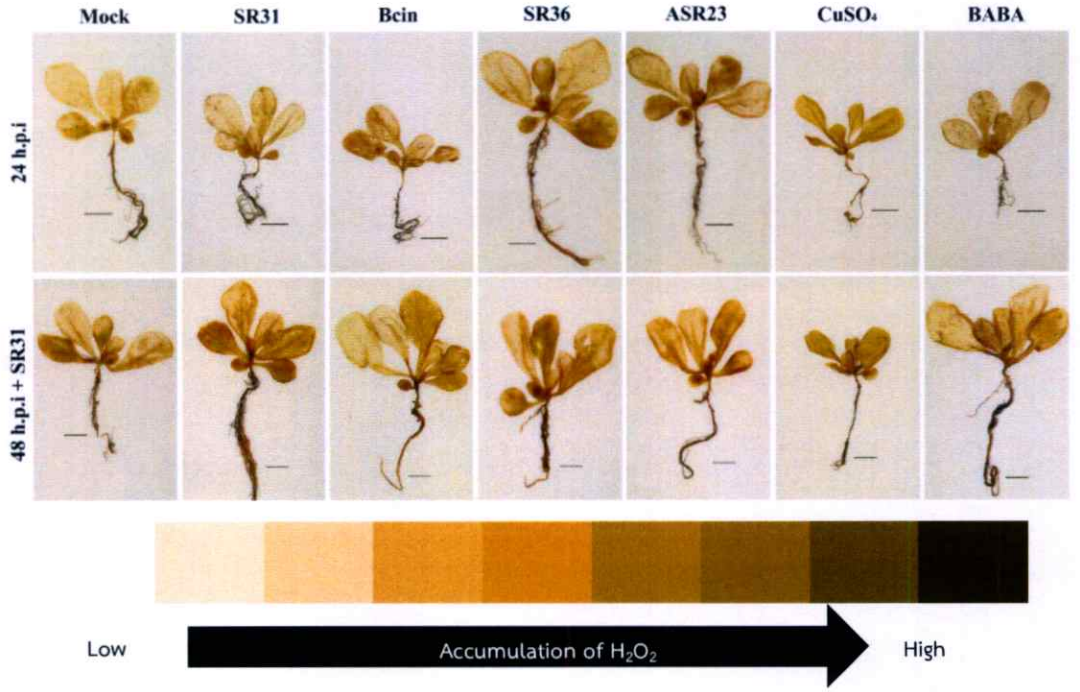

Figure 4.15 Accumulation of hydrogen peroxide (H_2O_2) was analyzed by DAB staining method. The photos were taken at 24 and 48 hpi. (Bar = 1 cm).

The results showed no significant accumulation of superoxide anions (O_2^-) on lettuce leaves within 24 and 48 hpi, while superoxide anions accumulation in roots produced a slight staining at 24 hpi. The roots accumulated more superoxide anion after inoculation with pathogenic *Pythium* sp. SR31 for 24 hours. In contrast, there was no accumulation in plants pre-treated with $CuSO_4$. Although the mock treatment produced stains indicating the presence of hydrogen peroxide and superoxide anions, the accumulation of those compounds were still relatively little when compared to samples with all other induced treatments, except for the ones involving $CuSO_4$. Treatment with $CuSO_4$ leads to plant death and thus, no stain could be observed (Figure 4.16).

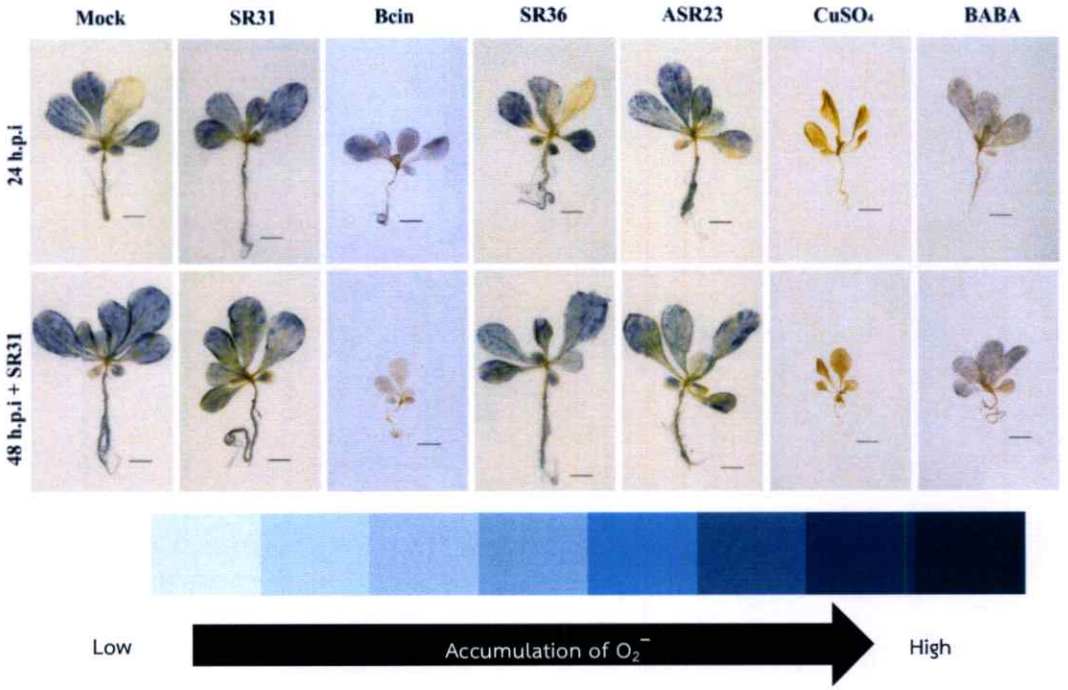

Figure 4.16 Accumulation of superoxide anions (O_2^-) was analyzed by NBT staining method. The photos were taken at 24 and 48 hpi. (Bar = 1 cm).

In order to examine ROS production, PAMPs were added to 7-day-old butterhead lettuce seedlings after pre-treatment with elicitors. The results show that the leaves and root parts (top, middle, bottom) of untreated plants (mock) had the highest response to flg22, especially the top and bottom parts of the root (Figure 4.17a). Only top roots showed clear ROS response to XyL and Chi response to HaNLP was very weak.

The same trend could be observed in other parts of the roots (especially at top roots), as they also responded strongly to flg22 and moderately chitin (Chi), and xylanase (XyL) (Figure 4.17c-e) upon stimulation with non-pathogenic *P. aphanidermatum* ASR23, *Pythium* sp. SR36, and pathogenic *P. myriotylum* SR31. These *Pythium* stimulated the host plant to react strongly when triggered by flg22, while only flg22 response was abundantly present in lettuce leaves treated with BABA, followed by exposure to *P. aphanidermatum* ASR23 (Figure 4.17b). These results indicated that the leaves and roots (top, middle, bottom) from butterhead lettuce seedlings pre-treated with difference elicitors involved in the activation of defense response by PAMPs, especially flg22. Pre-treatment with BABA, non-

pathogenic *P. aphanidermatum* ASR23, and *Pythium* sp. SR36 increased PAMPs response in leaves and roots. These elicitors may be used to activate defense responses to causal agents of the lettuce root rot disease.

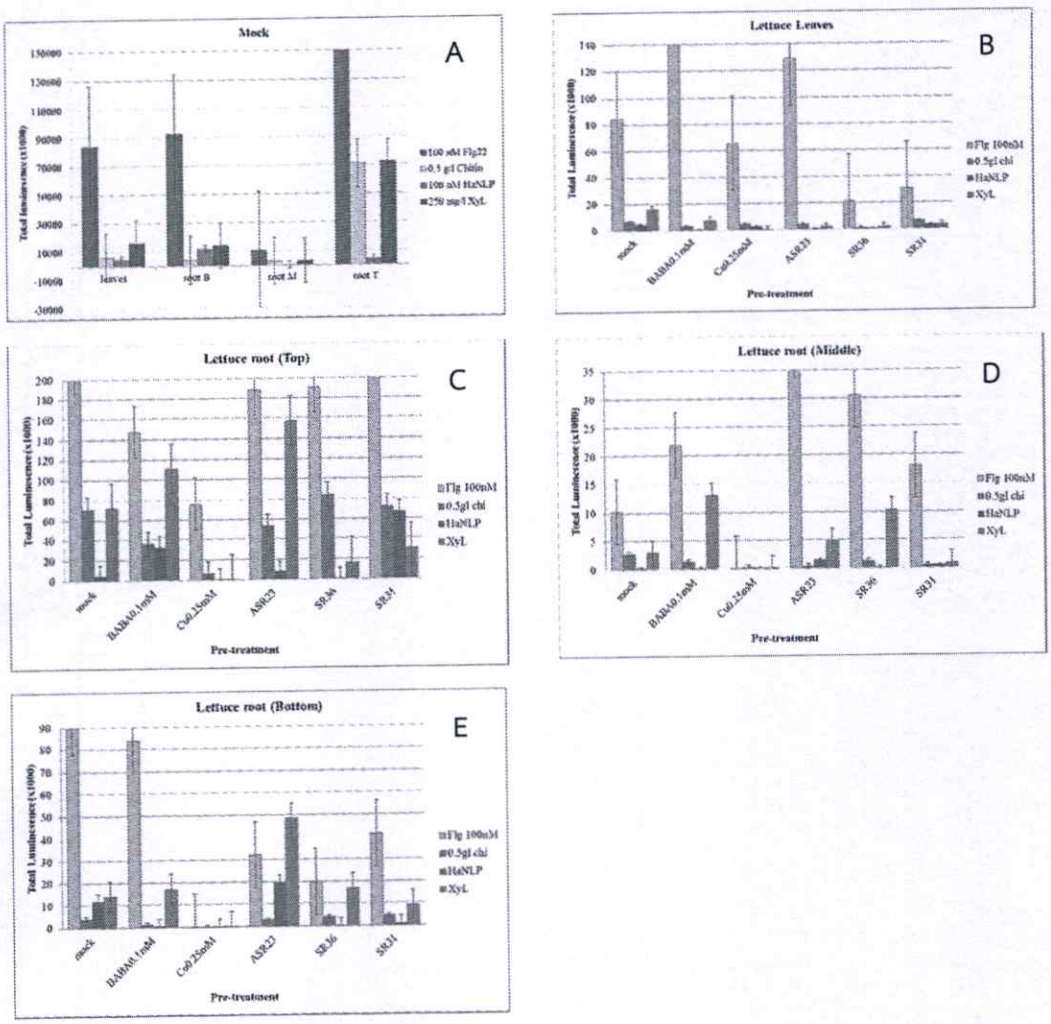

Figure 4.17 Oxidative burst in butterhead lettuce seedlings. Pre-treated with 0.1 mM BABA, 0.25 mM $CuSO_4$, non-pathogenic *P. aphanidermatum* ASR23, *Pythium* sp. SR36, and pathogenic *P. myriotylum* SR31. Luminescence of leaves and roots slices in a solution with peroxidase and luminol was measured after the addition of 100 nM flg22, 0.5g/L chitinase, 100 nM HaNLP, and 250 ng/L xylanase. Results were averaged with standard error bars. A = mock, B = leaves, C = top root, D = middle root, E = bottom root

