DIVERSITY OF *Cercospora canescens* AND RESISTANCE MECHANISMS OF MUNGBEAN TO CERCOSPORA LEAF SPOT

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ความหลากหลายของเชื้อ *Cercospora canescens* และกลไกการต้านทานของถั่วเขียวต่อโรคใบจุด

นางสาวธนา ใจชอบสันเทียะ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ธนา ใจชอบสันเทียะ: ความหลากหลายของเชื้อ *Cercospora canescens* และกลไกการต้านทานของถั่วเขียวต่อโรคใบจุด (DIVERSITY OF *Cercospora canescens* AND RESISTANCE MECHANISMS OF MUNGBEAN TO CERCOSPORA LEAF SPOT) อาจารย์ที่ปรึกษา: ศาสตราจารย์ ดร.ปิยะดา อลิฌาณ์ ตันตสวัสดิ์, 119 หน้า.

คำสำคัญ: โปรตีนที่เกี่ยวข้องกับการต้านทานโรค/เบต้ากลูคาเนส/ไคติเนส/พอลิฟีนอลออกซิเดส/การตอบสนองอย่างเฉียบพลัน/ความต้านทานในเนื้อเยื่อที่ห่างไกลบาดแผล

โรคใบจุด เป็นโรคที่มีความสำคัญในการผลิตถั่วเขียวในประเทศไทย โรคชนิดนี้ก่อให้เกิดความเสียหายอย่างมากต่อผลผลิตและคุณภาพของเมล็ดถั่วเขียว ดังนั้นการทดสอบระดับความต้านทานของพันธุ์/สายพันธุ์ถั่วเขียว และการพัฒนาวิธีการจัดการโรคจึงมีความจำเป็นเร่งด่วน การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อประเมินความหลากหลายของเชื้อสาเหตุโรคใบจุดในถั่วเขียว (*Cercospora canescens*) ศึกษากลไกความต้านทานของพันธุ์/สายพันธุ์ถั่วเขียวต่อการเข้าทำลายของโรคใบจุด และประเมินประสิทธิภาพของวิธีการปลูกถ่ายเชื้อด้วยเส้นใยของเชื้อ *C. canescens* ซึ่งการทดลองนี้แบ่งออกเป็น 3 ส่วน คือ 1) การศึกษาลักษณะทางสัณฐานวิทยา ความสามารถในการก่อโรค และความหลากหลายทางพันธุกรรมของ *C. canescens* ด้วยเครื่องหมาย randomly amplified polymorphic DNA (RAPD) 2) การตรวจสอบกลไกความต้านทานของถั่วเขียวพันธุ์/สายพันธุ์ต่าง ๆ ต่อโรคใบจุด และ 3) การประเมินประสิทธิภาพของวิธีการปลูกถ่ายเชื้อในสภาพห้องปฏิบัติการเปรียบเทียบกับการประเมินในสภาพไร่โดยใช้เส้นใยของเชื้อ *C. canescens* ในการศึกษาลักษณะทางสัณฐานวิทยา พบว่าอาหารสูตร mungbean leaf agar (MLA) และ lettuce leaf agar (LLA) มีสภาวะที่เหมาะสมต่อการส่งเสริมขนาดโคโลนีในการเจริญเติบโตของ *C. canescens* และพบว่าเชื้อแต่ละไอโซเลตมีลักษณะทางสัณฐานวิทยาที่แตกต่างกันเมื่อเลี้ยงบนอาหารต่างชนิดกัน การวิเคราะห์เครื่องหมาย RAPD แสดงว่าเชื้อ 20 ไอโซเลตมีความเหมือนกันทางพันธุกรรม 0.84-1.00 จากการวิเคราะห์ unweighted pair group method with arithmetic mean (UPGMA) สามารถแบ่งเชื้อออกได้เป็น 4 กลุ่มตามจังหวัดต้นกำเนิด จากการประเมินระดับความรุนแรงในการก่อโรคในถั่วเขียว พบว่า ถั่วเขียวสายพันธุ์ V4718 และ Super5 มีความต้านทานต่อไอโซเลตของเชื้อส่วนใหญ่ จากผลการวิจัยนี้ บ่งชี้ว่าเชื้อ *C. canescens* จากต่างแหล่งกันมีการเจริญเติบโต ลักษณะทางสัณฐานวิทยา พันธุกรรม และระดับความรุนแรงในการก่อโรคที่แตกต่างกัน ดังนั้นถั่วเขียวที่มียีนต้านทานแบบเดี่ยวอาจไม่มีประสิทธิภาพมากพอในการต้านทานต่อโรคชนิดนี้ในทุกพื้นที่ จากการทดสอบกลไกความต้านทานของถั่วเขียวหลากหลายพันธุ์/สายพันธุ์ พบว่าถั่วเขียวพันธุ์อ่อนแอสองสายพันธุ์ ได้แก่ EGMD-6D และ SUT1 มีระดับปฏิกิริยาของเอนไซม์ β-1,3-glucanase chitinase และ polyphenol oxidase (PPO) ต่ำกว่าในพันธุ์/สายพันธุ์อื่นในใบที่เชื้อเข้าทำลายในขณะที่สายพันธุ์ต้านทาน ได้แก่

V4718 และ Super5 มีปฏิกิริยาของเอนไซม์เหล่านี้ในระดับที่สูงกว่า นอกจากนี้ถั่วเขียวพันธุ์/สายพันธุ์ต่าง ๆ แสดงการเกิด hypersensitive response (HR) ในระดับแตกต่างกัน ซึ่งสอดคล้องกับระดับปฏิกิริยาของเอนไซม์ โดยการเกิด HR นั้น พบเฉพาะในสายพันธุ์ต้านทาน V4718 และ Super5 และไม่พบในพันธุ์/สายพันธุ์อ่อนแอหรือต้านทานปานกลาง อย่างไรก็ตาม จากการทดลองนี้ ไม่พบการกระตุ้นเพิ่มระดับปฏิกิริยาของเอนไซม์ β-1,3-glucanase chitinase และ PPO ในใบที่อยู่ด้านบนและด้านล่างซึ่งไม่ถูกเชื้อเข้าทำลาย รวมทั้งความต้านทานที่ถูกกระตุ้นในเนื้อเยื่อที่ห่างไกลบาดแผล (systemic acquired resistance; SAR) การวิเคราะห์ Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) ของโปรตีนรวมจากใบที่เชื้อเข้าทำลาย แสดงแถบโปรตีนขนาด 17 kDa ที่เพิ่มขึ้นเฉพาะในพันธุ์อ่อนแอ ได้แก่ EGMD-6D และ SUT1 และแถบโปรตีนขนาด 39 kDa ที่เพิ่มขึ้นในพันธุ์/สายพันธุ์ส่วนใหญ่หลังการปลูกถ่ายเชื้อ ยกเว้นสายพันธุ์ Super5 ที่มีการแสดงออกตั้งแต่ก่อนการปลูกถ่ายเชื้อ (0 วัน) การแสดงออกที่แตกต่างกันของโปรตีนเหล่านี้แสดงถึงกลไกความต้านทานที่แตกต่างกันระหว่างพันธุ์/สายพันธุ์ของถั่วเขียว จากการทดลองนี้บ่งชี้ว่า เอนไซม์ β-1,3-glucanase chitinase และ PPO มีบทบาทสำคัญต่อกลไกความต้านทานของถั่วเขียวต่อการเข้าทำลายของโรคใบจุด เพื่อพัฒนาวิธีการปลูกถ่ายเชื้อในระดับห้องปฏิบัติการโดยการใช้สายละลายแขวนลอยเส้นใยของเชื้อ *C. canescens* ทำการทดลองด้วยวิธีใบตัด (detached leaf inoculation) โดยใช้เส้นใยที่ได้จากเชื้อที่มีความรุนแรงในการก่อโรคมากที่สุดจำนวน 4 ไอโซเลต ได้แก่ ไอโซเลต SUT-1 SUT-4 PAK-1 และ PAK-2 ในการทดสอบระดับความต้านทาน/อ่อนแอของถั่วเขียวจำนวน 19 พันธุ์/สายพันธุ์ เมื่อเปรียบเทียบระหว่างพันธุ์/สายพันธุ์ พบว่าระดับความรุนแรงของโรคของถั่วเขียวแต่ละพันธุ์/สายพันธุ์ที่ประเมินโดยวิธีใบตัดให้ผลสอดคล้องกับการทดลองในสภาพไร่ โดยมีค่าสัมประสิทธิ์สหสัมพันธ์ เท่ากับ 0.822 ($p < 0.01$) จากวิธีการประเมินระดับความต้านทานทั้งในสภาพห้องปฏิบัติการและสภาพไร่ พบว่าถั่วเขียวสายพันธุ์ V4718 V4785 V4758 และ Super5 มีความต้านทานต่อโรคใบจุด ซึ่งจะเป็นประโยชน์ต่อการปรับปรุงพันธุ์ในอนาคต จากผลการทดลองนี้ บ่งชี้ว่าการประเมินระดับความต้านทานด้วยวิธีการปลูกถ่ายเชื้อลงบนใบตัดในระดับห้องปฏิบัติการโดยการใช้สายละลายแขวนลอยเส้นใยของเชื้อ *C. canescens* มีประสิทธิภาพในการกระตุ้นให้เกิดอาการของโรคและการระบุพันธุ์/สายพันธุ์ต้านทานเทียบเท่ากับการประเมินในสภาพไร่

สาขาวิชาเทคโนโลยีการผลิตพืช
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ลายมือชื่อนักศึกษา ชม ใจของสันเทียะ
ลายมือชื่ออาจารย์ที่ปรึกษา

TANA JAICHOPSANTHIA: DIVERSITY OF *Cercospora canescens* AND RESISTANCE MECHANISMS OF MUNGBEAN TO CERCOSPORA LEAF SPOT. THESIS ADVISOR: PROF. PIYADA ALISHA TANTASAWAT, Ph.D., 119 PP.

Keyword: Pathogenesis related proteins/β-1,3-glucanase/Chitinase/Polyphenol oxidase/Hypersensitive response/Systemic acquired resistance

Cercospora leaf spot (CLS) is a serious disease that poses a significant threat to mungbean cultivars in Thailand, leading to considerable damage, yield losses, and reduced seed quality. Assessing resistance levels in mungbean genotypes and developing effective disease management strategies are essential. The objectives of this study were to evaluate the diversity of *Cercospora canescens*, the causal agent of CLS, investigate the resistance mechanisms of mungbean genotypes against CLS, and determine the effectiveness of the laboratory inoculation using the mycelium of *C. canescens*. The experiment consisted of three main parts: 1) examination of the morphological characteristics, pathogenicity, and genetic diversity of *C. canescens* using randomly amplified polymorphic DNA (RAPD) markers, 2) investigation of the resistance mechanisms exhibited by various mungbean genotypes against CLS, and 3) evaluation of the effectiveness of the laboratory inoculation method compared with field evaluation by using the mycelium of *C. canescens.* In the morphological study of *C. canescens*, it was discovered that mungbean leaf agar (MLA) and lettuce leaf agar (LLA) provided optimal conditions for promoting colony diameter in the growth of *C. canescens*, and each isolate demonstrated variations in morphological traits when grown on different media. Analysis using RAPD revealed a genetic similarity ranging from 0.84 to 1.00 among the twenty isolates studied. Cluster analysis, employing the unweighted pair group method with arithmetic mean (UPGMA), classified these isolates into four distinct groups based on their location origins. The pathogenicity assessment demonstrated that mungbean genotypes V4718 and Super5 exhibited resistance against the majority of the isolates. The findings indicate that *C. canescens* isolates from various locations exhibit variations in growth, morphology, genetics, and virulence. Consequently, it suggests that a single resistance gene may not be effective across all geographical regions. The investigation of the resistance mechanisms of various mungbean genotypes to CLS showed that two susceptible genotypes, EGMD-6D and

SUT1 had lower levels of β-1,3-glucanase, chitinase, and polyphenol oxidase (PPO) activities in infected leaves. The resistant genotypes V4718 and Super5, on the other hand, had higher levels of β-1,3-glucanase, chitinase, and PPO activities. Moreover, the mungbean genotypes displayed varying levels of hypersensitive response (HR) symptoms, which was consistent with the enzyme activities. HR symptoms were observed in the resistant genotypes, V4718 and Super5, but not in the susceptible or moderately resistant genotypes. However, no evidence of systemic induction of β-1,3-glucanase, chitinase, and PPO activities in non-infected upper and lower leaves as well as systemic acquired resistance (SAR) was found in this study. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed the 17 kDa band that was only induced in the susceptible mungbean genotypes EGMD-6D and SUT1, and the 39 kDa band which was induced in most mungbean genotypes following inoculation, with the exception of Super5 that expressed this band since the pre-inoculation stage (0 DAI). The differential expression of these proteins suggests that resistance mechanisms differ among mungbean genotypes. This finding suggests that β-1,3-glucanase, chitinase, and PPO play important roles in the defense mechanisms of mungbean against CLS. To develop a laboratory inoculation method utilizing mycelium of *C. canescens*, the detached leaf inoculation was performed using mycelium obtained from the most virulent *C. canescens* isolates, namely SUT-1, SUT-4, PAK-1, and PAK-2 to assess the resistance/susceptibility levels of 19 mungbean genotypes. When comparing genotypes, the disease severity levels of mungbean genotypes evaluated by detached leaf inoculation were comparable to field inoculation with a correlation coefficient of 0.822 ($p < 0.01$). Both laboratory and field inoculation methods consistently identified genotypes V4718, V4785, V4758, and Super5 as CLS-resistant, which were useful for future breeding programs. These findings indicate that the laboratory *C. canescens* mycelium-inoculated detached leaf assay was just as effective as field inoculation at inducing disease symptoms and identifying resistant genotypes.

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Tana Jaichopsanthia

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LIST OF ABBREVIATIONS

AFLP	=	amplified fragment length polymorphism
CLA	=	carrot leaf agar
CRD	=	completely randomized design
CLS	=	cercospora leaf spot
DAI	=	days after inoculation
DAMPs	=	damage-associated molecular patterns
DLA	=	detached leaf assays
HR	=	hypersensitive response
LLA	=	lettuce leaf agar
MLA	=	mungbean leaf agar
OMA	=	oat meal agar
PAL	=	phenylalanine ammonia lyase
PAMPs	=	pathogen-associated molecular patterns
PDA	=	Potato Dextrose Agar
PDB	=	Potato Dextrose Broth
PIC	=	polymorphism information content
PM	=	powdery mildew
PO	=	peroxidase
PPO	=	polyphenol oxidase
PR	=	pathogenesis related
RAPD	=	random amplified polymorphic DNA
RFLP	=	restriction fragment length polymorphism
ROS	=	reactive oxygen species
SA	=	salicylic acid
SAR	=	systemic acquired resistance
SDS-PAGE	=	sodium dodecyl sulfate polyacrylamide gel electrophoresis
UPGMA	=	unweighted pair group method with arithmetic mean

CHAPTER 1
INTRODUCTION

1.1 Background problem and significance of the study

Mungbean (*Vigna radiata* (L.) Wilczek) is an important legume crop in South and Southeast Asia, as well as Thailand. It has short life cycle (approximately 60 days) and has good performance in heat and drought conditions. Mungbean roots can fix nitrogen from atmosphere through symbiosis with rhizobia. Therefore, it is suitable for use as rotate crop and soil amelioration, which can reduce nitrogen requirement. Moreover, mungbean is a nutrient source for human because it has high nutrition such as proteins, fat, vitamins and minerals e.g., potassium, phosphorous and calcium (Prabhavat, 1991). Thailand is one of the largest mungbean producers in Southeast Asia with approximately 0.2 million hectares of cultivated area and 0.3 tons of yield (Nair and Pepijn, 2020). It can grow during all seasons and in all parts of Thailand, but rainy season is recommended. Generally, mungbean suffers from many diseases but Cercospora leaf spot (CLS) which is caused by *Cercospora canescens* is a serious threat to mungbean, causing 50-70% yield reduction, and up to 96% losses if there is no disease control (Lal et al., 2001; Kaur et al., 2004; Chand et al., 2012). This disease spreads widely in Asia such as India, Bangladesh, the Philippines, Thailand and all parts of the world (Pandey et al., 2009).

There are several studies conducted on CLS characterization and diversity evaluation. Daub and Briggs (1983) reported that *Cercospora* spp. Produce cercosporin toxin, which generates singlet oxygen when exposed to plant cells, leading to membrane breakdown and cell death. Therefore, cercosporin production can be used for identification of *Cercospora* spp. together with morphological characters such as mycelium, conidiophores and conidia. Although cercosporin toxin may enhance virulence of the fungus but it is not produced by all *Cercospora* species, depending on isolates and environment (Wang et al., 1998; Dunkle and Levy, 2000). Moreover, Joshi et al. (2006) reported that several isolates of *C. canescens* have the same morphological characteristics even though collected from different locations.

Therefore, identification of *C. canescens* by their morphology and cercosporin production may not be accurate enough. The use of molecular markers such as amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD), will significantly improve the accuracy of population genetics study of *Cercospora* pathogen. RAPD markers have been widely used for evaluating diversity of many fungal species due to its simple, yielding a large number of polymorphic loci, and cost-effective features (not require any prior information of DNA sequences like other markers such as AFLP or RFLP). Joshi et al. (2006) evaluated the genetic diversity among *C. canescens* isolates collected from different locations in India, which had different morphological and cercosporin production using RAPD markers and ITS region of ribosomal DNA (rDNA). The RAPD data revealed that there were three major clusters according to location. However, the similarity coefficient ranging from 0.55-0.88 indicated no 100% similarity across all isolates studied. In addition, RAPD markers have been effectively used to evaluate the genetic diversity of *Cercospora* spp. The pathogen characterization and diversity based on these methods is one of the initial steps for breeding resistant mungbean varieties against CLS.

Use of resistant varieties is the most effective strategy for controlling plant diseases. Pradhan et al. (2015) evaluated resistance among rice lines/varieties having different resistance genes against 8 isolates of *Xanthomonas oryzae* pv. *oryzae* (Xoo), causal agent of bacterial blight. The results showed that 3 gene pyramided lines had higher resistance levels to all isolates than 2 genes pyramided lines and susceptible variety, respectively. The use of several pathogen isolates obtained from pathogen characterization and virulence determination may be helpful for selecting broad-range disease resistant plants for breeding programs. Plants possess two different resistance mechanisms; preformed and induced defense against diseases. Plant tissues contain various preformed structural barriers such as lignin, cutin, waxes, trichomes, and several chemicals such as alkaloids, phenolics, and saponins for defense against pathogen attachment, invasion and infection. Mir et al. (2012) found that thickness of upper cuticle, epidermis, and stomatal size of mulberry (*Morus* spp.) played an important role in *C. moricola* infection and development. Similarly, Ahmed (1949) found that resistant mungbean genotype against *Cercospora* spp. had lower stomatal

frequency and smaller pore size than susceptible genotype. Moreover, high phenol contents were found in resistant mungbean genotypes (Sunil and Benagi, 2013). While induced defense consists of induction of hypersensitive response (HR) which causes plant cell death at the site of infection and also systemic acquired resistance (SAR) in plant parts distant from the local site of infection. Induced defense both locally and systemically increased the production of several defense proteins such as pathogenesis related (PR) proteins and phenylalanine ammonia lyase (PAL) etc. (Jones and Dangl, 2006; Walters et al., 2009). These proteins were found to be associated with resistance to several fungal diseases of legumes (Vallad and Goodman, 2004; Abdel-Kader et al., 2013). Saravanakumar et al. (2007) reported that inoculating mungbean with *Pseudomonas fluorescens* and chitin could increase the accumulation of PAL, peroxidase (PO), polyphenol oxidase (PPO), two PR proteins (chitinase and β-1,3-glucanase) and phenolic compounds. Recently, Koche and Chaudhary (2019) reported that resistant mungbean genotype had higher accumulation of PR-proteins (chitinase and β-1,3-glucanase), phytoalexin (genistein) and PAL under both field and in vitro conditions after spraying with cercospora cell wall elicitor derived from *C. canescens*. Mercier and Kuć (1996) observed a reduction in the number of CLS lesions caused by *C. carotae* when newly emerged carrot leaves were subjected to secondary inoculation. This finding indicated that prior inoculation with *C. carotae* induced systemic resistance in carrot plants. The induced systemic resistance resulted in fewer CLS lesions compared to the control group, suggesting that the initial exposure to *C. carotae* triggered a protective response throughout the entire carrot plant. For these reasons, induction and accumulation of biochemical compounds may indicate the resistance status of mungbean varieties. However, these resistance mechanisms may be different due to diverse resistance genes from different sources. Therefore, study of resistance mechanisms in resistant mungbean varieties/lines is crucial. CLS and powdery mildew resistant lines (Super) derived from double cross of three resistant lines; V4718, V4758, and V4785 has higher resistance level which is more effective than those of parental lines. Study of resistance mechanisms in these resistant lines will be useful for developing mungbean resistant varieties in future breeding program.

In order to facilitate the development of resistant mungbean varieties, it is essential to establish a laboratory assay that is rapid, highly efficient, and dependable for screening a substantial number of mungbean genotypes for their resistance to CLS. While most screening methods employ conidia as an inoculum, the production of conidia on culture media is generally limited and may not be generated in certain isolates. Furthermore, the preparation of conidial inoculum is a time-consuming process, especially when significant quantities of inoculum need to be prepared from numerous culture plates, and sporulation capacity varies among different fungal isolates. Although mycelia demonstrate faster growth and larger quantities, their utilization as an inoculum has been limited to a few studies only (Singh et al., 2002; Chen and Wang, 2005). Utilizing mycelia as an inoculum for screening assays would simplify the process of inducing symptoms and preparing the inoculum. Additionally, the detached leaf assay proves to be an efficient method for swiftly screening a substantial number of genotypes and assessing resistance under controlled conditions. Nonetheless, the utility of the detached leaf assay is contingent upon its robust correlation with the results obtained from field evaluations. Therefore, the evaluation of the effectiveness of the laboratory inoculation method utilizing *C. canescens* mycelium, compared to field evaluation is required. This evaluation is crucial because it allows for a direct comparison between the laboratory-based method and the field evaluation. By examining the correlation between the two approaches, researchers can determine the reliability and relevance of the laboratory method in predicting the performance of the genotypes in real field conditions.

1.2 Research objectives

1.2.1 To study morphological characters, pathogenicity, and genetic diversity using RAPD markers of *C. canescens*, causing CLS in mungbean.

1.2.2 To study resistance mechanisms of various mungbean genotypes against CLS.

1.2.3 To evaluate the effectiveness of the laboratory inoculation method compared with field evaluation by using mycelium of *C. canescens*.

1.3 Research hypotheses

1.3.1 *C. canescens* are genetically diverse according to their origin which may differ in their morphology on culture media, their pathogenicity on different mungbean genotypes, and their genetics based on RAPD markers.

1.3.2 Resistant mungbean varieties may exhibit their resistance status by accumulation of β-1,3-glucanase, chitinase and PPO enzymes as well as eliciting HR cell death and SAR.

1.3.3 Different resistant mungbean genotypes may employ diverse resistance mechanisms which may be useful when pyramided into the same mungbean genotype.

1.3.4 The detached leaf assay using *C. canescens* mycelium exhibits a strong correlation with the results derived from field evaluations.

1.4 Research scope

This study focused on CLS resistance mechanisms of mungbean. The experiments were divided into 3 parts. The first experiment was carried out to isolate *C. canescens* from infected mungbean leaves collected from 4 provinces of Thailand (Nakhon Ratchasima, Nakhon Sawan, Phichit and Phetchabun provinces), and to study morphological characters, pathogenicity and genetic diversity using RAPD markers. The second experiment was carried out to study resistance mechanisms against CLS through HR cell death and accumulation of β-1,3-glucanase, chitinase and PPO enzymes locally among 6 mungbean genotypes (EGMD-6D, SUT1, CN84-1, B2, V4718 and Super 5), and systemically among 4 mungbean genotypes (EGMD-6D, CN84-1, V4718 and Super 5). And the last experiment was to compare CLS severity scores and resistance/susceptibility levels of 19 mungbean genotypes from detached leaf inoculation using mycelium from the most virulent *C. canescens* isolates with field inoculations.

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CHAPTER 2
LITERATURE REVIEW

2.1 Mungbean and their importance

Mungbean (*Vigna radiata* (L.) Wilczek var. *radiata*) is an important legume crop in South and Southeast Asia, as well as Thailand. It has short cultivation duration (approximately 60 days), and good performance in heat and drought conditions. Mungbean root can fix nitrogen from atmosphere through symbiosis with rhizobia. Therefore, it is suitable for use as rotated crop and for soil amelioration, which can reduce nitrogen requirement. Moreover, mungbean is a nutrient source for human because it has high nutrition such as proteins, fat, vitamins and minerals e.g., potassium, phosphorous and calcium (Prabhavat, 1991). Globally, there are four main segments of mungbean market including dry grain (common in South Asia, Kenya and China), bean sprouts (common in East Asia and Southeast Asia), vermicelli and starch (common in East and Southeast Asia), and bean paste and sweets (Asia) (Nair and Pepijn, 2020).

India is the largest producer estimated at 3.8 million hectares in 2016, with an average yield of 0.4 tons/hectares. Other large producers are Myanmar, China, Kenya, and Thailand. An average yield of mungbean around the world is 0.7 tons/hectares but yield can reach up to 1.9 tons/hectares from using elite varieties with good management. Thailand is one of the largest mungbean producers in Southeast Asia, about 0.2 million hectares production with 0.3 million tons of yield (Nair and Pepijn, 2020). Mungbean can grow in all seasons of the year and at all parts of Thailand, but growing in rainy season is recommended. Generally, mungbean suffers from several diseases such as mungbean yellow mosaic disease (MYMD), powdery mildew (PM), Cercospora leaf spot (CLS) and root rot. These plant diseases can reduce mungbean yield up to 10-100% (Rana et al., 2016). Several Thai certified varieties; Chai Nat 36, Khampang Saen 1 and Khampang Saen 2 are widely grown by farmers because they have high yield. However, an average yield of mungbean in Thailand is still low compared to the neighboring countries and this is not enough to keep with domestic requirement.

2.2 Cercospora leaf spot disease and its importance

Cercospora leaf spot disease in mungbean is caused by the *C. canesens*. It was first reported in 1960 by Munjal et al. from Delhi, India. It is a serious disease to mungbean which can cause 50-80% yield reduction depending on stage of infection. It can incur up to 96% yield losses in uncontrol conditions (Lal et al., 2001; Kaur et al., 2004; Chand et al., 2012). Moreover, it also causes yield losses in other crops; 20% in sesame, 25-43% in groundnut, 36-42% in cowpea, 50% in sugar beet and up to 100% in maize (Schneider et al., 1976; Ferry et al., 1977; Latterell and Rossi, 1974; Shane and Teng, 1992; Waliyar et al., 2000; Bhale et al., 2001). This disease spreads widely in Asia such as India, Bangladesh, the Philippines, Thailand and all parts of the world (Pandey et al., 2009).

2.2.1 Classification and morphological characters

C. canescens is a fungus in the genus *Cercospora* and is classified according to the taxonomy as follows (Braun et al., 1999)

Kingdom: Fungi

Phylum: Ascomycota

Class: Dothideomycetes

Order: Capnodiales

Family: Mycosphaerellaceae

This fungus is soil and air borne in nature, poor sporulating pathogen. Colonies of *C. canescens* are amphigenous and dark. Mycelium internal is smooth hyphae branched with 2-4 micron in width, septate and brownish or green-hyaline. Conidiophore of *C. canescens* is mostly straight to curved, pale to medium dark brown color, rarely branched, rather loose fascicles. Conidia are hyaline or rarely sub-hyaline, mostly acicular and with truncate base, acute to subacute tip, 2.5-6 microns in width (Phengsintham, 2013).

2.2.2 Disease cycle and habitat

The disease cycle of *Cercospora* spp. begins when conidia are drop onto plant leaves by wind or water. The favorite weather is wet, warm, and humid. Conidia germinate well during moderate to warm temperatures, with free moisture. The conidia

germinate until the end of the growing season. At the end of the growing season, conidia persist in weeds and plant leaf debris for 1-4 months and serve as sources of primary inoculum in the next season (Pool and McKay, 1916). Amin and Singh (1987) reported that *C. canescens* persists on infected plant debris. Rangaswami and Prasad (1961) stated that the natural soil is more conducive to survival of *C. capsica*, therefore it can survive on infected plant tissues in soil. *C. canescens* favorite temperature is about 22.5-23.5°C with 79-85% relative humidity (Sad and Singh, 1984). It is more destructive in humid tropical climate which can cause heavy defoliation at optimum temperatures of 25-30°C and 90-100% relative humidity (Shahbaz et al., 2014). Skaracis et al. (2010) reported that more than 90% humidity, 27-30°C day temperature and 17°C night temperature are favorite conditions for CLS disease in sugar beet. Host of this fungus is wide range. Legume family (Fabaceae) is the specific host, especially *Phaseolus* spp. *and Vigna* spp.

2.2.3 Disease symptoms

Conidia infection can cause spots on foliage with an average diameter of 3-15 mm. The symptoms are at first brown spot and then turn to grey with reddish brown margin, fruiting amphigenous and more abundant on lower surface (Munjal et al., 1960). The disease mostly infects plants at 30-40 days old and causes premature defoliation which affects size of pods and grains (Grewal et al., 1980). *C. canescens* can cause brown leaf spots on *V. catjang*, which turn to grey and dirty white with 5-10 mm wide subcircular to irregular shape. Symptoms are present on leaves, stems, pods, and cotyledons (Vasudeva, 1963). Vakili (1977) reported that symptoms of *C. canescens* and *C. cruenta* on cowpea are 8-15 mm spots with round shape, orange to light brown, turn grey when sporulating, and the lesions also appeared on petioles, peduncles and stems. Uddin et al. (2013) found that *C. canescens* symptoms on mungbean appeared as water-soaked spot on leaves at initial stage of infection, and older spots may merge together, causing large dead area on the infected leaves. Sometimes, heavy infections of *C. canescens* can cause unshaped and wrinkled leaves. *Cercospora* spp. can produce a non-selective toxin called cercosporin for successful pathogenesis of their hosts (Daub and Ehrenshaft, 2000). Cercosporin toxin is photoactivated and membrane sensitizer which produces singlet oxygen to kill the plant cells and tissues, by breaking down membrane and cells (Daub and Briggs, 1983).

2.3 Characterization of *Cercospora* spp. the causal pathogen of leaf spot in plants

2.3.1 Growth and sporulation of *Cercospora* spp. in culture media

Spore multiplication is a fundamental requirement for screening of several mungbean germplasms by artificial inoculation technique. However, growth and sporulation of *Cercospora* spp. in laboratory still face several problems such as slow growth and poor sporulation (Vathakos and Walters, 1979). Nevertheless, growth and sporulation of this fungus in culture media were subjected to pathogen isolates and several factors such as temperature, light, and nutrients (Chen et al., 1979; Cooperman and Jenkins, 1986). Potato Dextrose Agar (PDA) and potato Dextrose Broth (PDB) are the standard culture media for phytofungus because it consists of potato infusion and dextrose which are nutrient base for most fungi (Verma and Agnihotri (1972). Maximum mycelial growth of *C. solani*, *C. sorghi* and *C. nicotianae* was obtained when cultured in PDB (Lakshminarayana, 1981; Dinesha, 1984; Mallappa, 2007). Khandar et al. (1985) and Jamadar (1988) also reported that PDA supported mycelial growth of *C. canescens* but did not support sporulation. Similar result was reported by Poornima (2010) who found that *C. beticola* had maximum mycelial growth on PDA. Sathyaprasanth (2004) reported that *C. kikuchii* had maximum radial growth on malt extract agar. Verma and Agnihotri (1972) reported that *C. cruenta* and *C. beticola* had maximum vegetative growth on Czapeck's Dox agar and carrot leaf decoction media. Most of these studies found growth induction by PDA, PDB, malt extract agar, Czapeck's Dox agar, and carrot leaf decoction media. Using some of these media, Mishra and Bhattacharya (2002) observed that natural or semi-synthetic media (carrot leaf decoction and oatmeal extract agar) was best for *C. canescens* growth and sporulation. Similar with radial growth of *C. nicotianae* which was best on host extract dextrose agar (Mallappa, 2007). Moreover, it had high sporulation when placing the mycelium on host leaf surface (Poornima, 2010). Growth induction and sporulation may depend on several factors such as pathogen species, isolates and nutrient components etc.

Growth induction and sporulation may require combinations of media and temperature (Chen et al., 1979; Cooperman and Jenkins, 1986). The optimum temperature for growth of *C. canescens* and *C. cruenta* on agar media was 24-25°C (Ekpo and Esuruoso, 1978). Khandar et al. (1985) found the maximum growth of

C. canescens at 25°C, and it declined at 30°C. For *C. zebrine*, the optimal temperature was 24°C when incubated on solid media (Berger and Hanson, 1963). The temperature for maximum growth of *C. beticola* was 26°C (Dange and Patel, 1968). Sathyaprasanth (2004) reported that *C. kikuchii* had maximum growth at 25°C, little growth at 10°C and could not grow at 5°C.

2.3.2 Isolation and pathogenicity of *Cercospora* spp.

For studies on isolation and pathogenicity, *Cercospora* spp. causing leaf spot was successfully isolated by standard tissue isolation technique from infected plant leaves and assayed for their pathogenicity on host plants (Siddaramaiah, 1986; Jamadar, 1988). *C. beticola* isolated from infected spinach by standard tissue isolation technique was proved for its pathogenicity by smearing the mycelial bits on 30 days old leaves that the typical symptoms appeared at 7 days after inoculation (DAI) (Poornima, 2010).

Chand et al. (2012) isolated new variant of *C. canescens* (isolate NFCCI-2370) from Meha mungbean cultivar and the symptoms on susceptible mungbean cultivars were dry necrotic spots, grey and did not show reddish brown margin. Similar with Chan et al. (2013), *C. canescens* was isolated from mungbean infected leaves by using technique of Chupp (1953). The fungus spores were identified under the light microscope, collected and placed on PDA. The fungus was identified by its morphological characters with cercosporin production, typical conidia and conidiophores (Ellis and Martin, 1882; Daub, 1982). Pure isolate was proved for the pathogenicity on 35 days old Kopergaon mungbean cultivar.

2.4 Genetic diversity of *Cercospora* spp.

The focus of plant pathology study is to identify the causal agent, prove pathogenicity and develop a control strategy. Assessment of genetic variation within and among pathogenic populations called population genetics, is useful for inference about the origin, sources of inoculum and biology of plant pathogens which are the important basis for disease management. Numerous genetic/molecular and morphological markers are available for studying the population genetics of plant diseases. Among these markers, molecular markers are commonly used due to their

ability to unveil fungal genetics accurately. Molecular markers provide a reliable method for species identification by analyzing genetic sequences. They offer the advantages of distinguishing closely related species that may possess similar morphological features, which are helpful for understanding the functional behavior with the morphological and physiological characteristics (Chattopadhyay et al., 2017).

Nowadays, *Cercospora* spp. identification were done based on the fungus morphology and cercosporin toxin production (Daub, 1982), but genetics are not given much attention. Although cercosporin compound may enhance virulence but it is not produced by all species, often specific to isolates and environmental conditions (Wang et al., 1998; Dunkle and Levy, 2000). Therefore, the use of molecular markers such as amplified fragment length polymorphisms (AFLPs), restriction fragment length polymorphism (RFLP), and random amplified polymorphic DNA (RAPD) will significantly improve the accuracy of population genetics in *Cercospora* pathogen. RAPD markers have been widely used for evaluating diversity of many fungal species due to its simple, yielding a large number of polymorphic loci, and cost-effective features (not require any prior information of DNA sequences like other markers such as RFLP). Dunkle and Levy (2000) reported the analyses of AFLPs and internal transcribed spacer (ITS) sequence data in *C. zeae-maydis* (causal agent of gray leaf spot diseases) and found that *C. zeae-maydis* isolates were designated as group I and group II by their original location (Wang et al., 1998). Similarly, Goodwin et al. (2001) found that *C. zeae-maydis* groups I and II had different internal ITS sequences due to its origin from two species. Okori et al. (2003) used AFLP and RFLP to analyze the genetic diversity of *C. zeae-maydis* isolates collected from different countries, compared with isolates from Zimbabwe and the United States of America (USA). The AFLP data revealed that there are two major clusters; cluster II containing the isolates from African and USA and cluster I consisting of isolates from USA similar to RFLP data. Bradley et al., (2012) studied on genetic diversity of 62 *C. sojina* isolates from Brazil, China, Nigeria, and USA using AFLP markers. The clustering revealed two major clusters and seven sub-clusters. Joshi et al. (2006) evaluated genetic diversity of 11 *C. canescens* isolates collected from different locations in India having different morphological characters and cercosporin productions using RAPD markers and ITS region of ribosomal DNA (rDNA). The RAPD data reveal that there were three major clusters according to locations.

However, similarity coefficient ranging from 0.55-0.88 indicating no 100% similarity across all isolates studied. Similarly, the ITS data revealed that there were dissimilar isolates even though they were collected from the same host plant and location. In addition, RAPD has been effectively used to evaluate the genetic diversity of *Cercospora* spp. For example, Almeida et al. (2005) reported that RAPD could effectively cluster 72 isolates of *C. kikuchii* causing Cercospora leaf blight in soybean into 7 groups. Furthermore, RAPD could be efficiently applied for subsequent verification of *Cercospora* strains selected for mycoherbicide development for control of purple nutsedge (*Cyperus rotundus*) (Inglis et al., 2001). These results suggested that RAPD markers were suitable for determining the genetic diversity of *C. canescens* isolates. Due to its multi locus nature, RAPD markers can produce higher effective multiplex ratio (EMR) and marker index (MI) than single locus marker (Belaj et al., 2003). Higher EMR and MI values indicated marker appropriateness for better analysis of genetic diversity studies (Singh et al., 2014).

2.5 Cercospora leaf spot disease management

Recently, there are several methods to manage CLS disease. For example, cultural and physical practices, use of resistant varieties, use of synthetic fungicides and natural products.

2.5.1 Cultural and physical practices

Prevention and management of plant diseases by cultural and physical practices is a very effective method, especially in frequently found diseases. For example, cleaning seeds, pruning and selection of planting location, which are methods that farmers can do on their own by using simple tools or labor. An advantage of using cultural and physical practices is cheap, effective and practical, without harmful chemical residues in products and environment. In mungbean, these methods were effective to manage foliar and root rot disease under field conditions. Sharma et al. (2011) reported that using crop rotation, field sanitation, crop debris and weed removal around the field could reduce CLS incidence in mungbean. However, cultural and physical practices may be ineffective when occurrence of disease outbreak.

2.5.2 Biological methods

Few studies have been conducted on biological agents and botanical fungicides to manage this disease. Krishna (2005) found that foliar spray of chitinolytic *B. circulans* GRS 243 and *Serratia marcescens* GPS 5 could reduce CLS of groundnut. In 2008, Galletti et al. reported that *Trichoderma* can induces the systemic resistance of sugar beet to *C. beticola*. Poornima (2010) reported that *P. fluorescens* showed good results for controlling CLS of spinach. Derbalah et al, (2013) also reported that *P. fluorescens, B. pumilus, B. Subtilis* 1 and *B. Subtilis* 3 can control CLS in sugar beet. Hemachandra (2007) reported that *Allium* sp. could inhibit 100 % of mycelial growth. *Tridax procumbens* extract at 20 percent was best for *C. beticola* inhibition (Poornima, 2010). In mungbean, Uddin et al. (2013) reported that *Azadirachta indica* leaf extract was effective to reduce CLS disease incidence and severity.

2.5.3 Use of synthetic fungicides

Most studies evaluated fungicide efficacy in reduction of disease severity, disease incidence, and crop yield. Kapadiya and Dhruj (1999) reported that difenconozole could control CLS disease in mungbean by reducing disease intensity, thereby resulting in maximum yield in field condition. Khunti et al. (2002) found that hexaconazole and penconazole were effective to control this disease, whereas sulphur, propiconazole and carbendazim were moderately effective. Minimum CLS disease intensity (16.72) and maximum disease control (64.58%) in mungbean were found by using topsin-m followed by Bavistin (Kaur et al., 2004). More studies conducted on using fungicides to control this disease are shown in Table 2.1.

Table 2.1 Efficacy of fungicides for the control of Cercospora leaf spot diseases in mungbean.

Fungicides	Methods and frequency of application	Efficacy Impact (Disease reduction and yield)
Hexaconazole 5 EC (0.005%),	First foliar spray when disease appeared, repeated after 15 days	59% and 779 kg/ha yield, while 395 kg/ha in check (Khunti et al., 2005)
Difenconazole (25% EC) (0.0125 %)	Foliar spray after disease initiation, repeated twice at 15 DAS	61% (Kapadiya and Dhruj, 1999)
Carbendazim (0.10%)	First foliar spray when disease appeared, repeated after 15 days	61% and 690 kg/ha yield at 70 DAS (Khan et al., 2005)
Hexaconazole (0.1%)	Single foliar spray when disease appeared	81% with 752 kg/ha yield, while 525 kg/ha in check (Veena et al., 2014)
Carbendazim (0.1 %),	Single foliar spray when disease appeared	77% (Singh et al., 2013)
Metalaxyl (1.2 kg/ha)	Foliar spray after 50 days of sowing before disease appearance	55% (Shahbaz et al., 2014)
Propiconazole (0.10%),	Foliar spray after first disease appearance	86% with 908 kg/ha yield, while 746 kg/ha in check (Akhtar et al., 2014)
Carbendazim (0.1%) + Difenconazole (0.02 %)	First foliar spray when disease appeared, repeated after 15 DAS	82 and 72% leaf infection and 76 and 96% pod infection with 825 and 808 g/9 m^2 yield during 2009 and 2010, respectively, while in check yields were 691 and 680 g/9 m^2 (Bhat et al., 2015)
Carbendazim (12%) + Mancozeb (63%) 75% WP	First foliar spray when disease appeared, repeated after 15 DAS	70% and 990 kg/ha yield, while decreased in check (570 kg/ha) (Yadav et al., 2014)

Source: Modified from Pandey et al. (2018).

2.5.4 Use of resistant varieties

Although chemical control is the most popular method to control CLS disease, use of mungbean resistant varieties is an effective method with cheapest, practical and effective features for controlling CLS. The pathogen development for tolerance to resistant varieties is not acute because it takes long time and may affect its important metabolism processes. There are a lot of resistant status studies of mungbean and may be exploited in breeding program for development of CLS resistant mungbean varieties in the future. In 1989, Mathur et al. evaluated 297 mungbean cultivars against isolates of *C. canescens* and *C. cruenta* under field condition, but there was no mungbean resistant genotype observed. Wongpiyasatid et al. (1999) reported that 2 from 10 mutant lines (M5-22 and M5-25) obtained from chemical substances and irradiation were resistant to *C. canescens* isolated from Chiangmai under greenhouse conditions.