CHAPTER 5

DISCUSSION

5.1 Collection, Identification and Studies of *Pythium* Species from Asymptomatic and Symptomatic Lettuce

Of 40 isolates, 27 were recovered from asymptomatic lettuce roots, 9 from symptomatic lettuce roots, 2 from lettuce field containing soil naturally infested with root rot disease, and 2 from durian field containing soil naturally infested with root rot disease (which received from Dr. Veeranee Tongsri, Kasetsart University). Based on morphological comparison, only 2 species were identified. *Pythium aphanidermatum* was the pre-dominant species recovered from 24 isolates of asymptomatic roots, whereas only 3 isolates of *P. myriotylum* were detected. All 9 isolates gathered from symptomatic roots were identified as *P. myriotylum*. This *Pythium* species has been identified as the most aggressive and pathogenic species in the genus. It can also invade a wide range of hosts, thus causing root rot disease in many hydroponically-grown crops, which lead to huge economical losses in the agricultural industry (Sutton *et al.*, 2006). The most common species of *Pythium* are principle disease-causing agents, including *Pythium aphanidermatum*, *P. myriotylum*, *P. dissotocum*, members of *Pythium* group F, and *P. ultimum* var. *ultimum* (Utkhede *et al.*, 2000; Boshoff, 2005; Koohakan *et al.*, 2008; Garibaldi *et al.*, 2014). The results indicated that *P. aphanidermatum* and *P. myriotylum* were the most dominant *Pythium* species found in hydroponically-grown crops.

Based on ribosomal DNA sequences, the ITS regions of 40 Pythiaceae isolates were amplified and sequenced using ITS1 and ITS4 primers. The amplified rDNA-ITS region of all *Pythium* isolates ranged between 750-800 base pairs long, and upon BLAST search, yielded the highest similarity to oomycetes. Genetic comparisons of internal transcribed spacer (ITS) rDNA showed that all collected isolates belong to only 4 species of *Pythium*; *P. aphanidermatum, P. myriotylum*, *P. deliense*, and an unnamed *Pythium* species. This was in accordance with Nzungize *et al.* (2011)'s research, providing information that the ITS region containing 5.8S ribosomal DNA of *Pythium* vary between 750 and 1,050 bps in size. The genetic sequence of this region have been frequently used as reference data to identify and classify *Pythium* species

(Matsumoto *et al.*, 1999). The phylogenetic analysis indicated the grouping of *Pythium* isolates based on genetic sequence of rDNA-ITS analyses, was in agreement with grouping obtained from distinguished morphological characteristics. In this study, the phylogenetic tree contains two big clades consisting of *Pythium aphanidermatum* and *P. myriotylum* isolates, and four small clades consisting of *P. deliense*, *P. cucurbitacearum*, *Phytophthora palmivora* and *Pythium* sp. The experimental procedure, involving isolation of pathogens from lettuce plants of various sources, revealed that *P. aphanidermatum* was most frequently isolated species from hydroponically grown lettuce, while *P. myriotylum*, *P. deliense* and *Pythium* spp. could be found in both hydroponics and soil-grown lettuce. This is in agreement with Koohakan *et al.* (2008) and Ho *et al.* (2012) which reported that *P. aphanidermatum* and *P. myriotylum* are the most dominant *Pythium* species in hydroponically-grown crops. Furthermore, *Pythium aphanidermatum* and *P. deliense* have been reported to infest 14.1 and 1.3% of the agricultural soil in Iran, respectively (Babai-Ahary *et al.*, 2004). From the results, among all isolates similar to *P. aphanidermatum*, IS38 forms a distinct clade, only slightly differentiating itself from the *P. aphanidermatum* group. This isolate (IS38) cannot be classified by morphological characteristics due to the fact that its physical appearance is almost indistinguishable to the *Pythium aphanidermatum*. The ITS genetic sequences of *P. aphanidermatum* and *P. deliense* have been reported to contain huge similarities, which make them nearly identical (Moorman *et al.*, 2002; Nzungize *et al.*, 2011). van der Plaats-Niterink (1981) and Ho (2011) were able to determine distinct differences and similarities between the characteristics of both species. *P. deliense* produces oogonial stalks which branch off from the side and bend toward the antheridium, and contains a more slender antheridia. Furthermore, the sporangia formed by *P. deliense* branches off in a less complicated manner compared to those of *P. aphanidermatum*. In the present day, there has not yet been a report on the incidence of *P. deliense* contributing to the root rot disease infection of lettuce in Thailand. Hence, this is the first published report of *P. deliense* association with lettuce root rot, besides *P. aphanidermatum* and *P. myriotylum* (Koohakan *et al.*, 2008). The results of this phylogenetic tree showed that the ITS region could be used to identify the *Pythium* species, in accordance with Matsumoto *et al.* (1999) previous successful examination of the relationship between *Pythium* species via ITS

sequences and sporangia characteristics. In addition, *Pythium* and *Phytophthora* associated with durian in Indonesia have been identified by both molecular and morphological characteristics in a scientific study (Santoso *et al.*, 2015). The study suggested that molecular characteristics of *Pythium* support the morphological variations of its species and its association with lettuce.

5.2 *In vitro* Screening Pathogenicity of *Pythium* Isolates from Asymptomatic and Symptomatic Lettuce and Selection of Non Pathogenic Strains

The methods of testing pathogenicity in order to screen real non-pathogenic *Pythium* spp. from minor pathogens have been used previously in our study to examine *Pythium* diseases that occur in different crops (Thomson *et al.*, 1971; Foley, 1980; Christensen *et al.*, 1988; Zhang and Yang, 2000). From our study, the Petri-dish assay and seed inoculation method yielded similar results in terms of disease incidence of lettuce seedlings. However, a few variations were detected among both methods. The seed inoculation method was consistently more accurate on detecting and clarifying seed rot, while the Petri-dish assay was able to clearly differentiate levels of DI and DS of seedlings. The results obtained were in agreement with those in previously published studies. The Petri-dish method was more discriminating in detecting differences in aggressiveness among *Pythium* isolates than methods involving tests on infested soil (Thomson *et al.*, 1971). The seed inoculation technique was commonly used to introduce rhizobium to legume seeds by inoculation prior to sowing (Deaker *et al.*, 2004). Soak-inoculation of mature seeds in a spore suspension was also a technique employed in producing laboratory-inoculated seeds (Munkvold and Carlton, 1997). In addition, McCarter and Littrell (1970) has shown that *P. aphanidermatum* and *P. myriotylum* isolates were always obtained from different plant sources and locations, and the pathogenicity exhibited on plant species also differed. Moreover, infection of disease and disease severity of *Pythium* are dependent on the temperature of the nutrient solution (Bates and Stanghellini, 1984). Surprisingly, *Pythium* isolates can present in asymptomatic lettuce root without having it display any symptoms. The fact that *Pythium* can live in the roots of hydroponically grown plants leads to increased risks of *Pythium* becoming pathogenic under appropriate conditions in favour of the disease. The results corresponded with the findings of Koohakan *et al.* (2008), who discovered that *P.*

myriotylum can cause root rot of commercially grown lettuce and cucumber in hydroponic farms. *P. aphanidermatum* and *P. myriotylum* caused the root of rye to rot at 27 °C, *P. aphanidermatum* was the most virulent to tomato between 27 and 35 °C, and *P. myriotylum* almost completely infected tomato at 35 °C (Littrell and McCarter, 1970).

This scientific investigation revealed that only 3 out of 40 isolates of *Pythium* spp. were proven to be the real non-pathogenic isolates, displaying no characteristics of a minor pathogen. Among the 3 isolates obtained, 2 isolates, namely ASR23 and ASR4, were recovered from asymptomatic lettuce roots while unexpectedly, SR33 was the only isolate recovered from symptomatic roots. This interesting result could be explained by the fact that the *Pythium* spp. is a minor pathogen, especially in hydroponic environments. Based on our repetitious experimental procedure outcomes, we found that *Pythium* spp. in hydroponic systems are capable of unnoticeably switching from time to time from being a minor pathogen (without any obvious symptoms) to a non-pathogenic *Pythium*. When the plants were left to grow under optimal conditions, *Pythium*-infected roots were symptomless. Although roots looked generally healthy, the oomycete caused some changes in the root cortex (Rey *et al.*, 1998), and produced metabolites that may facilitate infection (Rey *et al.*, 2001). This means that mistakes can be easily made while isolating and screening non-pathogenic *Pythium* from symptomless plants. Ultrastructural and cytochemical investigation of asymptomatic infection by *Pythium* spp. could help elucidate several aspects of the relationships between non-pathogenic *Pythium* spp., minor pathogens and plant pathogens (Rey *et al.*, 1998). Immunoenzymatic staining procedures showed that *Pythium* spp. were the most frequent fungal invaders in asymptomatic roots of hydroponically grown tomato plants (Rafin and Tirilly, 1995; Vallance *et al.*, 2011). Quantitative real-time PCR assays were also developed for detection of the most prevalent oomycete groups, since pathogenic species of *Pythium* were also isolated from healthy looking vines in plant nurseries (Spies *et al.*, 2011). From our research, *in vitro* pathogenicity test on seeds could help screen for non-pathogenic isolates of *Pythium* spp. in asymptomatic plants.