Sindhan et al. (1999) found that 8 mungbean genotypes (EC27515, P281, P19, P334, P248, ML-1, E3-8 and H76- 14) were resistant, 28 genotypes were moderately resistant and 223 genotypes were moderately to highly susceptible against CLS. Iqbal et al. (2004) evaluated 58 mungbean genotypes under field condition with artificial inoculation of *C. canescens,* and found 12 mungbean genotypes as highly resistant, 15 genotypes as resistant, and 17 genotypes as moderately resistant. Saifulla et al. (2005) found 2 mungbean genotypes (LGG-460 and Panthmung 40) as CLS resistant lines. Other CLS resistant lines/varieties were also identified by several studies (Table 2.2).

Table 2.2 Resistant genotypes against Cercospora leaf spot in mungbean.

Countries where screening conducted	No. of genotypes evaluated	Resistant genotypes (R, Resistant; HR, highly resistant)
Taiwan	4000	R: V1471, V2757, V2773, V4718, V5036 (Hartman et al., 1993)
Thailand	27	R: M5-22 and M5-25 (Wongpiyasatid et al., 1999)
Pakistan	58	R: NCM 255-2, NCM 257-6, ML-267, NCM 251-1, NCM 259-2, NCM 251-13, NCM 257-2, NM-92, NCM 251-12, VC-3960-A88, NCM 257-10, NCM-209, Mung-6 C1/94-4-19, VC 3960-A89 HR: BRM-188, NM-98, C2/94-4-42, 98-cmg-003, NM-2, NM-1, 98cmg-018, Basanti, CO-3, PDM-11, VC3960-88, BARIMung-2 (Iqbal et al., 2004)
India	696	R: ML5, 443, 453, 515, 610, 611, 613, 682, 688, 713, 728, 735,746, 759 and 769 (Singh et al., 2004)
India	170	No infection: Vigna aconitifolia, V. glabrascence, V. sublobata, V. umbellata and a mutant PBM. R: 90 genotypes including PANT M103, PANT M3, PUSA 105, ML 613, PANT M2, ML 173, ML 347, ML 561, PANT M4, PDM 11 (Marappa, 2008)
India	65	R: GM-02-08, GM-02-13, GM-03-03 HR: LGG-460 (Yadav et al., 2014)
India	113	R: ML-5, ML-4, HUM-9, HUM-4, HUM-1, SM-9-124, LGG-450, and SM-9-107 (Singh and Singh, 2014)
India	136	R: 52 genotypes HR: 1224-52 and 12404 (Zhimo et al., 2013)
India	-	R: AKM 9910, IPM 02-5, ML 1299 and SML 668 (Akhtar et al., 2014)
India	63	MR: KMP-13 (Bhaskar, 2017)

Source: Modified from Pandey et al. (2018).

2.6 Mechanisms of resistance to *Cercospora* spp.

CLS resistance is controlled by a single dominant gene, a single recessive gene or quantitative genes (Lee, 1980; AVRDC, 1980; Chankaew et al., 2011). For resistance to diseases, there are two resistance mechanisms in plants; preformed resistance and induced defense. Plant tissues contain various preformed structural barriers such as lignin, cutin, waxes, trichomes and chemicals such as alkaloids, phenolics and saponins for defense against pathogen attachment, invasion and infection, and induced resistance. Kaur and Dhellon (1988) reported that groundnut varieties which were resistant to black leaf spot disease (*Cercosporidium personatum*) had higher palisade index, trichome, thick epidermis and higher calcium oxalate crystals compared with susceptible variety. In 2012, Mir et al. found that thickness of upper cuticle, epidermis and stomatal size of mulberry (*Morus* spp.) played an important role in *C. moricola* disease development. Similarly, Ahmed (1949) found that mungbean genotype resistant to *Cercospora* spp. had lower stomatal frequency and smaller pore size than those of susceptible genotype. Moreover, high phenol contents were found in resistant mungbean genotypes (Sunil and Benagi, 2013).

Induced defense consists of induction of hypersensitive response (HR) which causes plant cell death at the site of infection and also induced systemic acquired resistance (SAR) in plant parts distant from the local site of infection. HR is a biochemical defense mechanism which is mediated by an intracellular program, occurs only in incompatibility case of host and the pathogen (Staskawicz et al. 1995; Dempsey et al. 1999; Jain and Khurana, 2018). During a pathogen infection, plant cells recognize the presence of the pathogen through specific receptors that detect pathogen-associated molecular patterns (PAMPs) or the products of pathogen effectors. This recognition initiates a cascade of signaling events within the plant cells, leading to the activation of defense mechanisms. In HR, the plant mounts a rapid and localized response at the site of infection. The plant activates a programmed cell death response in the infected cells, effectively killing them. This localized cell death restricts the spread of the pathogen, preventing it from colonizing further tissues. The HR response also involves the production of toxic compounds and the reinforcement of cell walls in adjacent cells to further limit pathogen spread. HR cell death also can be seen with the naked eye or with the microscope. In 2008, Bernard et al. evaluated the

reaction of *Arabidopsis thaliana* to *Hyaloperonospora parasitica*, the causal agent of downy mildew. After inoculation and incubation for 7 days, plant leaves were stained with lactophenol-trypan blue and observed under light microscope. The leaves of susceptible plant showed hyphae-bearing haustoria of *H. parasitica*, whereas leaves from resistant plant showed HR necrotic lesions.

After the HR is triggered in plants upon pathogen recognition, signaling molecules like salicylic acid (SA) are synthesized, and some signal molecules are transported systemically throughout the plant or diffuse through air. This initiates a process called SAR, whereby the plant enhances its resistance not only at the site of infection but also in distant parts of the plant that were not initially infected. SAR is an induced defense response that occurs following the initial infection of a plant by a pathogen. The production and movement of signaling molecules, play a crucial role in SAR. These molecules are synthesized in response to pathogen infection and act as triggers for systemic defense responses throughout the plant. During SAR, various defense mechanisms are activated, including the production of pathogenesis-related (PR) proteins, which have antimicrobial properties, the synthesis of phytoalexins (antimicrobial compounds), and fortification of cell walls (Jones and Dangl, 2006; Walters et al., 2009). These defense responses collectively contribute to enhanced resistance against pathogens in both the infected site and other parts of the plant. The systemic nature of SAR allows the plant to prepare itself for potential future attacks by pathogens, even in areas distant from the initial infection. This coordinated systemic response provides a higher level of protection and contributes to the overall defense capacity of the plant. SAR is an important aspect of plant immune response and has significant implications for plant health and disease management.

Induced defense both locally and systemically increase the production of several defense proteins such as β-1,3-glucanse, chitinase and polyphenol oxidase (PPO). These proteins were associated with resistance to fungal diseases of leguminous species (Vallad and Goodman, 2004; Abdel-Kader et al., 2013). Pathogens can induce the production of β-1,3-glucanase, chitinase, and PPO in plants as part of their defense response. The induction of these enzymes is often triggered by the recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) by plant receptors. β-1,3-glucanase and chitinase production can be

induced by the recognition of fungal cell wall components, such as β-1,3-glucans and chitin, respectively. Pathogens release these cell wall components during infection, and their presence activates plant defense responses. Signaling molecules like SA, jasmonic acid (JA), and ethylene (ET) are involved in regulating the expression of genes encoding β-1,3-glucanase and chitinase, leading to their production. β-1,3-glucanase and chitinase directly target the structural components of the pathogens. They weaken pathogen defenses, inhibit growth, and limit disease. β-1,3-glucanase breaks down fungal cell wall β-1,3-glucans, while chitinase degrades chitin. Both enzymes release signaling molecules, triggering additional defense responses like antimicrobial compound production and cell wall reinforcement (Sharma, 2013; Kumar et al., 2018; Khakimzhanov et al., 2021). Similarly, PPO production can be induced by the presence of pathogen-derived molecules, toxins or wounding. Pathogens may release elicitors or toxins that directly stimulate PPO gene expression. The activation of defense signaling pathways, involving signal molecules like SA, JA, and ET can also lead to the expression of PPO genes and subsequent PPO production. PPO catalyzes the oxidation of phenolic compounds, forming antimicrobial quinones or reactive oxygen species (ROS) that inhibit pathogen growth (Duffey and Felton, 1991; Mayer and Harel, 1979; Peter, 1989). PPO also reinforces cell walls by generating ROS that strengthen cell wall components. The fortified cell walls act as physical barriers, impeding pathogen entry. PPO-generated products act as signaling molecules, activating defense-related genes and triggering various defense mechanisms. These responses, including antimicrobial compound synthesis and PR protein expression, enhance plant resistance to pathogens and contribute to overall defense. While both pathways contribute to plant defense against pathogens, they operate through different mechanisms and target different aspects of pathogen biology. The PPO pathway primarily focuses on producing toxic compounds and reinforcing cell walls, while the β-1,3-glucanase and chitinase pathway directly targets the fungal cell wall. These pathways often work in conjunction with other defense mechanisms to provide a comprehensive defense response against pathogens. Saravanakumar et al. (2007) reported that inoculation of mungbean with *P. fluorescens* and chitin could increase the accumulation of PAL, PO, PPO, chitinase, β-1,3-glucanse and phenolics compounds. Thilagavathi et al. (2007) found that mungbean plants treated with *P. fluorescens* and *Trichoderma* or *Bacillus* had high

levels of PO and PPO activities. Felipini and Piero (2013) reported that chitin elicitor could induce β-1,3-glucanase and peroxidase accumulation in beetroot against *C. beticola*. Recently, Koche and Chaudhary (2019) reported that resistant mungbean genotypes had higher accumulation of PR-proteins (chitinase and β-1,3-glucanase), phytoalexin (genistein) and PAL under both field and *in vitro* conditions after sprayed with Cercospora cell wall elicitor derived from *C. canescens*. Mercier and Kuć (1996) found fewer lesions of CLS (*C. carotae*) in newly emerged carrot leaves after inoculation with *C. carotae* when compared with controls, therefore they conclude that inoculation with *C. carotae* induced systemic resistance of carrot plants. However, there have been no reported findings indicating that inoculation of mungbean with *C. canescens* leads to an increase in the accumulation of β-1,3-glucanase, chitinase, and PPO enzymes both locally and systemically.

2.7 Detached leaf assay and field evaluation

The identification of resistant germplasm enables breeding programs to develop synthetic varieties that possess superior disease resistance and are well-suited to specific environments. This strategic approach played a crucial role in controlling disease outbreaks during the 1970s (Ullstrup, 1972). Therefore, the development of rapid and reliable assays for detecting disease-resistant plant germplasm holds great value for plant breeding programs worldwide. To assess the resistance or susceptibility of plant genotypes to diseases, they are typically evaluated through screenhouse, greenhouse, and/or field experiments (Gao et al., 2005). However, these evaluations come with drawbacks as they are time-consuming and require significant resources. Additionally, their effectiveness in screening for plant resistance or susceptibility is limited to disease variants prevalent in the specific region (Osorio et al., 2014; Smith, 2008). Furthermore, external factors can influence the outcomes of these evaluations, leading to inaccurate categorization of plant genotypes into resistance groups (Patial et al., 2017).

To successfully screen large plant populations for diseases, it is crucial to employ accurate and reproducible techniques for assessing disease response. Detached leaf assays (DLA) are rapid and cost-effective laboratory-based methods utilized for screening resistance to diseases caused by various pathogens (Green et al., 2000;

Michel et al., 2010). Due to the small amount of leaf material required for each DLA, this technique enables the rapid screening of a substantial number of genotypes within a relatively limited area. This approach offers the advantage of conducting screenings under controlled conditions, resulting in efficient utilization of planting space and conservation of resources. These assays involve the inoculation of detached leaves and are employed in determining the disease resistance of plant germplasm for various crops. For instance, DLA has been used to assess the resistance of tomato (*Solanum lycopersicum* L.) germplasm against late blight (Foolad et al., 2015), apple genotypes against Alternaria blotch (Abe et al., 2010), and American chestnut (*Castanea dentata*) germplasm against chestnut blight (Newhouse et al., 2014).

The detached leaf assay is considered useful when it exhibits a strong correlation with the results obtained from field evaluations. This is because the goal of any disease screening method is to accurately predict the performance and behavior of plant genotypes in real field conditions. If the detached leaf assay aligns well with the outcomes of field evaluations, it indicates that the observed resistance or susceptibility of genotypes in the laboratory setting can be reliably extrapolated to their performance in the field. According to Aregbesola et al. (2019), there was a positive correlation observed between the DLA and the results obtained from screenhouse and field experiments when assessing maize genotypes against various *Bipolaris maydis* strains. In a study conducted by Poolsawat et al. (2012), consistent results were obtained when evaluating the resistance levels of grape genotypes against *Sphaceloma ampelinum*, the causative agent of anthracnose in grapevine, under laboratory using DLA and field conditions. These findings suggest that the laboratory screening assay utilized in the study is an efficient, rapid, reliable, and cost-effective method for identifying resistant hybrids in grapevine breeding programs.

The majority of screening methods for assessing resistance to fungal diseases utilize conidia as an inoculum. However, the production of conidia on culture media is often limited and may not occur in certain isolates. Furthermore, the preparation of conidial inoculum can be a time-consuming process, especially when large quantities of inoculum need to be generated from numerous culture plates. Additionally, the sporulation capacity of fungal isolates can vary significantly. On the other hand, mycelia exhibit faster growth rates and can be produced in larger quantities.

Surprisingly, mycelia have only been used as an inoculum in a limited number of studies (Chen and Wang (2005). Guo et al. (2016) reported that inoculating plants with mycelium of *Calonectria pseudonaviculata* in boxwood was as effective as spore inoculation for causing disease symptoms. Chen and Wang (2005) reported that the results obtained with the spray-mycelium and drop-mycelium inoculation methods were significantly correlated with the results obtained with the cut-petiole inoculation method. These results indicate that the mycelium-inoculated was a reliable and practical alternative to conidial inoculation when it comes to evaluating disease susceptibility or resistance in plant genotypes.

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CHAPTER 3

MORPHOLOGICAL CHARACTERISTICS, PATHOGENICITY AND GENETIC DIVERSITY ANALYSIS OF *Cercospora canescens*, CASUAL PATHOGEN OF CERCOSPORA LEAF SPOT IN MUNGBEAN

3.1 Abstract

One of the main diseases affecting mungbean (*Vigna radiata*) cultivars in Thailand is cercospora leaf spot (CLS). CLS can cause significant damage to mungbean crops, resulting in yield losses and decreased seed quality. *Cercospora canescens* isolates were collected from four different provinces of Thailand. Twenty single-conidial isolates were studied for morphological characteristics, DNA patterns, and pathogenicity. On five different culture media, these isolates demonstrated variations in morphological characteristics including colony color, shape, and margin, cercosporin production, and colony diameter. Mungbean leaf agar (MLA) and lettuce leaf agar (LLA) were the best media for promoting colony diameter. An analysis of randomly amplified polymorphic DNA (RAPD) suggested that the twenty isolates shared 0.84–1.00 genetic similarity. These isolates were divided into four groups according to provinces by cluster analysis using the unweighted paired grouped mean arithmetic average. Pathogenicity evaluation of six mungbean genotypes and twenty *C. canescens* isolates showed that V4718 and Super5 were resistant to the majority of isolates, indicating their utility as resistant sources in future breeding programs.

3.2 Introduction

CLS is a common and economically significant disease in the cultivation of mungbean (*Vigna radiata* (L.) Wilczek var. *radiata*). It is caused by the fungus *C. canescens* and it affects the leaves, pods, stems, and seeds. CLS can reduce yield by 50-70% and cause up to 96% losses if disease control is not implemented (Lal et al., 2001; Kaur et al., 2004; Chand et al., 2012). This disease has spread throughout Asia, including India, Bangladesh, the Philippines, Thailand, and other countries (Pandey et al., 2009).

Various factors are often evaluated in pathogenicity study, such as the virulence of different *C. canescens* isolates, host specificity, and the interaction between the pathogen and host plant. These studies can help determine the susceptibility/ resistance of different plant species or cultivars to *C. canescens* and provide insights into the mechanisms underlying pathogenicity resistance. However, the pathogenicity study of *C. canescens* has been limited in previous research (Mahapatra et al., 2022). In morphological diversity studies, Joshi et al. (2006) indicated the presence of morphological variations among eleven isolates of *C. canescens*. In the screening of mungbean germplasms through artificial inoculation, the multiplication of spores or mycelia is an essential requirement. However, the growth of *C. canescens* in laboratory conditions can be challenging, characterized by slow growth and poor sporulation. Therefore, it is important to understand the culture media influencing mycelium growth and morphological characteristics of *C. canescens*. This understanding will facilitate the optimization of nutrient components in culture media and the development of effective screening techniques for studying CLS resistance in mungbean.

In addition to morphological diversity, genetic diversity among *C. canescens* isolates has also been investigated. Joshi et al. (2006) examined eleven isolates of *C. canescens* using RAPD markers and found that they clustered into three distinct groups. Significant genetic diversity was observed even among isolates from the same geographical locations, suggesting that RAPD markers can contribute to the identification and differentiation of closely related fungal isolates. The presence or absence of specific RAPD bands can serve as diagnostic markers, aiding in the rapid and reliable identification of fungal strains. Researchers can use RAPD markers to measure the genetic diversity of fungal populations, identify distinct genetic clusters, and examine interactions between various isolates or species. RAPD markers are also relatively easy to use, require minimal DNA sample preparation, and do not rely on prior knowledge of genomic sequences. This makes RAPD markers particularly useful in studying fungi where limited genomic information is available (Williams et al., 1990).

Understanding the pathogenicity and diversity of pathogens is a crucial initial step in the development of resistant cultivars. Therefore, the objectives of this study were to study morphological characters, pathogenicity, and genetic diversity using RAPD markers of *C. canescens*, causing CLS in mungbean.

3.3 Materials and methods

3.3.1 Isolation and identification of *C. canescens*

C. canescens isolation was performed by the following standard tissue isolation technique. The infected mungbean leaves were collected from the mungbean fields in 4 locations of Thailand, including Nakhon Ratchasima, Nakhon Sawan, Phichit and Phetchabun provinces. Infected leaves were surface sterilized in 0.5% (v/v) clorox for 30 seconds and were rinsed three times in sterile distilled water. Then, leaf pieces with 0.5 to 1.0 cm width were cut from the infected area along with some healthy tissues. The leaf pieces were transferred onto PDA medium (Verma and Agnihotri, 1972) in sterilized petri dishes by aseptic technique and were incubated at room temperature (27±2°C).

The isolation of a single-conidial isolate of *C. canescens* was performed to ensure the purity of the isolate. Once the fungus grew and produced conidia on the infected leaf area, a sterile inoculation loop was used to meticulously select a single-conidial from the leaf lesion under a compound microscope. This was achieved by gently touching the conidial with the loop and transferring it to a new, sterile Petri dish containing fresh culture media, using the streak plate technique. The single-conidial was then allowed to develop into a new colony on the fresh media. The colony was subsequently observed under a microscope, and it was confirmed that it consisted of a homogeneous population of *C. canescens*, thus indicating a successful single-conidial isolation. Twenty isolates of *C. canescens* were isolated from 4 locations, including 6 isolates from Nakhon Ratchasima, 5 isolates from Nakhon Sawan, 5 isolates from Phichit and 4 isolates from Phetchabun provinces.

The morphological characters of the fungus including mycelial morphology, conidiophores, conidia and cercosporin production were studied under a compound microscope based on the characters of *C. canescens* according to Ellis and Martin (1882). The fungus was sub-cultured on PDA slants and was incubated at 27±2°C for 14 days. Then, they were preserved in refrigerator at 4°C and were renewed once every month. The preserved culture was used in further studies and was transferred to room temperature before use.

3.3.2 Morphological characters of *C. canescens* and evaluation of morphological characters on different culture media

The growth characters of 20 isolates of *C. canescens* were studied on 5 solid media viz., PDA, carrot leaf agar (CLA), mungbean leaf agar (MLA), oat meal agar (OMA) and lettuce leaf agar (LLA). Media composition is described in Table 3.1. All media were sterilized at 1.1 kg/cm^2 pressure with 121°C for 20 minutes. After that, 20 ml of each of the media was poured in 90 mm diameter Petri dishes. Five mm diameter mycelial disc from *C. canescens* cultured on PDA was placed on each medium, and were incubated at 25°C with 12 light hours/day (1,500 lux) in growth chamber. Five replications per isolate were made. Morphological characteristics (color, shape and margin), cercosporin production and colony diameter were observed at 30 DAI. An experiment was performed with 5 replications per treatment (1 Petri dish per replication). The colony diameter was statistically analyzed by SPSS version 16.0 (Levesque and SPSS Inc., 2006), and mean comparison was performed by Duncan's multiple range test (DMRT).

Table 3.1 The composition of culture media.

Media	Composition	References
Potato dextrose agar (PDA)	200 g Potato + 20 g Dextrose + 20 g Agar	Verma and Agnihotri (1972)
Carrot leaf agar (CLA)	300 g Carrot leaf + 20 g Agar	Verma and Agnihotri (1972)
Mungbean leaf agar (MLA)	300 g Mungbean leaf + 20 g Agar	Khandar et al. (1985)
Oat meal agar (OMA)	30 g Oat meal + 20 g Agar	Modified from Mishra and Bhattacharya (2002)
Lettuce leaf agar (LLA)	300 g Lettuce leaf + 20 g Agar	Thomas and Duraisamy (2019)

3.3.3 Pathogenicity of *C. canescens*

Twenty single-conidial isolates from 4 provinces were evaluated for the pathogenicity to select for the most virulent isolates. The experiments were carried out at least 2 times with 4 replications. A completely randomized design (CRD) with 6 replications (1 leaf piece per replication) were used. Two susceptible mungbean

genotypes (EGMD-6D and SUT1), two moderately resistant to susceptible mungbean genotypes (CN84-1 and B2) and two resistant mungbean genotypes (V4718 and Super5) were grown in pots under greenhouse condition. Leaves from the second node (counting from top) of twenty-one days old plants were collected and used for detached leaf assay. Six leaf pieces were placed on 90 mm diameter petri dish and inoculated with one droplet of 5 µl of *C. canescens* inoculum (80 mycelial disks (5 mm diameter/5 mL), while control leaves were inoculated with sterile distilled water. The inoculated leaves were kept for 2-5 days at 27°C with 12 light hours/day (1,500 lux) in growth chamber to ensure successful penetration of the pathogen into plant tissues. After the symptoms appeared, re-isolation was made from disease spots compared with the original isolates for confirmation. Disease severity was assessed at 0, 1, 2, 3 and 4 DAI by rating the symptoms expression on a 1 to 5- point scale (Table 3.2). The disease severity score data was statistically analyzed by SPSS version 16.0 (Levesque and SPSS Inc., 2006). An isolate of *C. canescens* which was the most virulent with high growth rate was selected for further use in CHAPTER IV: DIFFERENTIAL INDUCTION OF β-1,3-GLUCANASE, CHITINASE AND POLYPHENOL OXIDASE ACTIVITIES IN MUNGBEAN GENOTYPES IN RESPONSE TO *Cercospora canescens*.

Table 3.2 Disease severity score for Cercospora leaf spot disease of mungbean.

Scale	Rating	Description
1	No infection	No visible symptoms
2	Low infection	1-25% of total leaf area is covered by lesions
3	Moderate infection	26-50% of the total leaf area is covered by lesions
4	Severe infection	51-75% of the total leaf area is covered by lesions
5	Very severe infection	76-100% of the total leaf area is covered by lesions

Source: Modified from Ngegba et al. (2017).

3.3.4 Genetic diversity of *C. canescens*

Genetic diversity of 20 isolates of *C. canescens* were studied using RAPD markers according to Joshi et al. (2006). Fungal mycelia were cut from the PDA medium with a sterile knife and were cultured on 50 ml of PDB in 250 ml conical flasks and were incubated with shaking incubator (120 rpm) at 21–23°C for 14 days. Mycelia were

harvested by filtration through Whatman filter paper No.1 and rinsed with sterile distilled water, blotted dry and were preserved in refrigerator at -20°C before used for DNA extraction. The mycelia were ground using a pre-chilled mortar and pestle, to a fine powder in liquid nitrogen. Fungal DNA was isolated using a method as modified from Stirling (2003). The DNA was quantified by ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and were diluted to a final concentration of 25 ng/ml for polymerase chain reaction (PCR). RAPD primers used in these studies were OPD20 (ACCCGGTCAC), OPK7 (AGCGAGCAAG), OPK11 (AATGCCCCAG), OPK12 (TGGCCCTCAC), OPK15 (CTCCTGCCAA), OPN4 (GACCGACCCA), OPL1 (GGCATGACCT), OPL19 (GAGTGGTGAC) and OPL20 (TGGTGGACCA), all of these showed high polymorphisms in genetic diversity study of *C. canescens* from various legume hosts observed by Joshi et al. (2006). The PCR reaction for RAPD markers was performed in 20 µL containing 150 ng of genomic DNA, 1 unit of Taq DNA polymerase, 1X buffer (50 mM KCl, 10 mM Tris-HCl pH 9.1, and 0.01% Triton™ X-100), 2 mM $MgCl_2$, 0.2 mM of each deoxyribonucleotide triphosphate (dNTP), and 0.5 µM of each primer. Amplification was conducted following the method described by Joshi et al. (2006) using a T100TM Thermal Cycler (Bio-Rad, California), with an initial denaturation at 94°C for 2 minutes, followed by 45 cycles of denaturation at 94°C for 1 minute, annealing at 37°C for 1 minute, and extension at 72°C for 2 minutes. A final extension step at 72°C for 5 minutes was performed. Amplified products were resolved in 6% polyacrylamide gel with 200V for 90 min. The gels were stained with silver nitrate (Sambrook and Russell, 2001). The 100 bp ladder served as the standard molecular weight marker. Only clear and reproducible bands were scored. The polymorphic RAPD bands were scored as binary digit code of 0 and 1 for the absence and presence of polymorphic RAPD band, respectively. Data was used for similarity-based analysis using the NTSYS-PC program (version 2.02). Similarity coefficients were used for construction of Unweighted Pair Group Method with Arithmetic Average (UPGMA) dendrogram.

3.4 Results and discussion

3.4.1 Isolation and identification of *C. canescens*

The *C. canescens* isolation was performed using the following standard tissue isolation technique. The infected mungbean leaves were collected from the mungbean fields in 4 provinces of Thailand, including Nakhon Ratchasima, Nakhon

Sawan, Phichit and Phetchabun. The *C. canescens* had mycelia that grew slowly on mungbean leaves at 3-5 DAI. The identification of the pathogen was performed under a compound microscope based on the characteristics of *C. canescens* according to Ellis and Martin (1882). Twenty isolates of *C. canescens* were isolated from 4 locations, including 6 isolates from Nakhon Ratchasima, 5 isolates from Nakhon Sawan, 5 isolates from Phichit and 4 isolates from Phetchabun provinces. The fungal isolates were cultured on PDA. The morphological variations were observed with the morphological parameters including colony color, shape and margin, cercosporin production and colony diameter among 20 isolates of *C. canescens* collected from 4 provinces of Thailand (Table 3.3).

3.4.2 Morphological characters of *C. canescens*

3.4.2.1 Evaluation of morphological characters on different culture media

I. Colony diameter

The growth characters of 20 isolates of *C. canescens* which were grown on various media (PDA, CLA, MLA, OMA and LLA) were characterized after 30 days. It was shown that the effects of isolates were highly significant ($p < 0.01$) on colony diameter. At 30 days, the most rapidly grown isolate was NW-5 with a colony diameter of 62.8, 68.2, 69.9, 72.6 and 77.0 mm on PDA, CLA, OMA, LLA and MLA, respectively, which were significantly higher than other isolates. Whereas the most slowly grown isolate was SUT-3 with a colony diameter of 27.0, 33.9, 34.0, 37.2 and 42.2 mm on PDA, CLA, OMA, LLA and MLA, respectively (Table 3.4).

It was found that different culture media affected colony diameter of all *C. canescens* isolates significantly ($p < 0.01$) at 30 days. Overall, the best media for promoting colony diameter were MLA and LLA. At 30 days, the average colony diameter of all isolates of *C. canescens* on MLA and LLA were 59.32 and 54.29 mm, respectively, which were significantly higher than that grew on PDA. (Table 3.4), suggesting that the culture media which contained host leaf extract (mungbean and carrot leaves) could promote the colony radial growth better than PDA. Similarly, previous reports showed that host leaf extract media was best for radial growth of *Cercospora* spp. (Mishra and Bhattacharya, 2002; Mallappa, 2007).

A highly significant interaction ($p < 0.01$) on colony diameter was observed among isolates of *C. canescens* grown on different culture media. When both factors were evaluated at 30 DAI, a maximum colony diameter was obtained in NW-5 when cultured on MLA (77.0 mm), which was not significantly different from PCH-4 cultured on MLA (75.3 mm). Each isolate of *C. canescens* had effectively grown on different culture media. The highest colony diameter was obtained in all isolates of *C. canescens* when cultured on MLA for 30 days. The lowest colony diameter was obtained in all *C. canescens* isolates when cultured on PDA (Table 3.4). The results were supported by Latterell and Rossi (1974) and Nega et al. (2016), who reported that different culture media support colony growth of *C. zeae-maydis* differently.

When comparing colony diameters throughout different locations on 5 culture media, we found that isolates from Nakhon Sawan had the highest average colony diameter (54.77) while isolates from Nakhon Ratchasima had the lowest average diameter (47.62 mm) (Table 3.4). The isolates from Nakhon Sawan had colony diameters that were 1.03, 1.05 and 1.15, fold larger than those from Phichit, Phetchabun, and Nakhon Ratchasima, respectively. It was observed that the isolates obtained from Nakhon Ratchasima revealed higher average colony diameter for the isolates originating from the Pak Thong Chai district (PAK-1, PAK-2, and PAK-3, average 59.07 mm) compared to those obtained from Suranaree University of Technology Farm (SUT-1, SUT-2, and SUT-3, average 40.9) indicating variation within province. Similarly, Poolsawat (2010) observed that isolates of *Sphaceloma ampelinum* obtained from different locations in Thailand exhibited varying growth rates in colony diameter.

II. Colony color, shape and margin

The colony color of 20 isolates of *C. canescens* which were grown on various media (PDA, CLA, MLA, OMA and LLA) were characterized after 30 days. It was shown that there was low variation of colony color among isolates of *C. canescens*. Colony color of most isolates was greyish-white (60%), and the remaining 40% was yellowish-white. It was also found that the colors of colonies within the same location varied. There was no variation on colony color of most isolates when cultured on different culture media, except for SUT-2, PAK-2, PCH-4 and PB-4. Interestingly, isolates from Petchabun had yellowish-white colonies when grown on most media (Table 3.5 and Figure 3.1).

When considering the colony shape and margin of 20 isolates of *C. canescens* which were grown on different culture media and were characterized after 30 days, there were circular (65%) and irregular (35%), and the shape of colonies within the same location differed. It was showed that there was no variation of colony shape of each isolate when cultured on different culture media (Table 3.6 and Figure 3.1). For colony margin, there were entire (65%) and undulate (35%). The variation of colony margin was found within the location. Additionally, it was demonstrated that when an isolate was cultured on various culture media, the colony margin did not vary (Tables 3.7 and Figure 3.1). The reason for the variation in morphology could be related to genetic and /or environmental factors. These results were supported by Kinyua et al. (2010) and Latterell and Rossi (1974) who reported that the colony colors of *C. zeae-maydis* isolates grown on agar media varied. Similarly, Lyimo et al. (2013) reported that colony color of most isolates of *C. zeae-maydis* were grey to light grey. Kinyua et al. (2010) also found that there was variation on colony color, shape and margin of *C. zeae-maydis* when cultured on different culture media.

III. Cercosporin production

The cercosporin production of 20 isolates of *C. canescens* which were grown on various media (PDA, CLA, MLA, OMA and LLA) were observed after 30 days. It was found that there was variation on cercosporin production among isolates of *C. canescens*. There were 8 isolates (40%) that produce cercosporin. Ability to produce cercosporin of the isolates depended on the culture media. The results showed that PDA was the best medium to produce cercosporin. Most isolates on MLA had a tendency to decrease the cercosporin production (Table 3.8 and Figure 3.1), suggesting that culture media affected cercosporin production. These results were supported by Daub and Chung (2009), who reported that cercosporin production was preferentially synthesized in some culture media such as PDA medium, and it was repressed when grown in some culture media such as V8 juice medium because of the different nutritional conditions of each culture medium. Jenns et al. (1989) stated that the best medium for toxin production of *C. asparagi, C. beticola, C. kikuchii, C. nicotianae, and C. zeae-maydis* was PDA. Chung (2003) also observed that PDA was the best medium to produce cercosporin for *C. nicotianae* and *C. zeae*.

Table 3.3 Morphology of isolates of *C. canescens* from mungbean fields in 4 provinces of Thailand, on PDA at 30 DAI.

Locations	Isolates	Colony diameter on PDA[1] (mm)	Colony characteristics			Cercosporin production
			Color	Shape	Margin	
Nakhon Ratchasima	SUT-1	32.6	Yellowish-white	Circular	Entire	-[2]
	SUT-2	33.6	Greyish-white	Circular	Entire	-
	SUT-3	27.0	Greyish-white	Irregular	Undulate	-
	PAK-1	54.0	Yellowish-white	Circular	Entire	++
	PAK-2	48.6	Greyish-white	Irregular	Undulate	++
	PAK-3	44.6	Greyish-white	Circular	Entire	+
Phichit	PCH-1	34.2	Greyish-white	Irregular	Undulate	-
	PCH-2	38.2	Yellowish-white	Circular	Entire	-
	PCH-3	50.4	Greyish-white	Circular	Entire	++
	PCH-4	60.8	Yellowish-white	Circular	Entire	+
	PCH-5	45.0	Greyish-white	Irregular	Undulate	-
Phetchabun	PB-1	30.4	Yellowish-white	Circular	Entire	-
	PB-2	47.0	Yellowish-white	Irregular	Undulate	-
	PB-3	56.6	Yellowish-white	Circular	Entire	-
	PB-4	43.6	Greyish-white	Irregular	Undulate	-
Nakhon Sawan	NW-1	49.8	Greyish-white	Circular	Entire	-
	NW-2	45.6	Yellowish-white	Circular	Entire	-
	NW-3	28.6	Greyish-white	Irregular	Undulate	++
	NW-4	53.4	Greyish-white	Circular	Entire	++
	NW-5	62.8	Greyish-white	Circular	Entire	+

[1] Culture media: PDA = potato dextrose agar.

[2] Cercosporin production; ++ = high cercosporin, + = low cercosporin, - = no cercosporin.

Table 3.4 Colony diameter of different isolates of *C. canescens* on various culture media after 30 days.

Locations	Isolates	Average of colony diameter (mm)					Average
		PDA[1]	MLA	OMA	CLA	LLA	
Nakhon Ratchasima	SUT-1	32.6 ij[2]	47.5 ij	39.2 ij	38.5 ij	42.1 ij	39.98 ij
	SUT-2	33.6 hij	48.4 hij	40.4 hij	39.7 hij	43.4 hij	41.10 hij
	SUT-3	27.0 k	42.2 k	34.0 k	33.9 k	37.2 k	34.86 k
	PAK-1	54.0 cd	69.1 cd	61.2 cd	60.4 cd	64.1 cd	61.76 cd
	PAK-2	48.6 d-g	63.3 d-g	55.5 d-g	54.2 d-g	58.4 d-g	56.00 d-g
	PAK-3	44.6 fg	59.5 fg	51.2 fg	50.2 fg	54.7 fg	52.04 fg
Average		**40.07**	**55.00**	**46.92**	**46.15**	**49.98**	**47.62**
Phichit	PCH-1	34.2 cd	49.1 hi	41.1 hi	40.1 hi	44.2 hi	41.74 hi
	PCH-2	38.2 hi	53.2 h	45.4 h	44.5 h	48.1 h	45.88 h
	PCH-3	50.4 de	65.8 de	57.3 de	56.2 de	60.9 de	58.12 de
	PCH-4	60.8 ab	75.3 ab	67.6 ab	66.5 ab	70.6 ab	68.16 ab
	PCH-5	45.0 efg	60.5 efg	52.3 efg	51.3 efg	55.2 efg	52.86 efg
Average		**45.72**	**60.78**	**52.74**	**51.72**	**55.80**	**53.35**
Phetchabun	PB-1	30.4 ijk	45.7 ijk	37.6 ijk	36.2 ijk	40.2 ijk	38.02 ijk
	PB-2	47.0 efg	62.2 efg	54.1 efg	53.1 efg	57.5 efg	54.78 efg
	PB-3	56.6 bc	71.8 bc	63.3 bc	62.3 bc	66.2 bc	64.04 bc
	PB-4	43.6 g	58.2 g	50.1 g	49.2 g	53.7 g	50.96 g
Average		**44.40**	**59.48**	**51.28**	**50.20**	**54.40**	**51.95**
Nakhon Sawan	NW-1	49.8 def	64.7 def	56.3 def	55.9 def	59.5 def	57.24 def
	NW-2	45.6 efg	60.7 efg	52.7 efg	51.5 efg	55.6 efg	53.22 efg
	NW-3	28.6 jk	43.2 jk	35.2 jk	34.3 jk	38.3 jk	35.92 jk
	NW-4	53.4 cd	68.9 cd	60.5 cd	59.3 cd	63.2 cd	61.06 cd
	NW-5	62.8 a	77.0 a	69.9 a	68.2 a	72.6 a	69.48 a
Average		**48.04**	**62.90**	**54.92**	**53.84**	**54.15**	**54.77**
Grand Average		**44.34 C[3]**	**59.32 A**	**51.25 BC**	**50.28 BC**	**54.29 AB**	

[1] Culture media: PDA = potato dextrose agar, CLA = carrot leaf agar, MLA = mungbean leaf agar, OMA = oat meal agar and LLA = lettuce leaf agar.

[2] Data followed by different letters in each column differ significantly ($p < 0.05$) according to Duncan's multiple range test.

[3] Data followed by different letters in each row differ significantly ($p < 0.05$) according to Duncan's multiple range test.

Table 3.5 Colony color among different isolates of *C. canescens* on 5 culture media (PDA, MLA, OMA, CLA and LLA).

Isolates	Colony color				
	PDA[1]	MLA	OMA	CLA	LLA
SUT-1	Yellowish-white	Yellowish-white	Yellowish-white	Yellowish-white	Yellowish-white
SUT-2	Greyish-white	Greyish-white	Yellowish-white	Greyish-white	Yellowish-white
SUT-3	Greyish-white	Greyish-white	Greyish-white	Greyish-white	Greyish-white
PAK-1	Yellowish-white	Yellowish-white	Yellowish-white	Yellowish-white	Yellowish-white
PAK-2	Greyish-white	Greyish-white	Greyish-white	Greyish-white	Yellowish-white
PAK-3	Greyish-white	Greyish-white	Greyish-white	Greyish-white	Greyish-white
PCH-1	Greyish-white	Greyish-white	Greyish-white	Greyish-white	Greyish-white
PCH-2	Yellowish-white	Yellowish-white	Yellowish-white	Yellowish-white	Yellowish-white
PCH-3	Greyish-white	Greyish-white	Greyish-white	Greyish-white	Greyish-white
PCH-4	Yellowish-white	Greyish-white	Yellowish-white	Greyish-white	Greyish-white
PCH-5	Greyish-white	Greyish-white	Greyish-white	Greyish-white	Greyish-white
PB-1	Yellowish-white	Yellowish-white	Yellowish-white	Yellowish-white	Yellowish-white
PB-2	Yellowish-white	Yellowish-white	Yellowish-white	Yellowish-white	Yellowish-white
PB-3	Yellowish-white	Yellowish-white	Yellowish-white	Yellowish-white	Yellowish-white
PB-4	Greyish-white	Greyish-white	Yellowish-white	Yellowish-white	Yellowish-white
NW-1	Greyish-white	Greyish-white	Greyish-white	Greyish-white	Greyish-white
NW-2	Yellowish-white	Yellowish-white	Yellowish-white	Yellowish-white	Yellowish-white
NW-3	Greyish-white	Greyish-white	Greyish-white	Greyish-white	Greyish-white
NW-4	Greyish-white	Greyish-white	Greyish-white	Greyish-white	Greyish-white
NW-5	Greyish-white	Greyish-white	Greyish-white	Greyish-white	Greyish-white

[1] Culture media: PDA = potato dextrose agar, CLA = carrot leaf agar, MLA = mungbean leaf agar, OMA = oat meal agar and LLA = lettuce leaf agar.

Table 3.6 Colony shape among different isolates of *C. canescens* on 5 culture media (PDA, MLA, OMA, CLA and LLA).

Isolates	Colony shape				
	PDA[1]	MLA	OMA	CLA	LLA
SUT-1	Circular	Circular	Circular	Circular	Circular
SUT-2	Circular	Circular	Circular	Circular	Circular
SUT-3	Irregular	Irregular	Irregular	Irregular	Irregular
PAK-1	Circular	Circular	Circular	Circular	Circular
PAK-2	Irregular	Irregular	Irregular	Irregular	Irregular
PAK-3	Circular	Circular	Circular	Circular	Circular
PCH-1	Irregular	Irregular	Irregular	Irregular	Irregular
PCH-2	Circular	Circular	Circular	Circular	Circular
PCH-3	Circular	Circular	Circular	Circular	Circular
PCH-4	Circular	Circular	Circular	Circular	Circular
PCH-5	Irregular	Irregular	Irregular	Irregular	Irregular
PB-1	Circular	Circular	Circular	Circular	Circular
PB-2	Irregular	Irregular	Irregular	Irregular	Irregular
PB-3	Circular	Circular	Circular	Circular	Circular
PB-4	Irregular	Irregular	Irregular	Irregular	Irregular
NW-1	Circular	Circular	Circular	Circular	Circular
NW-2	Circular	Circular	Circular	Circular	Circular
NW-3	Irregular	Irregular	Irregular	Irregular	Irregular
NW-4	Circular	Circular	Circular	Circular	Circular
NW-5	Circular	Circular	Circular	Circular	Circular

[1] Culture media: PDA = potato dextrose agar, CLA = carrot leaf agar, MLA = mungbean leaf agar, OMA = oat meal agar and LLA.