The ability of 6 isolates of *Pythium* species extracted from asymptomatic and symptomatic lettuce roots in producing cell wall degrading enzymes, eg. cellulase, pectinase, and chitinase on solid media was investigated. Sampled pathogens were

grouped according to their virulence, as non to weakly virulent or the moderately virulent group. Activities of cellulase and pectinase enzymes were determined by the hydrolysis capacity (HC) value, which is defined as the ratio of the hydrolysis zone over the colony diameter. Chitinase activity was categorized based on relative increase in diameter as well as the intensity of purple color on colloidal chitin medium. The results revealed that all tested isolates of *Pythium* species showed low cellulase production potential, with HC values of less than 1.00, while complete absence in cellulase was observed in *Phytophthora* IS40. Regarding pectinase enzyme production, absence as well as presence of activity with low HC value (<1.00) were observed in the non to weakly virulent group of *Pythium*. Meanwhile, only low HC values (<1.00) of pectinase could be detected in the moderately virulent group. Two isolates from the non to weakly virulent group presented moderate chitinolytic enzyme activity, while the rest did not show any activity. Our results indicated that most of *Pythium* species isolated from asymptomatic and symptomatic lettuce root could produce small amounts of cellulolytic and pectinase enzymes, but only in small quantities. This observation is in agreement with studies by Janardhanan and Husain (1974), and Sutton *et al.* (2006), which reported the ability of *P. aphanidermatum* in producing cellulolytic and pectolytic enzymes. The studies also mentioned that the age of *Pythium* cultures influence the quantity and activity of these enzymes. The reason that cellulolytic and pectinase enzymes could only be detected in small amounts in our study may be due to the fact that *Pythium* species are regarded as weak parasites. This hypothesis was confirmed by Chen *et al.* (1998), and Owen-Going *et al.* (2004), who reported that there is a high correlation between the levels of cell wall degrading enzymes and the levels of virulence. Interestingly, chitinase activity was detected only in *Pythium* species from the non to weakly virulent group.

5.3 Phytoalexin Production in Lettuce Grown Hydroponically with Elicitors

From our findings, phytoalexin detected in the lettuce samples was hypothesized to be lettucenin A, as it generates the same yellow fluorescent spot pattern with the same R_f value, developing a clear inhibition zone against *A. niger* (Ong and Chong, 2009). This claim was supported by the results showing highest phytoalexin lettucenin A accumulation at different plant ages ranging from week nine

to week 12 after elicitations with chemical elicitors, silver nitrate ($AgNO_3$) and copper sulfate ($CuSO_4$) (Ong and Chong, 2009). Moreover, phytoalexin was reportedly synthesized in lettuce plants as early as 5 day-old, up until harvest stage (Mai and Glomb, 2014). The present study revealed that among tested lettuce of different ages, 8 and 9 weeks-old lettuce samples seemed to produce phytoalexin more consistently in the presence of chemical abiotic elicitors. This also corresponds with the previous researches by Bestwick *et al.* (1995), suggesting that plants produce different amounts of phytoalexin at different ages. Ong and Chong (2009) also reported that lettucenin A significantly increased during week 9 to 12 of cultivation. With regards to this information, it can be concluded non-pathogenic *Pythium* do not function well as biotic elicitors, and therefore did not yield satisfactory results. On the contrary, there was a study by Liu *et al.* (1995) claiming to successfully use *Pythium ultimum* and *P. sylvaticum* as biotic elicitors in producing 3 kinds of phytoalexins (kievitone, phaseollinisoflavan and phaseollin) in the root tissue of bean seedlings (*Phaseolus vulgaris* L.). Moreover, there were also a few reports on phytoalexin accumulation in lettuce induced by biotic elicitors such as *Pseudomonas cichorii* (Takasugi *et al.*, 1985), *Botrytis cinerea*, *Bremia lactucae* and *Pseudomonas syringae* (Bennett *et al.*, 1994).

Observations of sporangium, zoospore and oospore germination by *P. aphanidermatum* were made, but no data could be obtained either in control or in crude extract treatments due to detection difficulties. However, changes in the structure of sporangium, oogonium, antheridium and oospore were detected such as lysis of sporangium and antheridium, and formation of the granular cytoplasm with an intense vacuolization in oogonium and oospore. Lysis was also detected on hyphae. The findings above suggest that the crude extract of hydroponically grown lettuce elicited with $CuSO_4$ and $AgNO_3$ possessed *in vitro* antifungal activity against *C. gloeosporioides*, *C. lunata*, *F. oxysporum* and *P. aphanidermatum*, probably due to the presence of phytoalexin (lettucenin A) within the extract. Our results are in accordance with studies reporting great inhibitory effect of phytoalexin against various plant pathogenic fungi (Huang, 2001). The sesquiterpene lactone phytoalexin, lettucenin A, possessed considerable activity against *Botrytis cinerea*, *B. lactucae* and *Pseudomonas syringae* pv. *phaseolicola* (Bennett *et al.*, 1994), *Ceratocystis fimbriata* (Takasugi *et al.*, 1985), whereas cichoralexin from chicory was reported to completely inhibit the conidial germination of *Bipolaris*

leerside (Monde *et al.*, 1990), *B. cinerea*, *Fusarium moniliform*, *P. ultimum*, *Phoma betae* and *Alternaria* sp. (Mares *et al.*, 2005). Other types of phytoalexins in various plants were also observed to have antifungal activities, for example, crucifer phytoalexin inhibited the activity of *Alternaria brassicicola* and *A. brassicae* (Sellam *et al.*, 2007); momilatones A and B (derived from rice plant) defended the plant against *B. cinerea*, *F. solani*, *F. oxysporum* and *C. gloeosporioides* (Fukuta *et al.*, 2007); daidzein (from cowpea) was used against *F. oxysporum* (Sundaresan *et al.*, 1993).

5.4 Induced Resistance to *Pythium* Root Rot in Lettuce Grown Hydroponically after Induction with Elicitors

There has not been many publish scientific papers involving the examination of plant response to *Pythium* infection and its defense mechanism. This research aims to examine elicitors effective in activating induced resistance in hydroponically grown lettuce after pathogenic infection (elicitor treatment). Quantitative RT-PCR was performed for the 7 defense response genes in order to examine the mechanism of gene expression between healthy and induced plants, challenged with pathogenic *Pythium* SR31. The expression profiles of phytoalexin, lettucenin A, PR protein and *Pythium* genes were compared using specific primers. The relative expressions of *LsPR1b_like*, *LsLTC1*, and *LsLTC2* were higher in lettuce leaves treated with *Pythium* sp. ASR23 than the expressions in lettuce roots as determined by qRT-PCR. The expression of *LsPR1b_like*, a gene involved in the SA pathway, was triggered by *B. cinerea* B05.10, 3 strains of *Pythium* sp., and BABA. The latter confirmed plant response by the plant's activity of reactive oxygen species production and histological analysis. Pre-treatment with BABA and non-pathogenic *Pythium* sp. ASR23 and SR36 activated PAMPs response in the leaves and roots (top, middle, bottom). During histological analysis, the accumulation of ROS left clear stains on the roots (middle and bottom). These elicitors may be used to activate defense responses against lettuce root rot. Bennett *et al.* (2002) reported that RT-PCR analysis showed consistent expression of *LsLTC1* and *LsLTC2* genes in roots, hypocotyls, and true leaves, but level of the *LsLTC1* transcript was much lower than *LsLTC2* in leaves. The expression of *LsPR1b_like*, a gene involved in the SA pathway, was stimulated by *B. cinerea* B05.10, 3 strains of *Pythium* sp., and BABA. However, the accumulation of pathogenesis-related proteins was not altered by BABA; it was found that only minor

accumulation occurred in BABA-treated plants. *LsPR1b_like* accumulation was observed in mock-inoculated plants at 4 dpi (days post inoculation), therefore, it can be concluded that PR-proteins are not responsible for triggering induced resistance as accumulation occurred too late (Cohen *et al.*, 2010). Of all of these elicitors, *Pythium* sp. ASR23 showed strongest gene expression on leaves. $CuSO_4$ exposure did not trigger any expression of *LsPR1b_like* genes in leaves and roots. The expression of *Pythium* genes, *PyITS1* and *PyITS2*, were strongly expressed by 3 strains of *Pythium* sp. (SR31, SR36, ASR23), and BABA, while such expressions could not be observed in mock and $CuSO_4$ treatments. The relative expressions of *LsPR1b_like*, *LsLTC1*, and *LsLTC2* were higher in lettuce treated with *Pythium* sp. ASR23 than in lettuce roots as determined by qRT-PCR. However, the relative expression of *LsLTC1* in roots after pre-treatment with 3 strains of *Pythium* sp., BABA, and infection with pathogenic *Pythium* sp. SR31 were not significantly different compared to the relative gene expression of *LsLTC2*. Cohen *et al.* (2010) also reported late accumulation of PR-proteins, resulting in the latter not being responsible for triggering induced resistance against pathogens. Therefore, changes in gene expression in accordance with *Pythium* defense response indicated that the gene expressions increased after pre-treatment with various elicitors for 24 hours. Furthermore, the expressions significantly increased along with the activation of defense responses against pathogenic *Pythium* sp. SR31 infection in lettuce. The results suggested that non-pathogenic *Pythium* sp. ASR23 could be used as an effective elicitor to trigger *Pythium* defense response. The increased accumulation of ROS is a general indicator of the initiation of defense responses in plants challenged with avirulent pathogens or elicitors (Lamb and Dixon, 1997). Some other researchers have also reported that the flg22-FLS2 interaction leads to production of reactive oxygen species (ROS) (Navarro *et al.*, 2004). Likewise, Sano *et al.* (2014) demonstrated the involvement of light in the activation of SA biosynthesis pathway by flg22 in their experimental study.

CHAPTER 6

CONCLUSION

The identification and characterization of *Pythium* isolates collected from hydroponic systems and cultivated soil was assessed by their morphological characteristics and molecular profiles using ITS regions. Based on morphological characteristics, *P. aphanidermatum* was shown to be the predominant species (24 isolates), followed by *P. myriotylum* (11 isolates). This morphological identification was supported by molecular profiles. The phylogenetic analyses of ITS rDNA region confirmed two genetically distinct groups of *Pythium*, namely *P. aphanidermatum* and *P. myriotylum*. Our results suggested that morphological identification with supporting molecular profiles allowed accurate identification of *Pythium*. Furthermore, this study suggested that isolates recovered from symptomatic roots were more likely to be the *P. myriotylum* species, which were also slightly more virulent than other isolates.