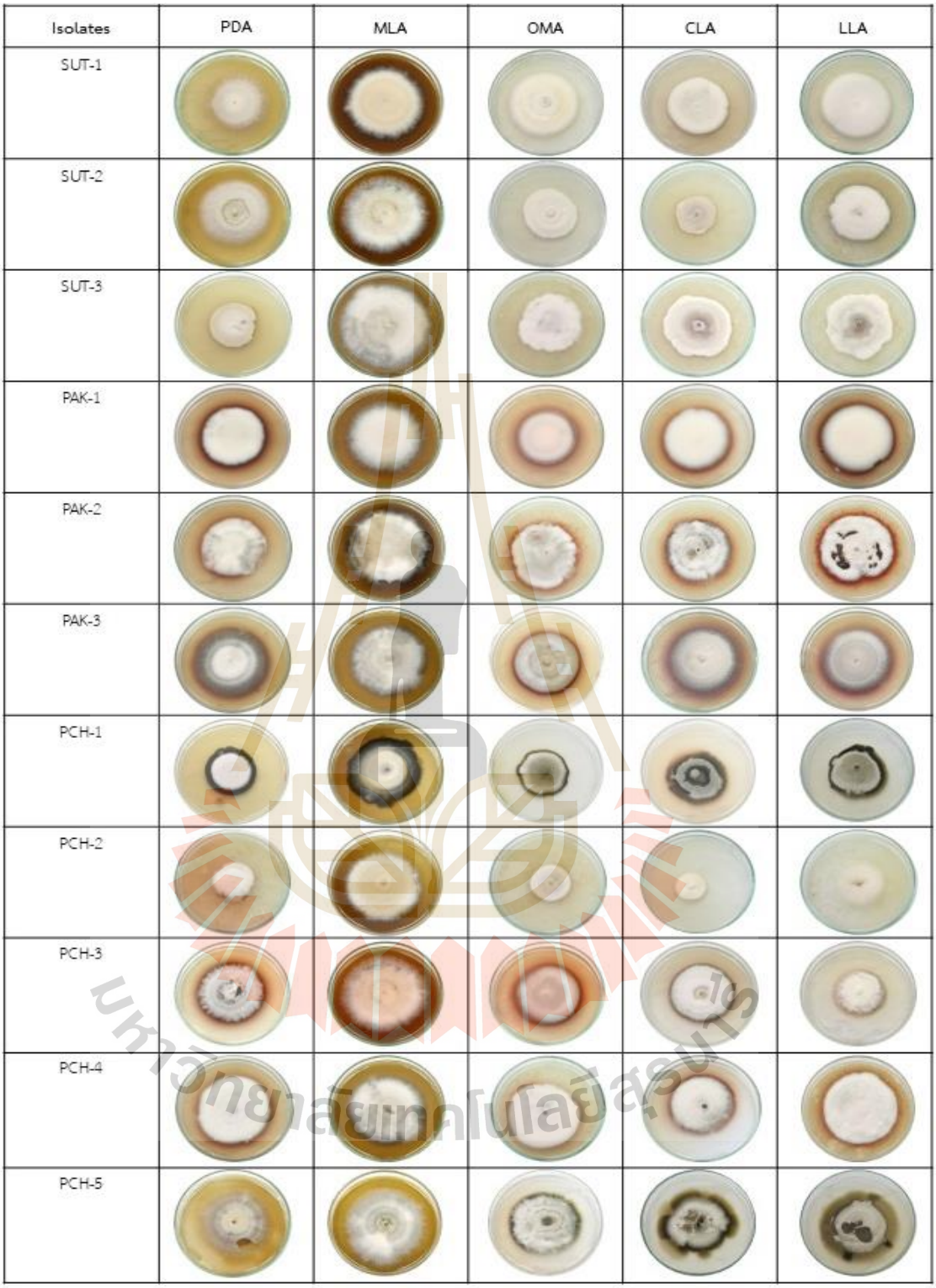


Figure 3.1 Morphological characters among different isolates of *C. canescens* on 5 culture media. Culture media: PDA = potato dextrose agar, CLA = carrot leaf agar, MLA = mungbean leaf agar, OMA = oat meal agar and LLA = lettuce leaf agar.

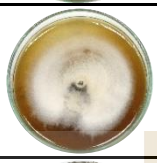
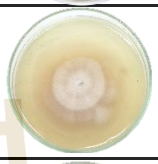
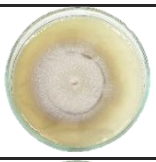
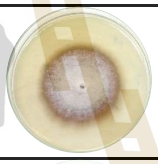
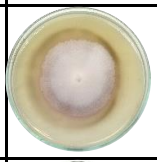
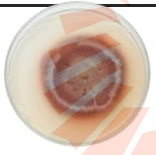
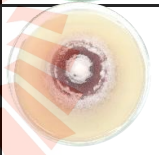
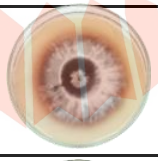
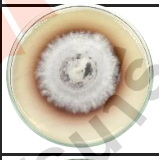
Isolates	PDA	MLA	OMA	CLA	LLA
PB-1					
PB-2					
PB-3					
PB-4					
NW-1					
NW-2					
NW-3					
NW-4					
NW-5					

Figure 3.1 (continued).

Table 3.7 Colony margin among different isolates of *C. canescens* on 5 culture media (PDA, MLA, OMA, CLA and LLA).

Isolates	Colony margin				
	PDA[1]	MLA	OMA	CLA	LLA
SUT-1	Entire	Entire	Entire	Entire	Entire
SUT-2	Entire	Entire	Entire	Entire	Entire
SUT-3	Undulate	Undulate	Undulate	Undulate	Undulate
PAK-1	Entire	Entire	Entire	Entire	Entire
PAK-2	Undulate	Undulate	Undulate	Undulate	Undulate
PAK-3	Entire	Entire	Entire	Entire	Entire
PCH-1	Undulate	Undulate	Undulate	Undulate	Undulate
PCH-2	Entire	Entire	Entire	Entire	Entire
PCH-3	Entire	Entire	Entire	Entire	Entire
PCH-4	Entire	Entire	Entire	Entire	Entire
PCH-5	Undulate	Undulate	Undulate	Undulate	Undulate
PB-1	Entire	Entire	Entire	Entire	Entire
PB-2	Undulate	Undulate	Undulate	Undulate	Undulate
PB-3	Entire	Entire	Entire	Entire	Entire
PB-4	Undulate	Undulate	Undulate	Undulate	Undulate
NW-1	Entire	Entire	Entire	Entire	Entire
NW-2	Entire	Entire	Entire	Entire	Entire
NW-3	Undulate	Undulate	Undulate	Undulate	Undulate
NW-4	Entire	Entire	Entire	Entire	Entire
NW-5	Entire	Entire	Entire	Entire	Entire

[1] Culture media: PDA = potato dextrose agar, CLA = carrot leaf agar, MLA = mungbean leaf agar, OMA = oat meal agar and LLA = lettuce leaf agar.

Table 3.8 Cercosporin production among different isolates of *C. canescens* on 5 culture media (PDA, MLA, OMA, CLA and LLA).

Isolates	Cercosporin production				
	PDA[1]	MLA	OMA	CLA	LLA
SUT-1	- [2]	-	-	-	-
SUT-2	-	-	-	-	-
SUT-3	-	-	-	-	-
PAK-1	++	-	++	++	++
PAK-2	++	-	++	++	++
PAK-3	++	-	++	+	+
PCH-1	-	-	-	-	-
PCH-2	-	-	-	-	-
PCH-3	++	-	++	+	+
PCH-4	+	-	+	++	+
PCH-5	-	-	-	-	-
PB-1	-	-	-	-	-
PB-2	-	-	-	-	-
PB-3	-	-	-	-	-
PB-4	-	-	-	-	-
NW-1	-	-	-	-	-
NW-2	-	-	-	-	-
NW-3	++	+	++	++	++
NW-4	++	+	+	++	++
NW-5	+	+	+	+	+

[1] Culture media: PDA = potato dextrose agar, CLA = carrot leaf agar, MLA = mungbean leaf agar, OMA = oat meal agar and LLA = lettuce leaf agar.

[2] Cercosporin production; ++ = high cercosporin, + = low cercosporin, - = no cercosporin.

3.4.3 Pathogenicity

Twenty isolates from 4 provinces were evaluated for the pathogenicity by a detached leaf assay. Two susceptible mungbean genotypes (EGMD-6D and SUT1), two moderately resistant to susceptible mungbean genotypes (CN84-1 and B2) and two resistant mungbean genotypes (V4718 and Super5) were used in this study. The results showed that mungbean leaves started to exhibit necrotic lesions at 2 DAI. At 5 DAI, necrotic lesions were formed differently among isolates and severity of symptoms was highly significantly different among mungbean genotypes ($p < 0.01$). Two mungbean resistant genotypes Super5 and V4718 were potentially resistant to most *C. canescens* isolates because they developed the least symptoms compared to those of other genotypes, with average severity scores of 2.6 and 2.7, respectively. The moderately resistant genotypes CN84-1 and B2 were moderately resistant to most *C. canescens* isolates, with average severity scores of 3.0 and 3.1, respectively. The susceptible mungbean genotype SUT1 was the most susceptible with average severity score of 3.5, which was significantly higher than those of CN84-1, V4718 and Super5 (Table 3.9).

To compare the disease reaction between mungbean genotypes and isolates. It was found that mungbean genotypes had highly significant differences ($p < 0.01$) in their CLS severity scores in response to different isolates. The highly significant interactions between mungbean genotypes and *C. canescens* isolates ($p < 0.01$) was also found (Table 3.10). The Mungbean genotype Super5 exhibited resistance or moderate resistance against the majority of pathogen isolates, with the exception of NW-3. Whereas, mungbean genotype V4718 displayed resistance or moderate resistance to nearly all pathogen isolates, except for PAK-3, NW-3, and NW-4. In the case of mungbean genotype B2, it demonstrated moderate resistance to most pathogen isolates, excluding NW-2 and NW-3. Similarly, mungbean genotype CN84-1 showcased resistance or moderate resistance against almost all pathogen isolates, except for NW-2 and NW-3. However, mungbean genotype SUT1 proved to be susceptible to 60% of pathogen isolates, except for SUT-2, PCH-1, PCH-4, PCH-5, PB-3, PB-4, NW-4, and NW-5. Furthermore, mungbean genotype EGMD-6D was also susceptible to 45% of isolates. An intriguing observation was made regarding the mungbean genotypes Super5 and V4718 in their responses to the NW-4 isolate.

Notably, the disease severity score of Super5 was 2.9, indicating a lower severity compared to mungbean genotypes V4718, which exhibited a score of 3.8. Furthermore, it was observed that the mungbean genotype Super5 displayed resistance or moderate resistance to 19 out of 20 *C. canescens* isolates. In comparison, the mungbean genotype V4718 exhibited resistance or moderate resistance to 17 out of 20 *C. canescens* isolates. These results indicate that Super5 which had CLS and powdery mildew (PM) resistance genes derived from the double cross [(CN72 × V4758) × (CN72 × V4718)] × [(CN72 × V4718) × (CN72 × V4785)], exhibited higher resistance levels against CLS disease compared to its resistant parent (V4718). These findings align with previous studies that reported Super5's superior resistance to both CLS and PM diseases when evaluated across various environments. They also highlight the potential of Super5 as an effective source of resistance in mungbean breeding programs, as supported by research conducted by Poolsawat et al. (2017) and Pookhamsak et al. (unpublished data). The significant interactions observed between different mungbean genotypes and isolates of *C. canescens* indicate the existence of different resistance gene(s) corresponding to various avirulent gene(s) of each isolate in each mungbean genotype, and suggest that relying on a single resistance gene may not be sufficient in the long term. This is due to the pathogen can evolve and produce new strains that can overcome the protections conferred by a single resistance gene. Combining multiple resistance genes, a process known as gene pyramiding, would therefore be a more effective strategy for ensuring long-lasting resistance to this disease. Papan et al. (2021) reported that two pyramided mungbean backcross lines, namely H3 and H4, had a moderate to high levels of CLS and PM resistance compared to the susceptible recurrent parent KING. Similarly, Pradhan et al. (2015) evaluated resistance among rice lines/varieties having different resistance genes against 8 isolates of *Xanthomonas oryzae* pv. *oryzae* (Xoo), causal agent of bacterial blight. The results showed that 3 gene pyramided lines had higher resistance levels to all isolates than 2 genes pyramided lines and susceptible variety, respectively. By pyramiding multiple resistance genes, plants can have a more robust and durable defense against the pathogen, thereby increasing the likelihood of successful cultivation in a variety of environments with different strains of pathogen.

When comparing the disease severity scores of *C. canescens* isolates throughout different locations on 6 mungbean genotypes, it was found that isolates had highly significantly difference ($p < 0.01$) in disease severity scores. The results showed that isolates from Nakhon Sawan and Nakhon Ratchasima had the highest disease severity with average severity scores of 3.15 and 3.06, respectively, which were significantly higher than that of Phichit (Table 3.10). Similarly, Almeida et al. (2005) reported that *C. kikuchii* isolates obtained from different geographic regions in Brazil are variable in relation to virulence when infected in soybean (*Glycine max*). When considering the disease severity scores of each *C. canescens* isolate on different mungbean genotypes at 5 DAI, it was found that they were highly significantly different ($p < 0.01$). Isolate NW-3 was the most virulent isolate with an average severity score of 3.93, which was significantly higher than other isolates. Moreover, the NW-3 isolate was also virulent to the resistant mungbean genotypes V4718 and Super5, which showed potentially resistant or moderately resistant in most *C. canescens* isolates (Table 3.10). Noted that this isolate may have broad-range of host, and have the ability to infect and cause disease in various mungbean genotypes. The least virulent isolate was PCH-1 with an average severity score of 2.30, and could not differentiate among mungbean genotypes. These results suggest that both *C. canescens* isolates and mungbean genotypes affected the severity of CLS symptoms, in agreement with previous studies, which reported that disease severity among mungbean genotypes varied according to the *C. canescens* isolates (Mattana, 2019; Singh et al., 2021). Different mungbean genotypes may respond differently to different *C. canescens* isolates due to variations in their resistance mechanisms. Therefore, an evaluation of disease severity and resistance levels should be assessed with more than one isolate to identify specific interactions between pathogen strains and different plant genotypes. This information is valuable for understanding the effectiveness of host resistance and identifying potential sources of resistance that can be utilized in breeding programs.

The crucial role of cercosporin in the pathogenicity of *C. kikuchii* was initially demon-strated by Upchurch et al. (1991), who found that the production of cercosporin was essential for the infection of soybean plants. They observed that mutants lacking cercosporin production, either spontaneous or UV-induced, were unable to cause infection when inoculated on soybean leaves. However, in our study,

the variation in virulence among the isolates was not linked to cercosporin production. Interestingly, the presence of a red color surrounding the colony proved to be an unreliable indicator of virulence for *C. canescens* isolates since several virulent isolates did not display this reddish coloration. Additionally, the production of more cercosporin did not necessarily correlate with increased virulence. This finding contrasts with the study conducted by Almeida et al. (2005), which reported a correlation coefficient of 83% between cercosporin content and disease severity of *C. kikuchii* when infecting soybean. While cercosporin is known to contribute to the pathogenicity of some *Cercospora* species, it is possible that other virulence factors or mechanisms are at play in *C. canescens*. The pathogen may utilize different strategies or produce additional toxins/enzymes that contribute to its virulence, independent of cercosporin production.

Table 3.9 Differential responses of selected mungbean genotypes against various isolates of *C. canescens* at 5 DAI.

Mungbean genotypes		Average severity score	Resistance levels[1]
Susceptible	EGMD-6D	3.2 ab[2]	MR
	SUT1	3.5 a	S
Moderately resistant	B2	3.1 ab	MR
	CN84-1	3.0 b	MR
Resistant	V4718	2.7 c	MR
	Super5	2.6 c	MR

[1] Cercospora leaf spot resistance levels based on disease severity scores are as follows: 1.0-2.4 = resistant (R), 2.5-3.4 = moderately resistant (MR), and 3.5-5.0 = susceptible (S).

[2] Data followed by different letters in each column differ significantly ($p < 0.05$) according to Duncan's multiple range test.

Table 3.10 Effects of isolates of *C. canescens* on severity scores of mungbean genotypes by detached leaf assay at 5 DAI.

Isolates	Mungbean genotypes												Average	Grand average
	Susceptible				Moderately resistant				Resistant					
	EGMD-6D		SUT1		B2		CN84-1		V4718		Super5			
SUT-1	3.8 a[1]	S[2]	3.9 ab	S	3.2 cd	MR	3.3 bc	MR	2.4 hij	R	2.4 efg	R	3.17 bcd	
SUT-2	3.1 c-f	MR	3.2 ef	MR	2.9 def	MR	2.7 e-h	MR	2.6 ghi	MR	3.0 bcd	MR	2.92 de	
SUT-3	3.8 a	S	3.6 b-e	S	3.0 efg	MR	3.2 bcd	MR	2.0 k	R	2.3 efg	R	2.98 de	**3.06 A[3]**
PAK-1	3.8 a	S	3.7 bcd	S	3.0 cde	MR	3.2 bcd	MR	2.2 jk	R	2.4 efg	R	3.05 cde	
PAK-2	3.5 abc	S	3.6 b-e	S	3.2 cd	MR	3.0 c-f	MR	2.1 jk	R	2.2 fg	R	2.93 de	
PAK-3	2.9 efg	MR	4.2 a	S	3.2 cd	MR	2.9 c-g	MR	3.5 bc	S	3.2 b	MR	3.32 b	
PCH-1	2.3 i	R	2.4 g	R	2.5 g	MR	2.2 i	R	2.4 hij	R	2.0 g	R	2.30 g	
PCH-2	2.8 fgh	MR	3.9 ab	S	3.2 cd	MR	2.8 d-h	MR	3.1 de	MR	3.0 bcd	MR	3.13 bcde	
PCH-3	3.5 abc	S	3.9 ab	S	3.2 cd	MR	3.2 bcd	MR	3.1 de	MR	2.7 cde	MR	3.27 bc	**2.88 B**
PCH-4	3.3 b-e	MR	3.4 c-f	MR	2.9 def	MR	2.9 c-g	MR	3.0 ef	MR	2.7 cde	MR	3.03 cde	
PCH-5	2.5 ghi	MR	3.2 ef	MR	2.8 abc	MR	2.4 hi	R	2.7 fgh	MR	2.4 efg	R	2.67 f	

[1] Data followed by different letters in each column differ significantly ($p < 0.05$) according to Duncan's multiple range test.

[2] Cercospora leaf spot resistance levels based on disease severity scores are as follows: 1.0-2.4 = resistant (R), 2.5-3.4 = moderately resistant (MR), and 3.5-5.0 = susceptible (S).

[3] Data followed by different capital letters differ significantly ($p < 0.05$) among various provinces according to Duncan's multiple range test.

Table 3.10 (continued).

Isolates	Mungbean genotypes												Average	Grand average
	Susceptible				Moderately resistant				Resistant					
	EGMD-6D		SUT1		B2		CN84-1		V4718		Super5			
PB-1	3.7 ab	S	3.5 b-e	S	3.3 bc	MR	3.3 bc	MR	2.0 k	R	2.2 fg	R	3.00 de	**2.98 AB**
PB-2	3.6 ab	S	3.6 b-e	S	3.0 cde	MR	3.0 c-f	MR	2.3 ijk	R	2.3 efg	R	2.97 de	
PB-3	3.1 c-f	MR	3.4 c-f	MR	3.2 cd	MR	2.8 d-h	MR	2.9 efg	MR	3.1 cb	MR	3.08 bcde	
PB-4	3.0 def	MR	3.3 def	MR	3.0 cde	MR	2.6 f-i	MR	2.7 fgh	MR	2.7 cde	MR	2.88 ef	
NW-1	3.6 ab	S	3.5 b-e	S	3.3 bc	MR	3.1 b-e	MR	2.2 jk	R	2.0 g	R	2.95 de	
NW-2	3.4 a-d	MR	3.8 bc	S	3.6 b	S	3.5 b	S	2.0 k	R	2.3 efg	R	3.10 bcde	
NW-3	3.7 ab	S	3.9 ab	S	4.3 a	S	3.9 a	S	3.9 a	S	3.9 a	S	3.93 a	**3.15 A**
NW-4	2.4 hi	R	3.1 f	MR	2.6 fg	MR	2.5 ghi	MR	3.8 ab	S	2.9 bcd	MR	2.88 ef	
NW-5	2.7 f-i	MR	3.4 c-f	MR	2.5 g	MR	2.8 d-h	MR	3.4 cd	MR	2.6 def	MR	2.90 ef	

[1] Data followed by different letters in each column differ significantly ($p < 0.05$) according to Duncan's multiple range test.

[2] Cercospora leaf spot resistance levels based on disease severity scores are as follows: 1.0-2.4 = resistant (R), 2.5-3.4 = moderately resistant (MR), and 3.5-5.0 = susceptible (S).

[3] Data followed by different capital letters differ significantly ($p < 0.05$) among various provinces according to Duncan's multiple range test.

3.4.4 Genetic diversity of *C. canescens*

Genetic diversity of 20 isolates of *C. canescens* were studied using RAPD markers according to Joshi et al. (2006). RAPD primers used in these studies were OPD20, OPK7, OPK11, OPK12, OPK15, OPN4, OPL1, OPL19 and OPL20, all of these showed high polymorphisms in *C. canescens* isolates (Table 3.11). It was found that there was a total of 83 reproducible DNA bands, ranging from 8 to 16 DNA bands/primer. The DNA bands varied in size from approximately 200 to 2000 bp, were amplified from 20 isolates using 9 RAPD primers. Out of these, 53 were polymorphic (63.77%) indicating a high degree of polymorphisms. The average number of DNA bands observed per primer was 9.22 and the average number of polymorphic bands was 5.88. This average number of polymorphic bands was comparable to that of Joshi et al. (2006) who used 33 RAPD primers and reported an average of 4.70 polymorphic bands among 11 *C. canescens* isolates collected from various locations in India. In this study, we found that primer OPN4 showed the highest percent of polymorphic bands (88.89%) and the OPK11 showed the lowest percent of polymorphic bands (14.28%). The average percentage of polymorphic bands was 63.77%. The occurrence of polymorphisms could be influenced by various factors, including the specific sequences of the primers used, the genetic backgrounds of the analyzed materials, and the number of samples included in the analysis (Keim et al., 1992). The polymorphism information content (PIC) value reflects the genetic diversity at a particular locus. The range of PIC values in this study was 0.297 (OPK12) to 0.400 (OPK15), with an average of 0.347. Since RAPD markers are dominant markers, the maximum PIC value for any primer is only 0.5. Therefore, the fact that the RAPD markers used in this experiment, specifically 7 out of the 9 markers, had a PIC value ≥ 0.30, indicates their usefulness for studying the genetic diversity of *C. canescens* pathogen.

Genetic similarity of various isolates was calculated by NTSYS-PC program (version 2.02), using 53 polymorphic DNA bands. The similarity coefficients among the isolates ranged from 0.74 to 1.00, revealing genetic diversity in the pathogen population (Fig. 3.2). Mantel's test for cophenetic correlation coefficient of 0.91 ($p < 0.01$) indicated that the grouping of the isolates in the dendrograms significantly correlated with genetic similarity. The dendrogram constructed separated the 20 isolates into four groups, at a similarity level of 0.84. Group I consisted of six isolates from

Nakhon Ratchasima provinces, genetic similarity within the group varied between 0.84-1.00. The three isolates from Pak Thong Chai, Nakhon Ratchasima (PAK-1, PAK-2 and PAK-3) demonstrated a high degree of similarity (0.95-1.00). The five isolates from Nakhon Sawan provinces formed group II with NW-2, NW-3, NW-4 and NW-5 closely related to each other. Group III was formed by five isolates from Phichit province with genetic similarity within the group of 0.84-1.00 The lower virulence observed in the pathogenicity of PCH-1, when compared to the other isolates in the group, may be associated with the finding that PCH-1 exhibited the greatest dissimilarity among all the isolates within the group. Group IV was formed by 4 isolates from Phetchabun province which were more closely related to each other than those of other groups. Similarly, Poolsawat (2010), revealed from RAPD data that there were four major clusters of *S. ampelinum* population in Thailand. It is apparent that there was a higher degree of genetic variation among isolates from different geographical regions than within the same region.

Principle coordinate analysis (PcoA) was used to identify multidimensional relationships that describe portions of the genetic variance in a data set. Three-dimensional plots of PCoA based on these 9 RAPD markers were generally consistent with the UPGMA cluster analysis with the three coordinates accounting for 27.58, 24.40 and 17.01 percent of the total variance, respectively, with a total of 68.99 percent (Fig. 3.3). PCoA could clearly separate *C. canescens* isolates into 4 different groups, which differed mainly by provinces, as in the UPGMA cluster analysis, indicating that it is effective to generate a good separation of these materials. These results suggested that RAPD markers were suitable for determining the genetic diversity of *C. canescens* isolates due to its ability to yield a large number of polymorphic loci, and cost-effective features. Besides, RAPD does not require any prior information of DNA sequences like some other markers such as RFLP (Inglis et al., 2001; Almeida et al., 2005, Joshi et al., 2006).

The RAPD analysis revealed significant genetic diversity among the isolates, which is an important finding of this study. Among the 20 isolates studied, four distinct RAPD groups were identified. However, these groups could not be related with the pathogenicity or morphology of the isolates. This observation aligns with the findings of Goodwin et al. (2001), who also reported that molecular techniques such as RAPD

provide strong evidence of genetic diversity among fungi. The use of molecular markers, specifically RAPD, has significantly improved the accuracy of population genetics studies of *Cercospora* pathogens (Almeida et al., 2005; Joshi et al., 2006). In addition, RAPD has been successfully utilized to assess the genetic variability of *Cercospora* species in studies conducted by Inglis et al. (2001) and Nagl et al. (2007).

Table 3.11 Primer sequences, number of total scoreable DNA bands, number of polymorphic bands, percentages of polymorphism, and amplified band size for each RAPD primer used for the analysis of 20 isolates of *C. canescens*.

Primers	Primer sequences	No. of total bands	No. of polymorphic bands	Polymorphism (%)	Amplified band size (bp)	PIC[1]
OPD20	ACCCGGTCAC	8	5	62.50	200-1,000	0.312
OPK7	AGCGAGCAAG	16	12	75.00	200-1,500	0.340
OPK11	AATGCCCCAG	7	1	14.28	200-900	0.375
OPK12	TGGCCCTCAC	10	5	50.00	200-1,500	0.297
OPK15	CTCCTGCCAA	9	7	77.78	200-900	0.400
OPN4	GACCGACCCA	9	8	88.89	200-800	0.326
OPL1	GGCATGACCT	9	6	66.67	200-1,000	0.374
OPL19	GAGTGGTGAC	7	3	42.86	200-800	0.398
OPL20	TGGTGGACCA	8	6	75.00	200-1,000	0.299
Total		83	53			
Average		9.22	5.88	63.77		0.347

[1] PIC = polymorphism information content

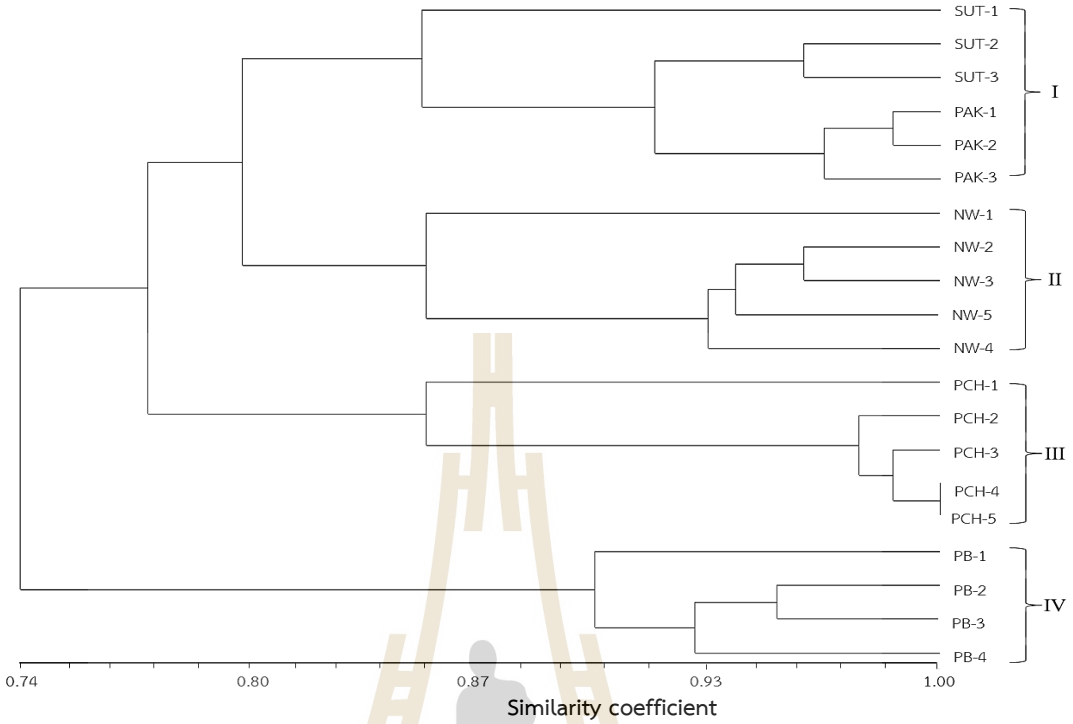


Figure 3.2 RAPD marker derived dendrogram of 20 isolates of *C. canescens*.

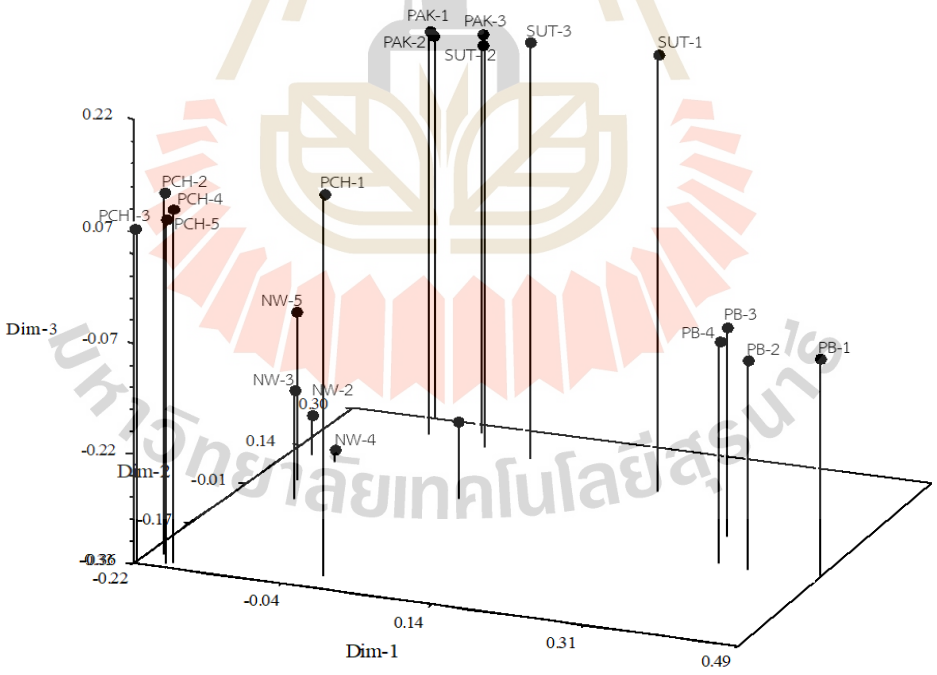


Figure 3.3 RAPD derived three-dimensional plot based on the first three principal coordinates from a principal coordinate analysis of 20 isolates of *C. canescens*.

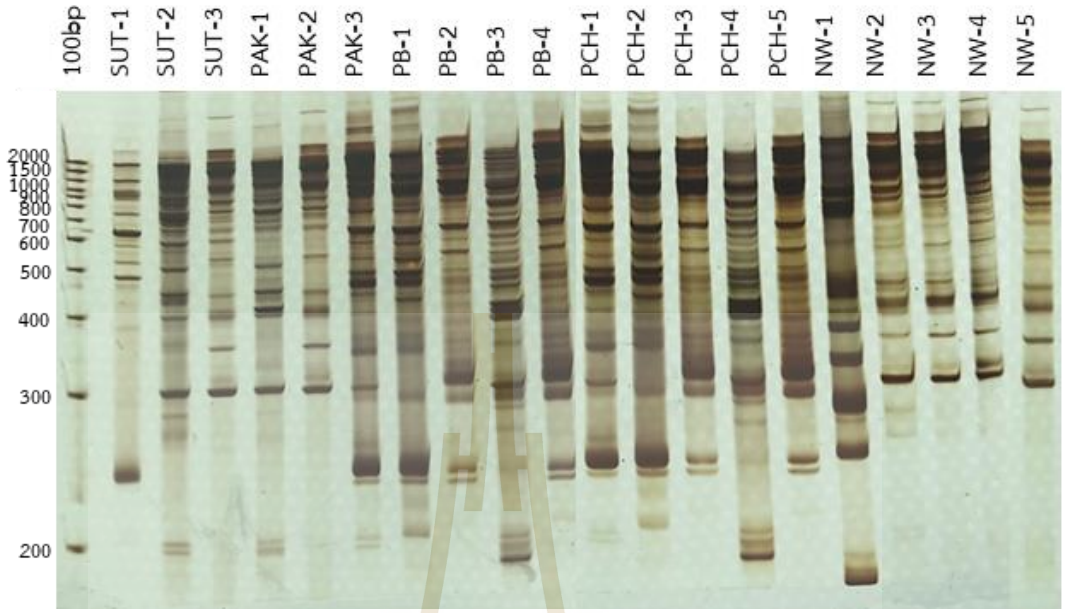


Figure 3.4 RAPD banding patterns amplified using RAPD OPK15 primer of 20 isolates of *C. canescens*.

3.5 Conclusion

This study provided valuable insights into the morphological characteristics, genetic diversity, and pathogenicity of 20 *C. canescens* isolates. The isolates demonstrated variations in morphological traits when grown on different media. The genetic similarity among the isolates was assessed using RAPD analysis, revealing a range of 0.85 to 1.00 genetic similarity among them. Through cluster analysis employing the UPGMA, the isolates were classified into four groups based on their respective provinces. Regarding pathogenicity, it was observed that the mungbean genotypes Super5 and V4718 exhibited resistance to the majority of the tested *C. canescens* isolates. This finding indicates that V4718 and Super5 can serve as valuable sources of resistance in future breeding programs. These insights will be instrumental in developing effective strategies to combat *C. canescens* disease in mungbean cultivation.

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CHAPTER 4

DIFFERENTIAL INDUCTION OF β-1,3-GLUCANASE, CHITINASE AND POLYPHENOL OXIDASE ACTIVITIES IN MUNGBEAN GENOTYPES IN RESPONSE TO *Cercospora canescens*

4.1 Abstract

Cercospora leaf spot (CLS) is a significant biotic stress that leads to reduced crop yield in mungbean. Consequently, it is crucial to investigate the resistance levels of different mungbean genotypes to CLS and develop effective strategies for disease management. In this study, the focus was on examining the role of biochemical defense in the resistance response of mungbean to CLS using a laboratory-grown plants. The defense responses of the six mungbean genotypes (EGMD-6D, SUT1, CN84-1, B2, V4718 and Super5) were analyzed by assessing the activities of three key enzymes: β-1,3-glucanase, chitinase, and polyphenol oxidase (PPO) in their infected leaves following CLS infection. CLS severity and hypersensitive response (HR) were observed, and the SDS-PAGE analysis was used for analyzing the protein profiles in infected leaves. The local and systemic induction of β-1,3-glucanase, chitinase and PPO after challenging with the pathogen was also compared using whole plant assay in four mungbean genotypes (EGMD-6D, CN84-1, V4718 and Super5). The systemic acquired resistance (SAR) of these four genotypes was evaluated in secondary CLS infection using detached leaf assay. In infected leaves of EGMD-6D, SUT1, CN84-1, B2, V4718 and Super5, the levels of β-1,3-glucanase and chitinase activities were significantly different ($p < 0.05$) among mungbean genotypes at every stages of inoculation (0, 1, 2, 3 and 4 DAI). The resistant genotypes V4718 and Super5 showed elevated levels of the three enzymes, which were significantly higher than those of susceptible and moderately resistant genotypes in infected leaves. Consistent with the enzyme activities, the mungbean genotypes demonstrated varying levels of hypersensitive response (HR) symptoms. The resistant genotypes, V4718 and Super5, exhibited HR symptoms, while the susceptible and moderately resistant genotypes did

not. In the SDS-PAGE analysis of infected leaves, two up-regulated protein bands were found: one at 17 kDa and the other at 39 kDa. The 17 kDa band was exclusively present in susceptible mungbean genotypes EGMD-6D and SUT-1. While The 39 kDa band was expressed in most mungbean genotypes, except for Super5, a resistant genotype that already showed this band at pre-inoculation stage (0 DAI). For the local and systemic induction of the three enzymes after challenging with the *C. canescens*, the results showed that all four mungbean genotypes exhibited local activation of β-1,3-glucanase, chitinase and PPO activities in the infected leaves. Nevertheless, it is crucial to note that this investigation did not uncover any indication of systemic induction of these three enzymes in the upper or lower non-inoculated leaves. At the peak induction stage, the activity levels of β-1,3-glucanase, chitinase, and PPO in infected leaves experienced noteworthy increments across all genotypes when compared to the levels at 0 DAI. Among these genotypes, the resistant genotype Super5 displayed the highest activities for all three enzymes, followed by the resistant genotype V4718 and the moderately resistant CN84-1. In contrast, the susceptible genotype EGMD-6D exhibited the lowest β-1,3-glucanase, chitinase, and PPO activities. Remarkably, these activity levels aligned with the observed resistance levels in each genotype. The assessment of SAR in secondary infection of CLS, conducted through a detached leaf assay, revealed that resistant genotypes exhibited lower disease severity scores in both upper and lower distal leaves compared to susceptible and moderately resistant genotypes. This observation suggests that the resistant genotypes possessed more effective defense mechanisms against the pathogen. However, the severity scores of all mungbean genotypes were not significantly different at 0, 1, 2, 3, and 4 DAI, indicating that prior inoculation with *C. canescens* does not induce systemic acquired resistance in mungbean genotypes. The differential expression of these proteins implies variations in resistance mechanisms among different mungbean genotypes. This observation suggests that β-1,3-glucanase, chitinase and PPO may play prominent roles in the local defense mechanisms of mungbean against CLS, serving as biochemical markers for disease resistance. The findings indicate the potential utility of β-1,3-glucanase, chitinase, and PPO as early screening markers for disease resistance in crop plants. These biochemical markers can help identify mungbean genotypes with enhanced resistance to CLS, facilitating the development of strategies for breeding programs to resist the disease effectively.

4.2 Introduction

Cercospora leaf spot (CLS) is a fungal disease that affects mungbean (*Vigna radiata* (L.) Wilczek var. *radiata*) plants. It is caused by the fungus *Cercospora canescens.* CLS is a major foliar disease that can significantly reduce crop yield and quality. The disease typically manifests as small, circular to irregular-shaped lesions on the leaves of infected mungbean plants. These lesions initially appear as tiny brown or dark spots, which later enlarge and turn gray or tan. As the disease progresses, the lesions may coalesce, leading to extensive leaf damage. Severe infections can cause defoliation, reducing the plant's photosynthetic capacity and overall productivity (Vasudeva, 1963; Vakili, 1977; Grewal et al., 1980). Early detection and proper management of CLS are crucial for decreasing its impact on mungbean and ensuring optimal yield and quality. Regular scouting and monitoring of the fields with appropriate disease management practices including the use of disease resistant cultivars, are essential for effectively controlling CLS in mungbean cultivation. Therefore, one of the key objectives of breeding programs for mungbean is to develop cultivars that are resistant to the CLS.

Plants possess two distinct disease resistance mechanisms, namely preformed and induced defense. For defense against pathogen attachment, invasion, and infection, plant tissues contain numerous preformed structural barriers such as lignin, cutin, waxes, trichomes, and several chemicals such as alkaloids, phenolics, and saponins (Ahmed, 1949; Sindhan et al., 1999; Garain et al., 2004; Mir et al., 2012; Sunil and Benagi, 2013). While induced defense mechanisms in plants involve: hypersensitive response (HR), locally induced defense and systemic acquired resistance (SAR). HR triggers localized plant cell death at the site of infection, while SAR induces resistance in distant plant parts from the initial infection site. These defense responses result in increased production of various defense proteins, including pathogenesis-related (PR) proteins such as β-1,3-glucanase and chitinase as well as polyphenol oxidase (PPO) (Jones and Dangl, 2006; Walters et al., 2009). These proteins have been associated with resistance against fungal diseases in legumes. Studies have demonstrated their involvement in plant defense against pathogens, highlighting their importance in enhancing resistance mechanisms in plants (Vallad and Goodman, 2004; Abdel et al., 2013). Koche and Chaudhary (2019) reported that resistant mungbean genotype had higher accumulation of PR-proteins (chitinase and β-1,3-glucanase), phytoalexin

(genistein) and PAL under both field and in vitro conditions after spraying with cercospora cell wall elicitor derived from *C. canescens*. Nevertheless, up until now, no study has investigated the higher accumulation of β-1,3-glucanase, chitinase, and PPO in mungbean following infection with *C. canescens.*

By focusing on inducing or enhancing the resistance of mungbean cultivars to CLS, plant breeders aim to harness the potential of these biochemical defenses. Through selective breeding, they seek to develop cultivars that possess higher levels of these enzymes. Therefore, present study is focused on investigating the role of β-1,3-glucanase, chitinase and PPO in the defense responses of mungbean against CLS. The study involves analyzing the presence and activities of these enzymes in the leaves of mungbean genotypes subjected to CLS infection, both locally and systemically. The systemic acquired resistance (SAR) was also evaluated in secondary CLS infection using detached leaf assay. The identification and understanding of these enzymes and their contribution to plant defense against CLS provide valuable insights for breeders. This knowledge enables breeders to select and cross plants with enhanced resistance traits, ultimately leading to the development of improved mungbean cultivars that are better equipped to combat the disease.

4.3 Materials and methods

4.3.1 Mungbean resistance mechanisms to *C. canescens* in infected leaves

The study of resistance mechanisms in infected leaves was performed in two susceptible mungbean genotypes (EGMD-6D and SUT1), two moderately resistant to susceptible mungbean genotypes (CN84-1 and B2) and two resistant mungbean genotypes (V4718 and Super5). Twenty-one days old mungbean plants were inoculated in whole plant assay with 6 droplets of 5 µl *C. canescens* mycelium inoculum (80 mycelium disks/5 mL) at the second node leaves from the top. At 0, 1, 2, 3 and 4 DAI, the infected leaves were collected, observed for disease severity and HR, and extracted for their total protein, β-1,3-glucanase, chitinase and PPO activity determination and SDS analysis. The experiments were carried out at least 2 times with 3 replications. A CRD with 3 replications (1 plant per replication) were used. The experimental data were analyzed by analysis of variance (ANOVA) and mean comparison was performed by Duncan's multiple range test (DMRT) using SPSS version 16.0 (Levesque and SPSS Inc., 2006).