A total of 38 oomycete isolates were obtained mainly from asymptomatic roots of lettuce grown hydroponically, symptomatic roots and infested soil containing pathogens that cause root rot disease. Only 3 isolates of *Pythium* spp. out of 38 isolates were shown to strong to be non-pathogenic isolates, while the rest could be minor pathogens. Among the 3 isolates obtained, 2 isolates, namely ASR23 and ASR4, were recovered from asymptomatic lettuce roots, while unexpectedly, SR33 was the only isolate recovered from symptomatic roots. Regarding the methods employed to test pathogenicity and detect real non-pathogenic *Pythium* spp. among minor pathogens in our study, the Petri-dish assay and seed inoculation method seemed to yield similar results in terms of disease incidence of lettuce seedlings.

While examining cell wall degrading enzyme production among *Pythium* species, it was found that most isolates from asymptomatic and symptomatic lettuce root could produce cellulolytic and pectinolytic enzymes in agar medium, but only in a small amount with HC value of less than 1. Low to medium chitinase activity was detected only in *Pythium* species from the non to weakly virulent group: more pectinase activity in medium pathogenic isolates than in non pathogenic.

Phytoalexin occurrence was observed in hydroponically grown butterhead lettuce after elicitation with 5% $CuSO_4$ and 1% $AgNO_3$ as abiotic elicitors at different plant ages (4-11 weeks). This also suggested that lettuce grown in hydroponics could produce phytoalexin in the same manner as in soil cultivated lettuce plants. Phytoalexin production was further studied on other varieties of lettuce with the use of non-pathogenic *Pythium* sp. as biotic elicitors. It was revealed that all 5 varieties of lettuce elicited with abiotic elicitors were able to effectively stimulate phytoalexin production during hydroponic cultivation for all experimental trials, whereas lettuce elicited with biotic elicitors did not produce satisfactory results in yielding the compound. From our results, the phytoalexin detected from hydroponically grown lettuce could probably be lettucenin A, since it produces the same pattern showing a yellow fluorescent spot with clear inhibition zone against *A. niger* on the TLC plate. The results of *in vitro* antifungal study involving 4 plant pathogenic fungi, namely *C. gloeosporioides*, *C. lunata*, *F. oxysporum* and *P. aphanidermatum*, suggested that the crude extract from lettuce elicited with abiotic elicitors possessed antifungal activity against all tested fungi, possibly due to the presence of phytoalexin (lettucenin A) within the extract. Thus, we have classified the obtained phytoalexin as the mentioned compound.

The quantitative RT-PCR of the 7 defense response genes was performed to explore the differences in gene expression between healthy plants and plants challenged with pathogenic *Pythium* SR31. The expression profiles of lettucenin A biosynthesis, PR protein and *Pythium* genes were compared by using specific primers. The relative expressions of *LsPR1b_like*, *LsLTC1*, and *LsLTC2* as determined by qRT-PCR were higher in lettuce leaves treated with *Pythium* sp. ASR23 than in lettuce roots. The expression of *LsPR1b_like*, a gene involved in the SA pathway, was triggered by *B. cinerea* B05.10, 3 strains of *Pythium* sp., and BABA. Along with gene expressions, the production of reactive oxygen species in sampled lettuce and histological analysis were key tools in confirming plant defense response. Treatment of lettuce with BABA and non-pathogenic *Pythium* sp., ASR23 and SR36, primed PAMP response in leaves and roots (top, middle, bottom). All gathered information corresponded to the results of histological analysis, by which strong stains were developed along the middle and bottom roots, indicating the accumulation of ROS. These elicitors could be used to activate defense responses against lettuce root rot.

REFERENCES

Abad, Z.G., Shew, H.D. and Lucas, L.T. 1994. "Characterization and pathogenicity of *Pythium* species isolated from turfgrass with symptoms of root and crown rot in North Carolina." **Phytopathology** 84 : 913-921.

Agrawal, T. and Kotasthane, A.S. 2012. "Chitinolytic assay of indigenous *Trichoderma* isolates collected from different geographical locations of Chhattisgarh in Central India." **SpringerPlus** 1(73) : 1-10.

Agrios, G.N. 1997. **Plant Pathology**. 4th ed. USA : Academic Press. 635 pp.

Ahuja, I., Kissen, R. and Bones, A.M. 2012. "Phytoalexins in defense against pathogens." **Trends in Plant Science** 17(2) : 73-90.

Alaei, H. and Rostami, F. 2013. "Identification of cucumber damping-off based on morphological and molecular characterizations in Rafsanjan." **Iranian Journal of Plant Pathology** 48(4) : 177-182.

Al-Sheikh, H. and Abdelzaher, H.M.A. 2012. "Occurrence, identification and pathogenicity of *Pythium aphanidermatum*, *P. diclinum*, *P. dissotocum* and *Pythium* 'Group P' isolated from Dawmat Al-Jandal Lake, Saudi Arabia." **Research Journal of Environmental Sciences** 6(6) : 196-209.

Angelova, Z., Georgiev, S. and Roos, W. 2006. "Elicitation of plants." **Biotechnology and Biotechnological Equipment** 20(2) : 72-83.

Aysan, Y., Sahin, S., Ulke, G. and Sahin, F. 2003. "Bacterial rot of lettuce caused by *Pseudomonas cichorii* in Turkey." **Plant Pathology** 52 : 782.

Babai-Ahary, A., Abrinnia, M. and Heravan, I.M. 2004. "Identification and pathogenicity of Pythium species causing damping-off in sugarbeet in northwest Iran." **Australasian Plant Pathology** 33 : 343-347.

Bahramisharif, A., Lamprecht, S.C., Spies, C.F.J., Botha, W.J., Calitz, F.J. and McLeod, A. 2014. "*Pythium* spp. associated with rooibos seedlings, and their pathogenicity toward rooibos, lupin, and oat." **Plant Disease** 98(2) : 223-232.

Bala, K., Gautam, N. and Paul, B. 2006. "*Pythium rhizo-oryzae* sp. nov. isolated from paddy fields: taxonomy, ITS region of rDNA, and comparison with related species." **Current Microbiology** 52 : 102-107.

Bates, M.L. and Stanghellini, M.E. 1984. "Root rot of hydroponically grown spinach caused by *Pythium aphanidermatum* and *P. dissotocum*." **Plant Disease** 68 : 989-991.

Bednarek, P., Schneider, B., Svatoš, A., Oldham, N.J. and Hahlbrock, K. 2005. "Structural complexity, differential response to infection, and tissue specificity of indolic and phenylpropanoid secondary metabolism in Arabidopsis roots." **Plant Physiology** 138 : 1058–1070.

Bennett, M.H., Gallagher, M.D.S., Bestwick, C.S., Rossiter, J.T. and Mansfield, J.W. 1994. "The phytoalexin response of lettuce to challenge by *Botrytis cinerea*, *Bremia lactucae* and *Pseudomonas syringae* pv. *phaseolicola*." **Physiological and Molecular Plant Pathology** 44 : 321-333.

Bennett, M.H., Mansfield, J.W., Lewis, M.J. and Beale, M.H. 2002. "Cloning and expression of sesquiterpene synthase genes from lettuce (*Lactuca sativa* L.)." **Phytochemistry** 60 : 255-261.

Benoit, F. 1992. **Practical Guide for Simple Soilless Culture Techniques**. Belgium : European Vegetable R&D Centre. 72 pp.

Bestwick, L., Bennett, M.H., Mansfield, J.W. and Rossiter, J.T. 1995. "Accumulation of the phytoalexin Lettucenin A and changes in 3-hydroxy-3-methylglutaryl coenzyme a reductase activity in lettuce seedlings with the red spot disorder." **Phytochemistry** 39(4) : 775-777.

Binagwa, P.H., Bonsi, C.K., Msolla, S.N. and Ritte, I.I. 2016. "Morphological and molecular identification of *Pythium* spp. isolated from common beans (*Phaseolus vulgaris*) infected with root rot disease." **African Journal of Plant Science** 10(1) : 1-9.

Blancard, D., Lot, H. and Maisonneuve, B. 2006. **A Colour Atlas of Diseases of Lettuce and Related Salad Crops Observation, Biology and Control**. UK : Manson Publishing. 375 pp.

Boshoff, J. 2005. "Biological control of Pythium wilt and root rot in hydroponically grown lettuce." M.Sc. Thesis. University of Pretoria.

Boudjeko, T., Andème-Onzighi, C., Vicré, M., Balangé, A.P., Ndoumou, D.O. and Driouich, A. 2006. "Loss of pectin is an early event during infection of cocoyam roots by *Pythium myriotylum*." **Planta** 223 : 271-282.

Buysens, S., Höfte, M. and Poppe, J. 1995. "Biological control of *Pythium* sp. in soil and nutrient film technique systems by *Pseudomonas aeruginosa* 7NSK2." **Acta Horticulturae** 382 : 238-243.

Calvo-Bado, L.A., Petch, G., Parsons, N.R., Morgan, J.A.W., Pettitt, T.R. and Whipps, J.M. 2006. "Microbial community responses associated with the development of oomycete plant pathogens on tomato roots in soilless growing systems." **Journal of Applied Microbiology** 100 : 1194-1207.

Campion, C., Massiot, P. and Rouxel, F. 1997. "Aggressiveness and production of cell-wall degrading enzymes by *Pythium violae*, *Pythium sulcatum* and *Pythium ultimum*, responsible for cavity spot on carrots." **European Journal of Plant Pathology** 103 : 725-735.

Chairat, Y. and Pasura, A. 2013. "Isolation and identification of rhizobacteria having inhibitory capability on pathogenic fungi, *Pythium* sp.." **Journal of Science, Technology, and Humanities** 11(2) : 117-127.

Chatterton, S., Sutton, J.C. and Boland, G.J. 2004. "Timing Pseudomonas chlororaphis applications to control *Pythium aphanidermatum*, *Pythium dissotocum*, and root rot in hydroponic peppers." **Biological Control** 30 : 360-373.