4.3.1.1 Protein extraction

Mungbean leaf total protein was extracted using prechilled mortar and pestle to a fine powder in liquid nitrogen. For determination of total protein, β-1,3-glucanase and chitinase activities, the samples were mixed with 1 ml extraction buffer [0.1 M sodium citrate (pH 5.0), 0.1 M KCl, 1% (v/v), Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml leupeptin, 1 mM EDTA and 3% (w/v) polyvinylpyrrolidone (PVPP)] (modified from Thipyapong et al., 1995). For PPO activity, the samples were mixed with 1 ml extraction buffer [0.1 M sodium phosphate buffer (pH 7.0), 0.1 M KCl, 1% (v/v), Triton X-100, 1 mM PMSF, 1 µg/ml leupeptin, 1 mM EDTA and 3% (w/v) PVPP] (Thipyapong et al., 1995). Then homogenate was centrifuged at 4°C, 12,000 rpm for 30 minutes and their supernatant were kept in Eppendorf tubes at -20°C for further use.

4.3.1.2 Total protein content and protein profile evaluation

The homogenates from 4.3.1.1 were used for estimation of total protein by using Bradford (1976) method with bovine serum albumin (BSA) as standard. Protein concentration was estimated from standard graph by the linear regression's equation and was calculated for total protein using the equation as described below;

$$\text{Protein concentration } (\mu g/\mu L) = \frac{\text{Total protein } (\mu g)}{\text{Volume of homogenate } (\mu L)} \quad (1)$$

The proteins were separated by molecular weight using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique as modified from Laemmli (1970); 12% separating gel and 4% stacking gel, run with 100 volts of electricity for 95 minutes. Protein bands were stained with 0.1% (w/v) silver nitrate. Prestained precision plus protein dual xtra (2-250 kDa) was used as protein standard. Protein bands were photographed and analyzed by Gel documentation program. The experiment was repeated at least two times.

4.3.1.3 β-1,3-glucanase and chitinase activities

β-1,3-glucanase activity was evaluated by techniques of Kauffman et al. (1987). The assay mixture contained 5 µl substrate (1% (w/v) laminarin) and 5 µl homogenate, and was incubated at 37°C for 30 minutes. Then 50 µl of stop solution

(1% (w/v) dinitrosalicylic acid (DNS) reagent) was added, and mixture was heated to 100°C for 8 minutes. The mixture was then cooled to room temperature and 300 µl of distilled water was added to each sample, and absorbance was recorded at 500 nm. The chitinase activity assay was conducted following the method outlined by Reissig et al. (1955). To perform the assay, a mixture consisting of 20 µl of substrate (1% (w/v) colloidal chitin) and 20 µl of homogenate was prepared. This mixture was then incubated at 37°C for a duration of 1 hour. After incubation, 60 µl of stop solution (1% (w/v) DNS reagent) was added, and the entire mixture was heated to 100°C for 20 minutes. Following the heating step, the mixture was cooled down to room temperature and subsequently subjected to centrifugation at 12,000 rpm for 5 minutes. The resulting clear supernatant was carefully collected, and each sample was supplemented with 200 µl of distilled water. Finally, the absorbance of the samples was measured at a wavelength of 520 nm. The experiment was repeated at least two times. Four replications were used for the experiment.

4.3.1.4 Polyphenol oxidase (PPO) activity

PPO activity was determined according to Thipyapong et al. (2004). The assay mixture contained 1 ml of 96 µM 2-nitro-5-thiobenzoic acid and 2 mM catechol in 100 mM Tris-HCl, pH 7.0. Catalase (280 units in 10 µl 100 mM Tris-HCl, pH 7.0) was add to eliminate peroxidase contribution to catechol oxidation. Then absorbance was recorded every 1-minute interval up to 3 minutes at 420 nm and the activity was expressed as µmol quinone formed/min/mg protein. An experiment was repeated at least two times.

4.3.1.5 Hypersensitive response (HR)

Infected mungbean leaves were stained with 1% (w/v) trypan blue solution [trypan blue, 85% w:w lactic acid, phenol TE buffer equilibrated, pH 7.5-8.0, glycerol ≥ 99% and distilled water] by using Fernández et al. (2016) method. At 0, 1, 2, 3 and 4 DAI, HR cell death was observed under light microscope for number and diameter of necrotic lesion using ocular micrometer. Four replications were used for the experiment and repeated at least two times.

4.3.2 Systemic acquired resistance (SAR)

The investigation of systemic acquired resistance was performed in four mungbean genotypes, one susceptible genotype EGMD-6D, one moderately resistant to susceptible genotype CN84-1, and the two resistant genotypes V4718 and Super5. In the whole plant assay, mungbean plants aged twenty-one days were inoculated with six droplets of 5 µl *C. canescens* mycelium inoculum (80 mycelium disks in 5 mL) at the second node leaves from the top. At 0, 1, 2, 3 and 4 DAI, mungbean leaves at the first, second and third leaf nodes were collected, extracted for their total protein, chitinase, β-1,3-glucanase and PPO activity determination to evaluate their local and systemic induction according to the methods as previously described. Mungbean leaves at 0, 1, 2, 3 and 4 DAI were also used to observe the reduction of necrotic lesions in secondary infection of *C. canescens* using detached leaf assay. Six leaf pieces (1 cm^2) were placed on 90 mm diameter petri dish and inoculate with 6 droplets of 3 µl of *C. canescens* inoculum (80 mycelium disks/5 mL). The inoculated leaf pieces were kept for 3 days at 27°C with 12 light hours/day (1,500 lux) in growth chamber. Disease severity was assessed by rating the symptoms expression on a 1 to 5-point scale according to Ngegba et al. (2017). The experiment was repeated at least two times. Three replications were used for the experiment (1 plant/replication). β-1,3-glucanase, chitinase and PPO activities, and disease severity scores were used to evaluate the resistance status and resistance mechanisms of mungbean genotypes against CLS.

4.4 Results and discussion

4.4.1 Changes in protein profiles of mungbean genotypes following *C. canescens* inoculation using SDS-PAGE

In the SDS-PAGE analysis of protein profiles in infected leaves using calibrated protein markers (ranging from 2-250 kDa), two distinct up-regulated protein bands were observed. These bands had molecular weights of 17 kDa and 39 kDa. The 17 kDa protein band was exclusively induced in the susceptible mungbean genotypes EGMD-6D and SUT-1, and its presence was detected in the leaves between 2-4 DAI in EGMD-6D and between 1-4 DAI in SUT1. This finding suggests that these two susceptible genotypes may possess different resistance mechanisms compared to the resistant and

moderately resistant genotypes (Figure 4.1). On the other hand, the 39 kDa protein band was induced in most mungbean genotypes, except for the mungbean genotype Super5, which had already exhibited the 39 kDa protein band before inoculation (0 DAI). Interestingly, the presence of the 39 kDa protein band at 0 DAI was unique to the resistant mungbean genotype Super5. This observation indicates that the resistance mechanisms employed by Super5 may differ from other genotypes. The differential expression of these proteins suggests variations in the resistance mechanisms employed by different mungbean genotypes.

The observed up-regulated proteins in this study may be associated with PR-proteins. Plants have developed diverse defense mechanisms to protect themselves against pathogens, including the production of small-molecule secondary metabolites, proteins, and peptides with antifungal activities (Singh et al., 2014). These findings align with previous researches that have reported the induction of β-1,3-glucanases, which are classified as "pathogenesis-related protein 2" (PR2) and typically have molecular weights ranging from 33 to 44 kDa (Hong and Meng, 2004; Saikia et al., 2005). In their study, Tonón et al. (2001) observed the expression of a 39 kDa protein and a significant increase in β-1,3-glucanase activity in potato tubers infected with *Phytophthora infestans*. Similarly, Kabir et al. (2016) reported the antifungal activity of a 39 kDa chitinase derived from *Trichosanthes dioica* seeds against *Aspergillus niger* and *Trichoderma* sp. Furthermore, Golshani et al. (2015) found that many chitinases with molecular weights ranging from 15 kDa to 43 kDa were induced in plants in response to disease infection. Jayasankar et al. (2000) reported that resistant grapevine lines derived from embryogenic cultures exhibited an up-regulated protein band at approximately 36 kDa, which was potentially related to chitinase and was not observed in the non-selected control group. Based on these findings, the 17 kDa protein observed in this study may be an isoform of chitinase which was specifically induced in susceptible genotypes, while the 39 kDa protein may be either a chitinase or a β-1,3-glucanase. Alternatively, these proteins could perform other functions. However, further analysis is required to confirm this hypothesis. This information will be invaluable for mungbean breeding programs aiming to enhance CLS resistance in future cultivars.

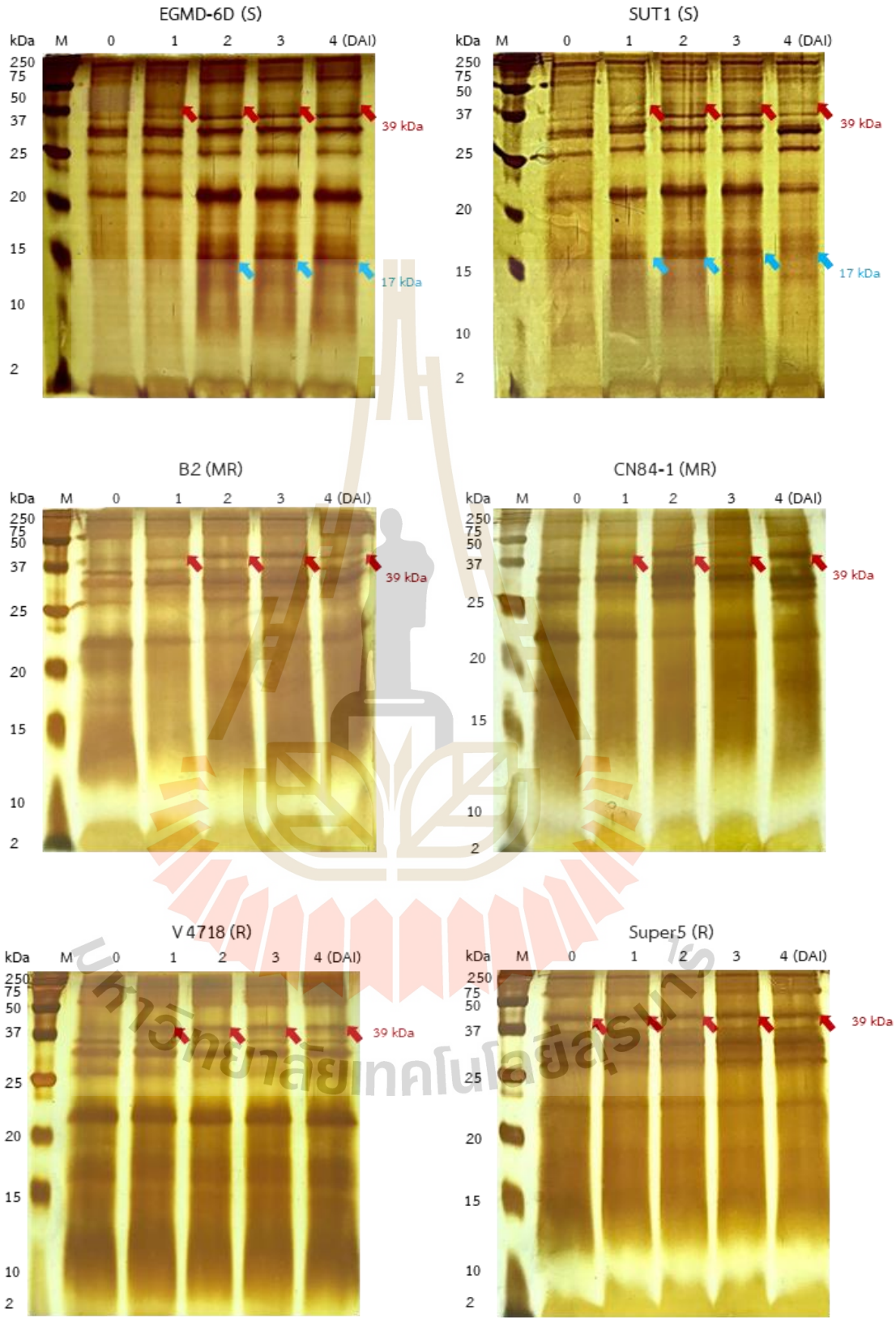

Figure 4.1 Protein profile changes of 6 mungbean genotypes at 0-4 DAI following *C. canescens* inoculation using SDS-PAGE.

4.4.2 β-1,3-glucanase and chitinase activities

The quantitative β-1,3-glucanase and chitinase activities were evaluated in six mungbean genotypes including EGMD-6D, SUT1, B2, CN84-1, V4718 and Super5 at 0-4 days after *C. canescens* inoculation. At the pre-inoculated stage (0 day), the levels of β-1,3-glucanase and chitinase activities were significantly different among mungbean genotypes ($p < 0.05$). In comparison to all other mungbean genotypes, the CLS resistant genotype Super5 showed the highest constitutive β-1,3-glucanase activity level (1,336.58 μmoles/min/mg protein (Figure 4.2). When the chitinase activity was considered, the highest constitutive chitinase activity level was also observed in CLS resistant genotype Super5 (81.40 μmoles/min/mg protein), which were significantly higher than all other mungbean genotypes (Figure 4.3).

β-1,3-glucanase activity levels slightly increased 1 DAI in all mungbean genotypes. The moderately resistant genotypes CN84-1 demonstrated the highest increased rate of 1.56-fold, and the susceptible genotype EGMD-6D showed the lowest increase rate of 1.13-fold when compared with the initial time (0 day) (Figure 4.2). At 2 DAI, β-1,3-glucanase activity level was continuously increased in all mungbean genotypes and the highest β-1,3-glucanase activity increased rate of 1.92-fold when compared with the initial time was observed in the moderately resistant genotype B2 (1,043.35 μmoles/min/mg protein). The lowest increased rate of 1.22-fold was observed in the susceptible genotype EGMD-6D (538.21 μmoles/min/mg protein). At 3 DAI, β-1,3-glucanase activity levels was still continuously increased in most mungbean genotypes, except for the susceptible genotype EGMD-6D that showed the decreased rate of activity. The moderately resistant genotypes CN84-1 demonstrated the highest increased rate of 2.41-fold when compared with the initial time (1,744.61 μmoles/min/mg protein). The susceptible genotype EGMD-6D was observed with the lowest increased rate of 1.15-fold (503.72 μmoles/min/mg protein). At 4 DAI, β-1,3-glucanase activity levels decreased from 3 DAI in all mungbean genotypes, but still remained significantly higher than those of 0 DAI.

In summary, the highest induction of β-1,3-glucanase activity levels was observed at 3 DAI in all mungbean genotypes, except for the susceptible genotype EGMD-6D that showed the highest induced activity at 2 DAI and started to decrease at 3 DAI. During the peak induction stage, the β-1,3-glucanase activity levels of all

mungbean genotypes were significantly higher than those at 0 DAI ($p < 0.05$). At the highest β-1,3-glucanase activity levels, the resistant genotype Super5 demonstrated the highest activity (2,495.80 μmoles/min/mg protein), followed by V4718, CN84-1, B2 and SUT1 (2191.81, 1744.61, 1227.03 and 662.51 μmoles/min/mg protein, respectively). The lowest β-1,3-glucanase activity was observed in susceptible genotype EGMD-6D (503.72 μmoles/min/mg protein). Moreover, maximum levels of β-1,3-glucanase activities of both resistant genotypes (Super5 and V4718) were significantly ($p < 0.05$) higher than those of moderately resistant and susceptible genotypes (1.43 to 4.64-folds and 1.26 to 4.07-folds, respectively).

The chitinase activity levels in most mungbean genotypes were induced within 1 DAI except for SUT1, B2 and CN84-1, ranging from 1.22- to 1.46-fold increase (Figure 4.3). Among the genotypes, the resistant genotype V4718 exhibited the highest increase in chitinase activity at 1 DAI (1.46-fold), followed by EGMD-6D and Super5 with fold increases of 1.22 and 1.21, respectively. The chitinase activity levels continued to increase in all genotypes at 2 DAI, with the highest increase observed in the resistant genotype V4718 (2.40-fold increase, 145.48 μmoles/min/mg protein). The moderately resistant genotype CN84-1 showed the lowest induction rate (1.21-fold increase, 62.58 μmoles/min/mg protein). At 3 DAI, most genotypes showed a continuous increase in chitinase activity levels, except the susceptible genotype EGMD-6D, which exhibited a decrease in activity. By 4 DAI, the chitinase activity levels had decreased in all mungbean genotypes to the levels comparable or lower than those of the initial time (0 DAI).

The results indicated that the highest induction of chitinase activity levels occurred at 3 DAI in all mungbean genotypes, except the susceptible genotype EGMD-6D, which exhibited the highest induction at 2 DAI. At the highest induction, the chitinase activity levels of all mungbean genotypes were significantly higher than those at 0 DAI ($p < 0.05$). During the peak induction stage, the resistant genotype V4718 displayed the highest chitinase activity (183.80 μmoles/min/mg protein), followed by Super5, CN84-1, B2, and SUT1 (129.80, 102.46, 73.93, and 58.04 μmoles/min/mg protein, respectively). The susceptible genotype EGMD-6D exhibited the lowest chitinase activity (47.59 μmoles/min/mg protein). Similar to the β-1,3-glucanase activities, the levels of chitinase activities in the resistant genotypes (V4718 and Super5)

were significantly ($p < 0.05$) higher compared to the moderately resistant and susceptible genotypes, with fold increases ranging from 1.79 to 3.86 and 1.27 to 2.73, respectively.

Before inoculation, the constitutive levels of β-1,3-glucanase and chitinase activities varied significantly among mungbean genotypes. The resistant genotypes Super5 and V4718 had the highest β-1,3-glucanase activities, which were significantly higher compared to those of the moderately resistant and susceptible genotypes. Similarly, Solanki et al. (2015) reported that fusarium wilt resistant tomato varieties had the highest average β-1,3-glucanase activity at the pre-inoculation stage, followed by moderately resistant and susceptible varieties. Similar to the findings of Ebrahim et al. (2011), resistant mango cultivars had the highest β-1,3-glucanase and chitinase activities, which were higher than those of the susceptible mango cultivar. Our results suggest that both Super5 and V4718 resistant genotypes have higher amounts of constitutively expressed PR proteins. During the initial stages of infection, β-1,3-glucanase and chitinase activities were found to be higher in resistant genotypes than in moderately resistant and susceptible genotypes. Particularly at 3 DAI, the β-1,3-glucanase activity of the resistant genotype Super5 was 1.14 to 4.95-fold higher than those of the rest of mungbean genotypes. And at 3 DAI, the resistant genotype V4718 exhibited the highest chitinase activity level, which was 1.42 to 3.07 -fold higher than the rest of mungbean genotypes. In summary, induced β-1,3-glucanase and chitinase activities were higher in two resistant mungbean genotypes than they were in moderately resistant and susceptible genotypes. Such changes usually occur from the up- and down-regulation of a broad variety of plant genes that mainly results in the production of PR-proteins. Compared to susceptible genotypes, these changes appeared to be induced to higher levels in resistant ones (Sudisha et al. 2011).

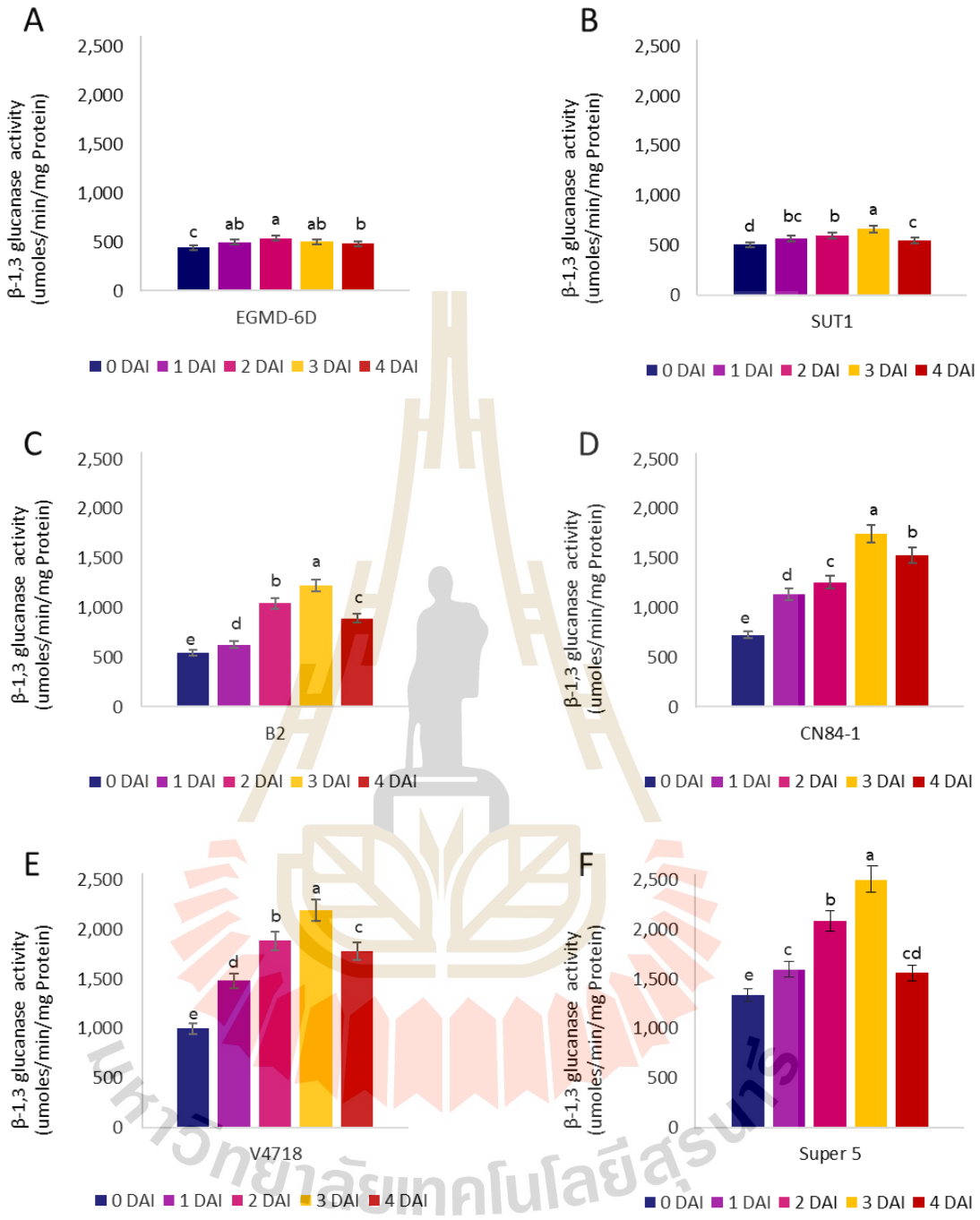

Figure 4.2 Changes in β-1,3-glucanase activities (μmoles/min/mg protein) of 6 mungbean genotypes (EGMD-6D (A), SUT1 (B), B2 (C), CN84-1(D), V4718 (E) and Super5 (F)) after inoculation with *C. canescens* for 0, 1, 2, 3 and 4 days. Data followed by different letters in each genotype differ significantly ($p < 0.05$) according to Duncan's multiple range test.