Chen, W.C., Hsieh, H.J. and Tseng, T.C. 1998. "Purification and characterization of a pectin lyase from *Pythium splendens* infected cucumber fruits." **Botanical Bulletin of Academia Sinica** 39 : 181-186.

Chitrampalam, P., Cox, C.A., Turini, T.A. and Pryor, B.M. 2010. "Efficacy of *Coniothyrium minitans* on lettuce drop caused by *Sclerotinia minor* in desert agroecosystem." **Biological Control** 55(2) : 92-96.

Cho, J.J. 1983. "Soft rot of crisphead lettuce incited by *Erwinia carotovora* subsp. *carotovora* in Hawaii." **Phytopathology** 73 : 1206-1209.

Christensen, M.J., Falloon, R.E. and Sklpp, R.A. 1988. "A petri plate technique for testing pathogenicity of fungi to seedlings and inducing fungal sporulation." **Australasian Plant Pathology** 17(2) : 45-47.

Clustal Omega. 2015. Multiple Sequence Alignment. [online]. Available : http://www.ebi.ac.uk/Tools/msa/clustalo/.

Cohen, Y., Rubin, A.E. and Kilfin, G. 2010. "Mechanisms of induced resistance in lettuce against *Bremia lactucae* by DL-β-amino-butyric acid (BABA)." **European Journal of Plant Pathology** 126 : 553-573.

Dan, S., Qiang, H., Zhaonan, D. and Zhengquan, H. 2014. "Genetic transformation of lettuce (*Lactuca sativa*): A review." **African Journal of Biotechnology** 13(16) : 1686-1693.

Davey, M.R., Anthony, P., Van Hooff, P., Power, J.B. and Lowe, K.C. 2007. "Lettuce in biotechnology in agriculture and forestry." In Pua, E.C. and Davey, M.R. (Ed.). **Transgenic Crops IV**. Vol. 59. Springer-Verlag Berlin Heidelberg.

de Boer, R., Petkowski, J. and Minchinton, E. 2013. "Review of *Pythium* induced root rots, cavity spots and cankers of Apiaceae vegetable crops." P. 5-36. In Minchinton, E. (ed.). **Identification of IPM Strategies for *Pythium* Induced Root Rots in Apiacae Vegetable Crops**. Horticulture Australia.

Deaker, R., Roughley, R.J. and Kennedy, I.R. 2004. "Legume seed inoculation technology-a review." **Soil Biology and Biochemistry** 36 : 1275-1288.

Deep, S., Sharma, P. and Behera, N. 2014. "Optimization of extracellular cellulase enzyme production from *Alternaria brassicicola*." **International Journal of Current Microbiology and Applied Sciences** 3(9) : 127-139.

Dhingra, O.D. and Sinclair, J.B. 1994. **Basic Plant Pathology Methods**. 2 nd. The United States of America : CRC Press. 448 pp.

Ebrahim, S., Usha, K. and Singh, B. 2011. "Pathogenesis related (PR) proteins in plant defense mechanism." p. 1043-1054. In Méndez-Vilas (ed.). **Science against Microbial Pathogens: Communicating Current Research and Technological Advances**. Formatex.

Ehret, D.L., Alsanius, B., Wohanka, W., Menzies, J.G. and Utkhede, R. 2001. "Disinfestation of recirculating nutrient solutions in greenhouse horticulture." **Agronomie** 21 : 323-339.

FAO. 2016. "**Crops and Livestock Products.**" [Online]. Available : http://faostat.fao.org/beta/en/.

Fernando, T.H.P.S., Jayasinghe, C.K. and Wijesundera, R.L.C. 2001. "Cell wall degrading enzyme secretion by *Colletotrichum acutatum*, the causative fungus of secondary leaf fall of *Hevea brasiliensis*." **Mycological Research** 105(2) : 195-201.

Florencio, C., Couri, S. and Farinas, C.S. 2012. "Correlation between agar plate screening and solid-state fermentation for the prediction of cellulase production by *Trichoderma* strains." **Enzyme Research** 2012 : 1-7.

Foley, D.C. 1980. "Resistance to *Pythium debaryanum* in *Zea mays* seedlings." *In* **Proceedings of the Iowa Academy of Science** 87(4) : 134-138.

Garibaldi, A., Gilardi, G., Ortu, G. and Gullino, M.L. 2014. "First report of root rot caused by *Pythium aphanidermatum* on bell pepper (*Capsicum annuum* L.) in Italy." **Plant Disease** 98(6) : 854.

Garzón, C.D., Geiser, D.M. and Moorman, G.W. 2005. "Diagnosis and population analysis of *Pythium* species using AFLP fingerprinting." **Plant Disease** 89 : 81-89.

Godfrey, S.A.C., Monds, R.D., Lash, D.T. and Marshall, J.W. 2003. "Identification of *Pythium oligandrum* using species-specific ITS rDNA PCR oligonucleotides." **Mycological Research** 107(7) : 790-796.

Gold, S.E. and Stanghellini, M.E. 1985. "Effects of temperature on Pythium root rot of spinach grown under hydroponic conditions." **Phytopathology** 75 : 333-337.

Gómez-Alpízar, L., Saalau, E., Picado, I., Tambong, J.T. and Saborío, F. 2011. "A PCR-RFLP assay for identification and detection of *Pythium myriotylum*, causal agent of the cocoyam root rot disease." **Letters in Applied Microbiology** 52 : 185-192.

Gutierrez, M.C., Parry, A., Tena, M., Jorrin, J. and Edwards, R. 1995. "Abiotic elicitation of coumarin phytoalexins in sunflower." **Phytochemistry** 38(5) : 1185-1191.

Hall, C., Heath, R. and Guest, D.I. 2011. "Rapid and intense accumulation of terpenoid phytoalexins in infected xylem tissues of cotton (*Gossypium hirsutum*) resistant to *Fusarium oxysporum* f.sp. *vasinfectum*." **Physiological and Molecular Plant Pathology** 76 : 182-188.

Hall, T.A. 1999. "Bioedit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT." **Nucleic Acids Symposium Series** 41 : 95-98.

Hammerschmidt, R. 2007. "Introduction : definitions and some history." *In* : Walters, D., Newton, A. and Lyon, G. eds. **Induced Resistance for Plant Disease Control : A Sustainable Approach to Crop Protection**. Oxford : Blackwell Publishing Ltd. 352 pp.

Hanawa, F., Kanauchi, M., Tahara, S. and Mizutani, J. 1995. "Lettucenin A as a phytoalexin of dandelion and its elicitation in dandelion cell cultures." **Journal of the Faculty of Agriculture, Hokkaido University** 66(2) : 151-162.

Haritha, V., Gopal, K., Madhnsudhan, P., Viswanath, K. and Rao, S.V.R.K. 2010. "Integrated management of damping-off disease incited by *Pythium aphanidermatum* (Edson) Fitzp. in tobacco nursery." **Journal of Plant Disease Sciences** 5(1) : 41-47.

Hernández-Silva, L., Piñón-Escobedo, C., Cano-Camacho, H., Zavala-Páramo, Ma.G., Acosta-Rodríguez, I. and López-Romero, E. 2007. "Comparison of fungal growth and production of extracellular pectin lyase activity by pathogenic and non-pathogenic races of *Colletotrichum lindemuthianum* cultivated under different conditions." **Physiological and Molecular Plant Pathology** 70 : 88-95.

Herrera-Estrella, A. and Chet, I. 1999. "Chitinases in biological control." **Experientia Supplementum** 87 : 171-184.

Herrero, M.L. and Klemsdal, S.S. 1998. "Identification of *Pythium aphanidermatum* using the RAPD technique." **Mycological Research** 102(2) : 136-140.

Herrero, M.L., Hermansen, A. and Elen, O.N. 2003. "Occurrence of *Pythium* spp. and *Phytophthora* spp. in Norwegian greenhouses and their pathogenicity on cucumber seedlings." **Journal of Phytopathology** 151 : 36-41.

Ho, H.H. 2011. "The genus *Pythium* in Taiwan (2)-an illustrated diagnostic key." **Mycotaxon** 116 : 33-47.

Ho, H.H., Chen, X.X, Zeng, H.C. and Zheng, F.C. 2012. "The occurrence and distribution of *Pythium* species on Hainan Island of South China." **Botanical Studies** 53 : 525-534.

Ho, W.C. and Ko, W.H. 1997. "A simple method for obtaining single-spore isolates of fungi." **Botanical Bulletin of Academia Sinica** 38 : 41-44.

Hubballi, M., Sornakili, A., Nakkeeran, S., Anand, T. and Raguchander, T. 2011. "Virulence of *Alternaria alternata* infecting noni associated with production of cell wall degrading enzymes." **Journal of Plant Production Research** 51(1) : 87-92.

Janardhanan, K.K. and Husain, A. 1974. "Production of a toxic metabolite and pectolytic enzyme by *Pythium butleri.*" **Mycopathologia et Mycologia Applicata** (52)3-4 : 325-330.

Jee, H.J., Cho, W.D. and Kim, C.H. 2002. "Effect of potassium phosphonate on the control of Phytophthora root rot of lettuce in hydroponics." **Plant Pathology Journal** 18(3) : 142-146.

Jee, H.J., Nam, K.W. and Cho, W.D. 2001. "Severe root rot on hydroponically-grown lettuce caused by *Phytophthora drechsleri.*" **Plant Pathology Journal** 17(5) : 311-314.

Jenkins, S.F., Jr. and Averre, C.W. 1983. "Root diseases of vegetables in hydroponic culture systems in North Carolina greenhouses." **Plant Disease** 67 : 968-970.

Kageyama, K., Suzuki, M., Priyatmojo, A., Oto, Y., Ishiguro, K., Suga, H., Aoyagi, T. and Fukui, H. 2003. "Characterization and identification of asexual strains of *Pythium* associated with root rot of rose in Japan." **Journal of Phytopathology** 151 : 485-491.

Karthik, N., Akanksha, K., Binod, P. and Pandey, A. 2014. "Production, purification and properties of fungal chitinases-A review." **Indian Journal of Experimental Biology** 52 : 1025-1035.

Kasana, R.C., Salwan, R., Dhar, H., Dutt, S. and Gulati, A. 2008. "A rapid and easy method for the detection of microbial cellulases on agar plates using gram's iodine." **Current Microbiology** 57 : 503-507.

Kielak, A.M., Cretoiu, M.S., Semenov, A.V., Sørensen, S.J. and van Elsas, J.D. 2013. "Bacterial chitinolytic communities respond to chitin and pH alteration in soil." **Applied and Environmental Microbiology** 79(1) : 263-272.

Kim, J.S., Lee, J., Lee, C.H., Woo, S.Y., Kang, H., Seo, S.G. and Kim, S.H. 2015. "Activation of pathogenesis-related genes by the rhizobacterium, *Bacillus* sp. JS, which induces systemic resistance in tobacco plants." **Plant Pathology Journal** 31(2) : 195-201.

Kitajima, S. and Sato, F. 1999. "Plant pathogenesis-related proteins : molecular mechanisms of gene expression and protein function." **Journal of Biochemistry** 125(1) : 1-8.

Koleosho, B., Ikotun, T. and Faboya, O. 1987. "The role of oxalic acid and polygalacturonase in the pathogenicity of *Pythium aphanidermatum* on different cowpea varieties." **Phytoparasitica** 15(4) : 317-323.

Koohakan, P., Jaenaksorn, T. and Nuntagij, I. 2008. "Major diseases of lettuce grown by commercial nutrient film technique in Thailand." **KMITL Science Technology Journal** 8(2) : 56-63.

Kuć, J. 1972. "Phytoalexins." **Annual Review of Phytopathology** 10 : 207-232.

Kuć, J. 1995. "Phytoalexins, stress metabolism, and disease resistance in plants." **Annual Review of Phytopathology** 33 : 275-297.

Kwon, H.W., Yoon, J.H., Kim, S.H., Hong, S.B., Cheon, Y. and Ko, S.J. 2007. "Detection of extracellular enzyme activities in various *Fusarium* spp.." **Mycobiology** 35(3) : 162-165.

Lee, S. and Moorman, G.W. 2008. "Identification and characterization of simple sequence repeat markers for *Pythium aphanidermatum*, *P. cryptoirregulare*, and *P. irregulare* and the potential use in *Pythium* population genetics." **Current Genetics** 53 : 81-93.

Lehotai, N., Pető, A., Weisz, M., Erdei, L. and Kolbert, Z. 2011. "Generation of reactive oxygen and nitrogen species in pea cultivars under copper exposure." **Acta Biologica Szegediensis** 55(2) : 273-278.

Lévesque, C.A. and de Cock, A.W.A.M. 2004. "Molecular phylogeny and taxonomy of the genus *Pythium*." **Mycological Research** 108(12) : 1363-1383.

Lévesque, C.A., Vrain, T.C. and de Bore, S.H. 1994. "Development of a species-specific probe for *Pythium ultimum* using amplified ribosomal DNA." **Phytopathology** 84 : 474-478.

Lin, Y.S., Huang, J.H. and Gung, Y.H. 2002. "Control of *Pythium* root rot of vegetable pea seedlings in soilless cultural system" **Plant Pathology Bulletin** 11(4) : 221-228.

Lionetti, V., Cervone, F. and Bellincampi, D. 2012. "Methyl esterification of pectin plays a role during plant-pathogen interactions and affects plant resistance to diseases." **Journal of Plant Physiology** 169 : 1623-1630.

Littrell, R.H. and McCarter, S.M. 1970. "Effect of soil temperature on virulence of *Pythium aphanidermatum* and *Pythium myriotylum* to rye and tomato." **Phytopathology** 60 : 704–707.

Liu, L., Punja, Z.K. and Rahe, J.E. 1995. "Effect of *Pythium* spp. and glyphosate on phytoalexin production and exudation by bean (*Phaseolus vulgaris* L.) roots grown in different media." **Physiological and Molecular Plant Pathology** 47(6) : 391-405.

Liu, W., Sutton, J.C., Grodzinski, B., Kloepper, J.W. and Reddy, M.S. 2007. "Biological control of *Pythium* root rot of chrysanthemum in small-scale hydroponic units." **Phytoparasitica** 35(2) : 159-178.

Lloyd, S.R., Schoonbeek, H.J., Trick, M., Zipfel, C. and Ridout, C.J. 2014. "Methods to study PAMP-triggered immunity in *Brassica* species." **Molecular Plant-Microbe Interactions** 27(3) : 286-295.

Maccheroni Jr., W., Araújo, W.L. and Azevedo, J.L. 2004. "Ambient pH-regulated enzyme secretion in endophytic and pathogenic isolates of the fungal genus *Colletotrichum*." **Scientia Agricola** 61(3) : 298-302.

Mai, F. and Glomb, M.A. 2014. "Lettucenin sesquiterpenes contribute significantly to the browning of lettuce." **Journal of Agricultural and Food Chemistry** 62 : 4747-4753.

Marino, D., Dunand, C., Puppo, A. and Pauly, N. 2012. "A burst of plant NADPH oxidases." **Trends in Plant Science** 17(1) : 9-15.

Martin, F.W. and Ruberte, R.M. 1975. **Edible Leaves of the Tropics**. Puerto Rico : Antillian College Press. 238 pp.

Mathew, R., Singh, K.K. and Paul, B. 2003. "*Pythium campanulatum* sp. nov., isolated from the rhizosphere of maize, its taxonomy, ITS region of rDNA, and comparison with related species." **FEMS Microbiology Letters** 226 : 9-14.

Matsumoto, C., Kageyama, K., Suga, H. and Hyakumachi, M. 1999. "Phylogenetic relationships of *Pythium* species based on ITS and 5.8S sequences of the ribosomal DNA." **Mycoscience** 40 : 321-331.

Matsuura, K., Kanto, T., Tojo, M., Uzuhashi, S. and Kakishima, M. 2010. "Pythium wilt of lettuce caused by *Pythium uncinulatum* in Japan." **Journal of General Plant Pathology** 76 : 320-323.

McCarter, S.M. and Littrell, R.H. 1970. "Comparative pathogenicity of *Pythium aphanidermatum* and *Pythium myriotylum* to twelve plant species and intraspecific variation in virulence." **Phytopathology** 60 : 264-268.

Mccombs, C.L. 1961. "Pectinolytic and cellulolytic enzyme production by *Pythium aphanidermatum*." **Phytopathology** 270-273.

Menzies, J., Ehret, D.L. and Stan, S. 1996. "Effect of inoculum density of *Pythium aphanidermatum* on the growth and yield of cucumber plants grown in recirculating nutrient film culture." **Canadian Journal of Plant Pathology** 18(1) : 50-54.

Mihail, J.D., Hung, L.F. and Bruhn, J.N. 2002. "Diversity of the *Pythium* community infecting roots of the annual legume *Kummerowia stipulacea*." **Soil Biology and Biochemistry** 34 : 585-592.

Mishra, A.K., Sharma, K. and Misra, R.S. 2012. "Elicitor recognition, signal transduction and induced resistance in plants." **Journal of Plant Interactions** 7(2) : 95-120.

Monde, K., Oya, T., Shirata, A. and Takasugi, M. 1990. "A guaianolide phytoalexin, cichoralexin, from *Cichorium intybusl.*" **Phytochemistry** 29(11) : 3449-3451.

Moorman, G.W., Kang, S., Geiser, D.M. and Kim, S.H. 2002. "Identification and characterization of *Pythium* species associated with greenhouse floral crops in Pennsylvania." **Plant Disease** 86 : 1227-1231.

Moreno-Vázquez, S., Ochoa, O.E., Faber, N., Chao, S., Jacobs, J.M.E., Maisonneuve, B., Kesseli, R.V. and Michelmore, R.W. 2003. "SNP-based codominant markers for a recessive gene conferring resistance to corky root rot (*Rhizomonas suberifaciens*) in lettuce (*Lactuca sativa*)." **Genome** 46 : 1059-1069.

Munkvold, G.P. and Carlton, W.M. 1997. "Influence of inoculation method on systemic *Fusarium moniliforme* infection of maize plants grown from infected seeds." **Plant Disease** 81(2) : 211-216.

National Center for Biotechnology Information. 2015. Nucleotide Blast. [Online]. Available : http://www.ebi.ac.uk/Tools/msa/clustalo/.

Navarro, L., Zipfel, C., Rowland, O., Keller, I., Robatzek, S., Boller, T. and Jones, J.D.G. 2004. "The transcriptional innate immune response to flg22 interplay and overlap with Avr gene-dependent defense responses and bacterial pathogenesis." **Plant Physiology** 135 : 1113-1128.

Neethu, K., Rubeena, M., Sajith, S., Sreedevi, S., Priji, P., Unni, K.N., Josh, M.K.S., Jisha, V.N., Pradeep, S. and Benjamin, S. 2012. "A novel strain of Trichoderma viride shows complete lignocellulolytic activities." **Advances in Bioscience and Biotechnology** 3 : 1160-1166.

Nzungize, J., Gepts, P., Buruchara, R., Buah, S., Ragama, P., Busogoro, J.P. and Baudoin, J.P. 2011. "Pathogenic and molecular characterization of *Pythium* species inducing root rot symptoms of common bean in Rwanda." **African Journal of Microbiology Research** 5(10) : 1169-1181.

Okunowo, W.O., Gbenle, G.O., Osuntoki, A.A., Adekunle, A.A. and Ojokuku, S.A. 2010. "Production of cellulolytic and xylanolytic enzymes by a phytopathogenic *Myrothecium roridum* and some avirulent fungal isolates from water hyacinth." **African Journal of Biotechnology** 9(7) : 1074-1078.

Ong, W.D. and Chong, K.P. 2009. "Aging effect to accumulation of lettucenin A in lettuce after elicitation with various abiotic elicitors." **Modern Applied Science** 3(2) : 66-70.

Ongena, M., Daayf, F., Jacques, P., Thonart, P., Benhamou, N., Paulitz, T.C. and Bélanger, R.R. 2000. "Systemic induction of phytoalexins in cucumber in response to treatments with fluorescent pseudomonads." **Plant Pathology** 49 : 523-530.

Onuh, O.M. and Ohazurike, N.C. 2008. "Effects of culture ages on the production and activities of polygalacturonase and cellulase (cx) enzymes produced by *Pythium aphanidermatum* (Edson Fitzpat.) isolated from soft stem rot disease of cowpea." **Science World Journal** 3(2) : 5-9.

Owen-Going, T.N., Beninger, C.W., Christie, B., Sutton, J.C. and Hall, J.C. 2004. "Role of phenolic compounds in epidemics of *Pythium* root rot (*Pythium aphanidermatum*) of hydroponic pepper." **Canadian Journal of Plant Pathology** 26(1) : 410-419.

Palmucci, H.E. and Grijalba, P.E. 2007. "Root and stem rot caused by *Pythium aphanidermatum* on poinsettia in a soil-less culture system in Buenos Aires province, Argentina." **Australasian Plant Disease Notes** 2 : 139-140.

Park, J.M. and Paek, K.H. 2007. "Recognition and response in plant-pathogen interactions." **Journal of Plant Biology** 50(2) : 132-138.

Paul, B. 2000. "ITS1 region of the rDNA of *Pythium megacarpum* sp. nov., its taxonomy, and its comparison with related species." **FEMS Microbiology Letters** 186 : 229-233.

Paul, B. 2001. "ITS region of the rDNA of *Pythium longandrum*, a new species; its taxonomy and its comparison with related species." **FEMS Microbiology Letters** 202 : 239 : 242.

Paulitz, T.C. 1997. "Biological control of root pathogens in soilless and hydroponic systems." **HortScience** 32(2) : 193-197.

Perrone, S.T., McDonald, K.L., Sutherland, M.W. and Guest, D.I. 2003. "Superoxide release is necessary for phytoalexin accumulation in *Nicotiana tabacum* cells during the expression of cultivar-race and non-host resistance towards

Phytophthora spp.." **Physiological and Molecular Plant Pathology** 62 : 127-135.

Pornsuriya, C., Wang, H.K., Lin, F.C. and Soytong, K. 2008. "First report of pineapple root rot caused by *Pythium graminicola*." **Journal of Agricultural Technology** 4(1) : 139-150.

Priya, V. and Sashi, V. 2014. "Pectinase enzyme producing microorganisms." **International Journal of Scientific and Research Publications** 4(3) : 1-4.

Rafin, C. and Tirilly, Y. 1995. "Characteristics and pathogenicity of *Pythium* spp. associated with root rot of tomatoes in soilless culture in Brittany, France." **Plant Pathology** 44 : 779-785.

Reanprayoon, P. and Pathomsiriwong, W. 2012. "Tropical soil fungi producing cellulase and related enzymes in biodegradation." **Journal of Applied Sciences** 12(18) : 1909-1916.

Rey, P., Benhamou, N. and Tirilly, Y. 1998. "Ultrastructural and cytochemical investigation of asymptomatic infection by *Pythium* spp." **Phytopathology** 88 : 234-244.

Ryder, E.J. 1999. **Lettuce, Endive, and Chicory**. The university press, Cambridge, Uk. 208 pp.

Saleem, A., Ei-Said, A.H.M., Maghraby, T.A. and Hussein, M.A. 2012. "Pathogenicity and pectinase activity of some facultative mycoparasites isolated from *Vicia faba* diseased leaves in relation to photosynthetic pigments of plant." **Journal of Plant Pathology and Microbiology** 3(6) : 1-7.

Sano, S., Aoyama, M., Nakai, K., Shimotani, K., Yamasaki, K., Sato, M.H., Tojo, D., Suwastika, I.N., Nomura, H. and Shiina, T. 2014. "Light-dependent expression of flg22-induced defense genes in *Arabidopsis*." **Frontiers in Plant Science** 5 : 1-12.

Santoso, P.J., Aryantha, I.N.P., Pancoro, A. and Suhandono, S. 2015. "Identification of *Pythium* and *Phytophthora* associated with durian (*Durio* sp.) in Indonesia : their molecular and morphological characteristics and distribution." **Asian Journal of Plant Pathology** 9(2) : 59-71.

Sarig, P., Zutkhi, Y., Monjauze, A., Lisker, N. and Ben-Arie, R. 1997. "Phytoalexin elicitation in grape berries and their susceptibility to *Rhizopus stolonifer*." **Physiological and Molecular Plant Pathology** 50 : 337-347.

Schroeder, K.L., Okubara, P.A., Tambong, J.T., Lévesque, C.A. and Paulitz, T.C. 2006. "Identification and quantification of pathogenic *Pythium* spp. from soils in eastern Washington using real-time polymerase chain reaction." **Phytopathology** 96 : 637-647.

Schuerger, A.C. and Hammer, W. 2009. "Use of cross-flow membrane filtration in a recirculating hydroponic system to suppress root disease in pepper caused by *Pythium myriotylum*." **Phytopathology** 99(5) : 597-607.

Schurko, A.M., Mendoza, L., Lévesque, C.A., Désaulniers, N.L., de Cock, A.W.A.M. and Klassen, G.R. 2003. "A molecular phylogeny of *Pythium insidiosum*." **Mycological Research** 107(5) : 537-544.

Sedlářová, M., Luhová, L., Petřivalský, M. and Lebeda, A. 2007. "Localisation and metabolism of reactive oxygen species during *Bremia lactucae* pathogenesis in *Lactuca sativa* and wild *Lactuca* spp." **Plant Physiology and Biochemistry** 45 : 607-616.

Seidl, V. 2008. "Chitinases of filamentous fungi : a large group of diverse proteins with multiple physiological functions." **Fungal Biology Reviews** 22 : 36-42.

Simko, I., Zhou, Y. and Brandl, M.T. 2015. "Downy mildew disease promotes the colonization of romaine lettuce by *Escherichia coli* O157:H7 and *Salmonella enterica*." **BMC Microbiology** 15 : 19-27.

Singh, K.K., Mathew, R., Masih, I.E. and Paul, B. 2003. "ITS region of the rDNA of *Pythium rhizosaccharum* sp. nov. isolated from sugarcane roots: taxonomy and comparison with related species." **FEMS Microbiology Letters** 221 : 233-236.

Sivasankar, S., Sheldrick, B. and Rothstein, S.J. 2000. "Expression of allene oxide synthase determines defense gene activation in tomato." **Plant Physiology** 122 : 1335-1342.

Sobolev, V.S., Khan, S.I., Tabanca, N., Wedge, D.E., Manly, S.P., Cutler, S.J., Coy, M.R., Becnel, J.J., Neff, S.A. and Gloer, J.B. 2011. "Biological activity of peanut

(*Arachis hypogaea*) phytoalexins and selected natural and synthetic stilbenoids." **Journal of Agricultural and Food Chemistry** 59 : 1673-1682.

Souza, R.F., Gomes, R.C., Coelho, R.R.R., Alviano, C.S. and Soares, R.M.A. 2003. "Purification and characterization of an endochitinase produced by *Colletotrichum gloeosporioides*." **FEMS Microbiology Letters** 222 : 45-50.

Stanghellini, M.E. and Rasmussen, S.L. 1994. "Hydroponics a solution for zoosporic pathogens." **Plant Disease** 78(12) : 1129-1138.

Stanghellini, M.E. and Rasmussen, S.L., Kim, D.H. and Rorabaugh, P.A. 1996. "Efficacy of nonionic surfactants in the control of zoospore spread of *Pythium aphanidermatum* in a recirculating hydroponic system." **Plant Disease** 80(4) : 422-428.

Staub, T. 1991. "Fungicide resistance : practical experience with antiresistance strategies and the role of integrated use." **Annual Review of Phytopathology** 29 : 421-442.

Sutton, J.C., Sopher, C.R., Owen-Going, T.N., Liu, W., Grodzinski, B., Hall, J.C. and Benchimol, R.L. 2006. "Etiology and epidemiology of *Pythium* root rot in hydroponic crops: current knowledge and perspectives." **Summa Phytopathologica** 32(4) : 307-321.

Taechapoempol, K., Sreethawong, T., Rangsunvigit, P., Namprohm, W., Thamprajamchit, B., Rengpipat, S. and Chavadej, S. 2011. "Cellulase-producing bacteria from Thai higher termites, *Microcerotermes* sp.: enzymatic activities and ionic liquid tolerance." **Applied Biochemistry and Biotechnology** 164 : 204-219.

Takasugi, M., Okinaka, S., Katsui, N., Masamune, T., Shirata, A. and Ohuchi, M. 1985. "Isolation and structure of lettucenin A, a novel guaianolide phytoalexin from *Lactuca sativa* var. *capitata* (Compositae)." **Journal of the Chemical Society, Chemical Communications** 10 : 621-622.

Talubnak, C., Parinthawong, N. and Jaenaksorn, T. 2014. "In-vitro screening for non-pathogenic isolates of *Pythium* spp. from asymptomatic and symptomatic lettuce in hydroponics." In **The 12th International Symposium on Biocontrol**

and Biotechnology, 11-13 December 2014 at KMITL Chumphon campus, Chumphon Thailand.

Tambong, J.T., de Cock, A.W.A.M., Tinker, N.A. and Lévesque, C.A. 2006. "Oligonucleotide array for identification and detection of *Pythium* species." **Applied and Environmental Microbiology** 72(4) : 2691-2706.

Tamura, K., Stecher, G., Peterson, D., Filipski A. and Kumar, S. 2013. "MEGA6: Molecular evolutionary genetics analysis version 6.0." **Molecular Biology and Evolution** 30 : 2725-2729.

Teymoori, S., Shahri, M.H., Rahnama, K. and Afzali, H. 2012. "Identification and pathogenicity of *Pythium* species on cantaloupe in Khorasan Razavi province of Iran." **Journal of Crop Protection** 1(3) : 239-247.

Thomson, T.B., Athow, K.L. and Laviollete, F.A. 1971. "The effect of temperature on the pathogenicity of *Pythium aphanidermatum*, *P. degaryanum* and *P. ultimum* on soybean." **Phytopathology** 61 : 933-935.

Tongaram, D. 2008. **Hydroponics : Principles of Manufacturing and Process Technology Business in Thailand.** (3rd ed.). Bangkok : Pimdee. (in Thai). 816 pp.

Utkhede, R.S., Lévesque, C.A. and Dinh, D. 2000. "*Pythium aphanidermatum* root rot in hydroponically grown lettuce and the effect of chemical and biological agents on its control." **Canadian Journal of Plant Pathology** 22 : 138-144.

Vallance, J., Le Floch, G., Déniel, F. and Rey, P. 2009. "Biocontrol management in soilless culture: impact of the antagonist *Pythium oligandrum* on native fungal populations." **Integrated Control of Plant Pathogens IOBC/wprs Bulletin** 43 : 189-192.

Van Beneden, S., Pannecoucque, J., Debode, J., De Backer, G. and Höfte, M. 2009. Characterisation of fungal pathogens causing basal rot of lettuce in Belgian greenhouses." **European Journal of Plant Pathology** 124 : 9-19.

Van der Plaats-Niterink, A.J. 1981. "Monograph of the Genus *Pythium*." **Studies in Mycology** 21 : 1-244.

Veit, S., Wörle, J.M., Nürnberger, T., Koch, W. and Seitz, H.U. 2001. "A novel protein elicitor (PaNie) from *Pythium aphanidermatum* induces multiple defense responses in carrot, arabidopsis, and tobacco." **Plant Physiology** 127 : 832-841.

Wang, P.H., Wang, Y.T. and White, J.G. 2003. "Species-specific PCR primers for *Pythium* developed from ribosomal ITS1 region." **Letters in Applied Microbiology** 37 : 127-132.

Watanabe, H., Taguchi, Y., Hyakumachi, M. and Kageyama, K. 2007. "*Pythium* and *Phytophthora* species associated with root and stem rots of kalanchoe." **Journal of General Plant Pathology** 73 : 81-88.

White, T.J., Bruns, T., Lee, S. and Taylor, J. 1990. "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics." p. 315-322. In Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. (Ed.). **PCR Protocols, A Guide to Methods and Applications**. San Diego : Academic Press.

Yakoby, N., Kobiler, I., Dinoor, A. and Prusky, D. 2000. "pH Regulation of pectate lyase secretion modulates the attack of *Colletotrichum gloeosporioides* on avocado fruits." **Applied and Environmental Microbiology** 66(3) : 1026-1030.

Yean, H.C., Atong, M. and Chong, K.P. 2009. "Lettucenin A and its role against *Xanthomonas campestris*." **Journal of Agricultural Science** 1(2) : 66-70.

Yigit, F. 2011. "Acibenzolar-S-methyl induces lettuce resistance against *Xanthomonas campestris* pv. *vitians*." **African Journal of Biotechnology** 10(47) : 9606-9612.

Yoshikawa, M. 1978. "Diverse modes of action of biotic and abiotic phytoalexin elicitors." **Nature** 275 : 546-547.

Yun, S.C. 2003. "Control of soybean sprout rot caused by *Pythium deliense* in recirculated production system." **Plant Pathology Journal** 19(6) : 280-283.

Zamski, E. and Peretz, I. 1996. "Cavity spot of carrots : II cell-wall-degrading enzymes secreted by *Pythium* and pathogen-related proteins produced by the root cells." **Annals of Applied Biology** 128(2) : 195-207.

Zhang, B.Q. and Yang, X.B. 2000 "Pathogenicity of *Pythium* populations from corn-soybean rotation fields." **Plant Disease** 84(1) : 94-99.

Zhang, J., Du, X., Wang, Q., Chen, X., Lv, D., Xu, K., Qu, S. and Zhang, Z. 2010. "Expression of pathogenesis related genes in response to salicylic acid, methyl jasmonate and 1-aminocyclopropane-1-carboxylic acid in *Malus hupehensis* (Pamp.) Rehd." **BMC Research Notes** 3(208) : 1-6.

Zhang, W. and Tu, J.C. 2000. "Effect of ultraviolet disinfection of hydroponic solutions on Pythium root rot and non-target bacteria." **European Journal of Plant Pathology** 106 : 415-421.

Zhang, Z., Schwartz, S., Wagner, L. and Miller, W. 2000. "A greedy algorithm for aligning DNA sequences." **Journal of Computational Biology** 7(1-2) : 203-214.

Zhao, Z.H., Kusakari, S.I., Okada, K., Miyazaki, A. and Osaka, T. 2000. "Control of *Pythium* root rot on hydroponically grown cucumbers with silver-coated cloth." **Bioscience, Biotechnology, and Biochemistry** 64(7) : 1515-1518.

Zipfel, C. 2009. "Early molecular events in PAMP-triggered immunity." **Current Opinion in Plant Biology** 12 : 414-420.

Zipfel, C. and Felix, G. 2005. "Plants and animals: a different taste for microbes?." **Current Opinion in Plant Biology** 8 : 353-360.

Zipfel, C., Kunze, G., Chinchilla, D., Caninard, A., Jones, J.D.G., Boller, T. and Felix, G. 2006. "Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation." **Cell** 125 : 749-760.

Zitnick-Anderson, K.K. and Nelson Jr., B.D. 2015. "Identification and pathogenicity of *Pythium* on soybean in North Dakota." **Plant Disease** 99(1) : 31-38.

Złotek, U. and Gawlik-Dziki, U. 2015. "Selected biochemical properties of polyphenol oxidase in butter lettuce leaves (*Lactuca sativa* L. var. *capitata*) elicited with DL-β-amino-n-butyric acid." **Food Chemistry** 168 : 423-429.

AUTHOR BIOGRAPHY

Name: Miss Chulalak Talubnak

Date of birth: 21 September 1984

Address: 26 Moo 1 Bankluay, Banmi, Lopburi Thailand 15110

Telephone: 086-413-1353

E-mail: chula_om@yahoo.com, chulalakkmitl@gmail.com

Education:

In 2005: Bachelor's degree at King Mongkut's Institute of Technology Ladkrabang and thesis title is "Isolation of fungi from bat guano, animal dungs and screening for enzyme production" and special problem title "Testing of new microbial fertilizer for the growth of kale (*Brassica oleracea*)

In 2010: Master's degree at King Mongkut's Institute of Technology Ladkrabang and thesis title is "Study on fungal diversity and biological control of anthracnose in vanilla

In 2010-2016: Doctor's degree in Biotechnology in Plant Pathology, International College from King Mongkut's Institute of Technology Ladkrabang. I'm a scholarship student from The Royal Golden Jubilee Ph.D. Program. The thesis title is "Induction of phytoalexin biosynthesis and its role against *Pythium* root rot of lettuce (*Lactuca sativa* L.) grown in hydroponics"

Training:

In 2007: Workshop for Disease Management and Good Agricultural Practice at Son La, Ha Noi, Viet Nam during 5-12 February 2007

In 2011: Participation in a mini-course on Molecular Basis of Plant Defense: Plant-Pathogen Interactions on 25-28 January 2011 at Center for Agricultural Biotechnology Kasetsart University, Kamphaeng Saen, Nakhon Pathom, Thailand.

In 2011: Student Exchange Research Program at School of Agriculture, Tokai University, Japan during 16 October – 16 November 2011 under the

scholarship from Tokai University. The research title is "Epidemiology, ecology and biology of plant pathogenic Streptomyces spp., particularly studies on disease of root tumor of melon" at laboratory of Professor Dr. Masahiro Yoshida

In July 2015-December 2015: student worker at John Innes Centre, Norwich Research Park, Norwich, UK

Conferences and Publications

Talubnak, C., Soytong, K. and Kanokmedhakul, S. 2007. Bioactive compounds from fungi against root rot of pomelo and tomato wilt. *In*: Proceedings of the International Conference on Integration of Science & Technology for Sustainable Development, 26-27 April 2007 at KMITL, Bangkok Thailand pp. 263-265.

Talubnak, C. and Soytong, K. 2007. Preliminary study on endophytic fungi from vanilla. *In*: International Conference on Engineering, Applied Sciences, and Technology. November 21-13, 2007 at The Swissotel Le Concorde, Bangkok, Thailand pp. 613-614.

Talubnak, C. and Soytong, K. 2008. Isolation of fungi from bat guano and animal dungs and screening for their enzyme production. Journal of Thai Phytopathology 22: 16-25.

Talubnak, C. and Soytong, K. 2009. Controlling anthracnose of *Vanilla pilifera* Holttum using *Emericella nidulans*. Journal of Science Ladkrabang 18(2): 25-34 (Publish in Thai).

Talubnak, C. and Soytong, K. 2010. Biological control of vanilla anthracnose using *Emericella nidulans*. Journal of Agricultural Technology 6(1): 47-55.

Talubnak, C., Koohakan, P., Parinthawong, N. and Jaenaksorn, T. 2010. Effect of the indigenous non-pathogenic *Pythium* spp. on growth of lettuce (*Lactuca sativa* L.) in hydroponic. *In*: 16th Asian Agricultural Symposium, 25-27 August 2010 at KMITL, Bangkok Thailand.

Talubnak, C., Parinthawong, N. and Jaenaksorn, T. 2014. In-vitro screening for non-pathogenic isolates of *Pythium* spp. from asymptomatic and symptomatic lettuce in hydroponics. *In*: The 12th International Symposium on Biocontrol and Biotechnology, 11-13 December 2014 at KMITL Chumphon campus, Chumphon Thailand.

Talubnak, C., Parinthawong, N. and Jaenaksorn, T. 2015. *In vitro* production of cell wall degrading enzymes by Pythium species isolated from asymptomatic and symptomatic lettuce root. *In*: 2nd International Symposium on Agricultural Technology, 1-3 July 2015 at A-One The Royal Cruise Hotel Pattaya, Thailand.

Talubnak, C., Parinthawong, N. and Jaenaksorn, T. Phytoalexin production of lettuce (*Lactuca sativa* L.) grown in hydroponics and its *in vitro* inhibitory effect on plant pathogenic fungi. Songklanakarin Journal of Science and Technology. (Accepted)

Talubnak, C., Schoonbeek, H.J., Parinthawong, N. and Jaenaksorn, T. Identification of diversity in *Pythium* spp. found in hydroponically-grown lettuce using morphological, pathogenic and molecular approaches. (In process of manuscript preparation)

Talubnak, C., Schoonbeek, H.J., Parinthawong, N. and Jaenaksorn, T. *Pythium* resistance in lettuce grown in hydroponics. (In process of manuscript preparation)