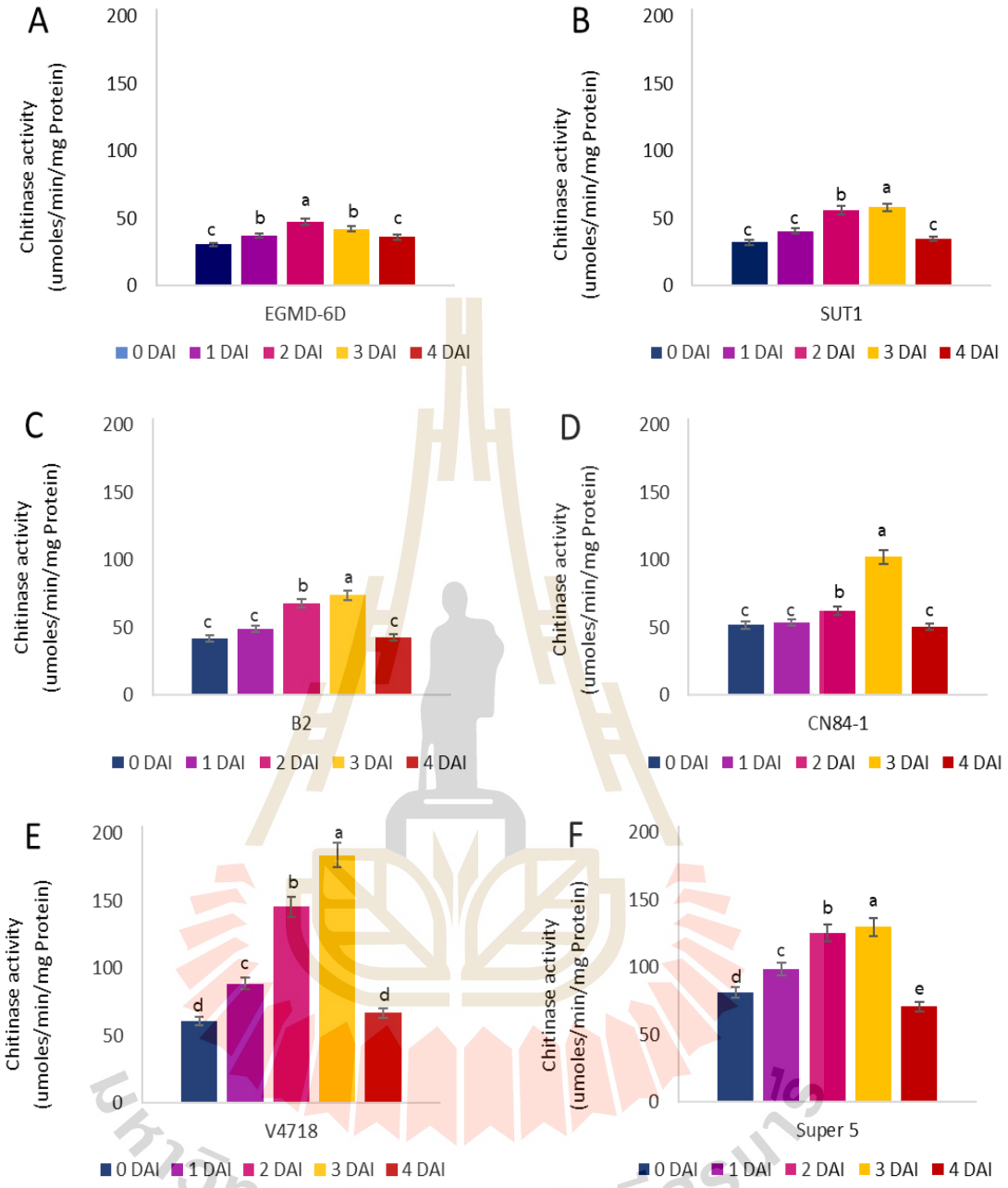

Figure 4.3 Changes in chitinase activities (μmoles/min/mg protein) of 6 mungbean genotypes (EGMD-6D (A), SUT1 (B), B2 (C), CN84-1(D), V4718 (E) and Super5 (F)) after inoculation with *C. canescens* for 0, 1, 2, 3 and 4 days. Data followed by different letters in each genotype differ significantly ($p < 0.05$) according to Duncan's multiple range test.

4.4.3 Polyphenol oxidase (PPO) activities

The PPO activities were evaluated in six mungbean genotypes EGMD-6D, SUT1, B2, CN84-1, V4718 and Super5 at 0-4 days after *C. canescens* inoculation. The levels of PPO activity at the pre-inoculated stage (0 day) were significantly different among mungbean genotypes ($p < 0.05$). The CLS resistant genotype V4718 showed the highest PPO activity level (0.81 μmoles quinone formed/min/mg protein), which was significantly higher than all other mungbean genotypes (Figure 4.4). At 1 DAI, PPO activity slightly increased in most mungbean genotypes excepted for CN84-1. The moderately resistant genotypes B2 showed the highest increased rate of 1.29-fold when compared with the initial time (0 day). The PPO activity levels continuously increased in all mungbean genotypes at 2 DAI. The highest PPO activity increased rate of 1.40-fold was observed in moderately genotype B2 when compared with the initial time. The lowest increased rate of 1.06-fold was observed in the moderately resistant genotype CN84-1. At 3 DAI, PPO activity levels tended to decrease or remained the same as those at 2 DAI in all mungbean genotypes, except for susceptible genotype SUT1 that still showed the increase in PPO activities. At 4 DAI, the PPO activity levels decreased from those at 3 DAI in all mungbean genotypes.

Our results demonstrated that the highest induction of PPO activity levels was observed at 2 DAI in all mungbean genotypes, except for the susceptible genotype SUT1 and resistant genotype Super5 that showed the highest induced activity at 3 DAI and started to decrease at 4 DAI. During the peak induction stage, all mungbean genotypes had significantly higher PPO activity levels than those at 0 DAI ($p < 0.05$). The resistant genotype V4718 showed the highest PPO activity (0.92 moles quinone formed/min/mg protein), followed by Super5, B2, EGMD-6D and CN84-1 (0.85, 0.81, 0.75 and 0.72 μmoles quinone formed/min/mg protein, respectively). The lowest PPO activity was observed in susceptible genotype SUT1 (0.70 μmoles quinone formed/min/mg protein). Moreover, maximum levels of PPO activities of both resistant genotypes (V4718 and Super5) were significantly ($p < 0.05$) higher than those of moderately resistant and susceptible genotypes (1.14 to 1.28-folds and 1.06 to 1.19-folds, respectively).

Before inoculation, the levels of PPO activities varied significantly among mungbean genotypes. The resistant genotypes V4718 and Super5 had the highest constitutive PPO activities, which were significantly higher compared to those of the moderately resistant and susceptible genotypes. Similar with the study of Li and Steffens (2002), which reported that transgenic resistant tomato that produced 5- to 10-fold more PPO activities had increased *Pseudomonas syringae* resistance, and had 15-fold fewer lesions and 100-fold fewer bacteria in infected leaves than control and susceptible plants. PPOs have frequently been hypothesized to play a role in plant defense against pests and pathogens due to their wound and pathogen inducibilities. (Mayer and Harel 1979; Thipyapong and Steffens 1997). In response to fungal infection, plants may also increase the production and accumulation of phenolic compounds, which serve as substrates for PPO. These compounds can act as chemical barriers against fungi by inhibiting their growth and development. In addition, localized inoculation of tomato leaflets with *P. syringae* resulted in a significant increase in PPO activities and systemic resistance to subsequent *P. syringae* infection (Stout et al., 1999). During the initial stage of infection, the mungbean genotypes V4718 and Super5, which exhibit resistance, displayed elevated levels of PPO activities. This observation suggests that resistant genotypes may possess a heightened ability to restrict plant disease development through increased PPO-mediated phenolic oxidation. Specifically, at 2 DAI, the PPO activity in the resistant genotype V4718 was found to be 1.03 to 1.24 times higher compared to the moderately resistant and susceptible mungbean genotypes. The differences observed in PPO induction patterns among these genotypes may be attributed to genetic variation, similar to the variances observed in β-1,3-glucanase and chitinase activities. This suggests that the diverse responses in PPO, β-1,3-glucanase, and chitinase activities are likely influenced by genetic variations among the mungbean genotypes.

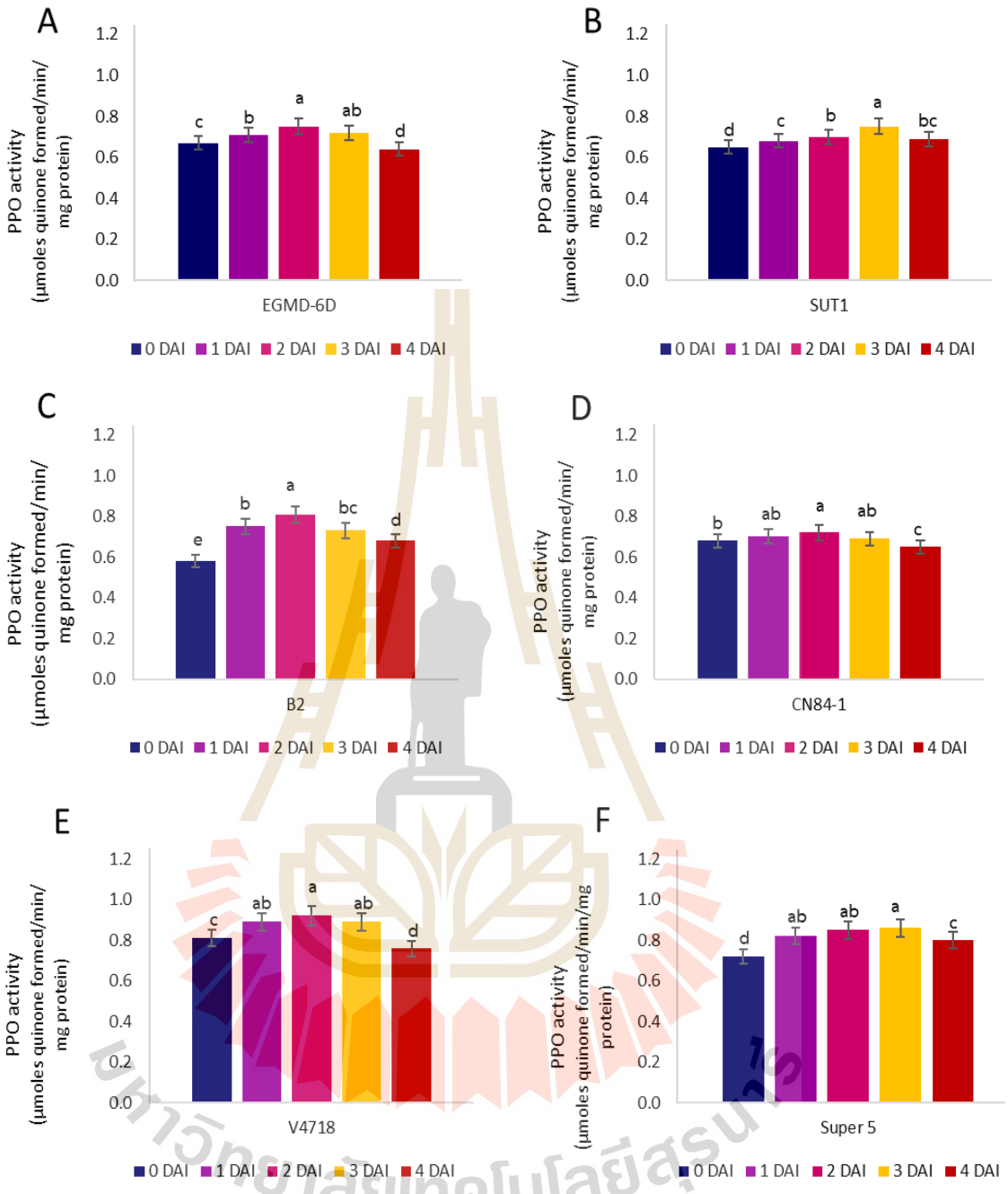

Figure 4.4 Changes in PPO activities (µmoles quinone formed/min/mg protein) of 6 mungbean genotypes (EGMD-6D (A), SUT1 (B), B2 (C), CN84-1(D), V4718 (E) and Super5 (F)) after inoculation with *C. canescens* for 0, 1, 2, 3 and 4 days. Data followed by different letters in each genotype differ significantly ($p < 0.05$) according to Duncan's multiple range test.

4.4.4 Hypersensitive response (HR)

The disease severity scores, HR and diameter of necrotic lesions were evaluated in six mungbean genotypes EGMD-6D, SUT1, B2, CN84-1, V4718 and Super5 at 0-4 days after *C. canescens* inoculation. The results showed that mungbean leaves started to show necrotic lesions at 1 DAI in those of moderately resistant and susceptible genotypes, but not appeared in resistant genotypes (V4718 and Super5) (Table 4.1). At 2 DAI, the disease severity scores continued to slightly increase in those of moderately resistant and susceptible genotypes. The resistant genotype V4718 started to exhibit the necrotic lesions, but the resistant genotype Super5 still did not show the disease symptom. All mungbean genotypes showed necrotic lesions at 3 DAI and the susceptible genotypes EGMD-6D showed the highest disease severity score of 3.42. While the resistant genotypes Super5 and V4718 showed much lower disease severity scores of 1.67 and 1.75, respectively. At 4 DAI, severity of symptoms was highly significantly different among mungbean genotypes (p <0.01). Two mungbean resistant genotypes Super5 and V4718 were potentially resistant to *C. canescens* isolate PAK-1 because they were developing least symptoms than those of other genotypes, with average severity scores of 2.0 and 2.17, respectively. The susceptible mungbean genotypes EGMD-6D and SUT1 were the most susceptible with average severity score of 3.83 and 3.75, respectively, whose severity score was significantly higher than those of other genotypes.

The HR was observed at 2 DAI in resistant genotype V4718 while the other genotypes did not show the HR symptom (Table 4.1). At 3 DAI, the number of HR continued to increase in V4718 and started to appear in resistant genotype Super5, but still did not show in those of moderately resistant and susceptible genotypes. At 4 DAI, we found that resistant genotypes Super5 and V4718 showed high percentage of HR (91.67 and 83.33 percent), while those of moderately resistant and susceptible genotypes did not show the HR symptom. The occurrence of HR corresponded with disease severity score and diameter of necrotic lesions which were much smaller in the resistant genotypes Super5 and V4718 than other genotypes. This study revealed that HR occurred exclusively in the resistant genotypes (Super5 and V4718), characterized by significantly higher levels of β-1,3-glucanase, chitinase, and PPO activities compared to the moderately resistant and susceptible genotypes in previous

study (4.4.2 and 4.4.3). The formation of HR lesions is a critical defence strategy employed by plants to limit the damage caused by pathogens. By sacrificing a small area of tissues through cell death, the plant contains the infection and prevents it from spreading to healthy tissues. Additionally, study of Garza et al. (2001) reported that the resistant genotypes of *Phaseolus vulgaris* showed the HR on the infected area of *Apion godmani*, but did not show in susceptible genotypes. The different defense mechanisms among genotypes may affect the disease severity when the pathogen attacked the plant.

Table 4.1 Changes in disease severity scores, number of HR and diameter of necrotic lesions of 6 mungbean genotypes after inoculation with *C. canescens* (isolate PAK1) for 0, 1, 2, 3 and 4 days.

Mungbean genotypes	Disease severity scores[1]								Number of HR[2]					Diameter of necrotic lesions (mm)[3]				
	Days after inoculation								Days after inoculation					Days after inoculation				
	0	1		2		3		4	0	1	2	3	4	0	1	2	3	4
EGMD-6D	1	1.67 b[4]	R	2.75 a	MR	3.42 a	MR	3.83 a	S	0/36	0/36	0/36	0/36	0/36	0	0.58 a	0.78 a	1.53 a	1.77 a
SUT1	1	1.75 b	R	2.25 b	R	2.83 b	MR	3.75 a	S	0/36	0/36	0/36	0/36	0/36	0	0.59 a	0.77 a	1.56 a	1.78 a
B2	1	1.42 c	R	2.17 b	R	2.75 b	MR	3.42 b	MR	0/36	0/36	0/36	0/36	0/36	0	0.45 a	0.78 a	1.19 b	1.53 b
CN84-1	1	2.17 a	R	2.92 a	MR	3.17 a	MR	3.58 b	S	0/36	0/36	0/36	0/36	0/36	0	0.43 a	0.79 a	1.12 b	1.48 b
V4718	1	1.00 d	R	1.25 c	R	1.75 c	R	2.17 c	R	0/36	0/36	12/36	26/36	30/36	0	0.00 b	0.33 b	0.38 c	0.64 c
Super5	1	1.00 d	R	1.00 c	R	1.67 c	R	2.00 c	R	0/36	0/36	0/36	27/36	33/36	0	0.00 b	0.00 c	0.32 c	0.56 c

[1] Cercospora leaf spot resistance levels based on disease severity scores as follows: 1.0-2.4 = resistant (R), 2.5-3.4 = moderately resistant (MR), and 3.5-5.0 = susceptible (S). Numbers represent average for two independent experiments.

[2] Number of leaf spot in which HR was detected/total number of leaf spot inoculated. Numbers represent totals for two independent experiments.

[3] Diameter of necrotic lesions. Numbers represent average for two independent experiments.

[4] Data followed by different letters in each column differ significantly ($p < 0.05$) according to Duncan's multiple range test.

4.4.5 Systemic acquired resistance (SAR)

The β-1,3-glucanase, chitinase and PPO activities were evaluated in four mungbean genotypes EGMD-6D, CN84-1, V4718 and Super5 at 0-4 days after *C. canescens* inoculation. Mungbean leaves were inoculated on leaf node 2 and β-1,3-glucanase, chitinase and PPO activities were measured to evaluate for both local (leaf node 2) and systemic induction (leaf nodes 1 and 3). Mungbean leaves at nodes 1 and 3 were also used to observe the reduction of necrotic lesions in secondary infection of *C. canescens* using detached leaf assay. The levels of β-1,3-glucanase, chitinase and PPO activities at the pre-inoculated stage (0 day) were significantly different among mungbean genotypes ($p < 0.05$). Among all the mungbean genotypes, the CLS resistant genotype Super5 exhibited the highest levels of enzyme activities in all leaf nodes. These activity levels were significantly higher ($p < 0.05$) compared to the other mungbean genotypes. In leaf node 1, Super5 exhibited significantly ($p < 0.05$) higher β-1,3-glucanase activities (1.42- to 2.88-fold), chitinase activities (1.18- to 2.39-fold), and PPO activities (1.06- to 1.32-fold) compared to other genotypes (Figures 4.5, 4.6 and 4.7). This trend was consistent in leaf node 2, where Super5 displayed higher β-1,3-glucanase, chitinase, and PPO activities (1.39- to 3.04-fold, 1.25- to 2.23-fold and 1.07- to 1.43-fold, respectively) than other genotypes. Similarly, in leaf node 3, Super5 exhibited elevated β-1,3-glucanase activities (1.40- to 3.04-fold), chitinase activities (1.27- to 2.52-fold), and PPO activities (1.07- to 1.52-fold) compared to other genotypes. Specifically, in leaf node 1, the resistant genotype Super5 exhibited the highest levels of β-1,3-glucanase (1,402.21 μmoles/min/mg protein), chitinase (81.31 μmoles/min/mg protein), and PPO (0.75 μmoles quinone formed/min/mg protein) activities. Conversely, the susceptible genotype EGMD-6D displayed the lowest levels of β-1,3-glucanase, chitinase, and PPO activities (487.52 and 34.04 μmoles/min/mg protein, and 0.57 μmoles quinone formed/min/mg protein, respectively). This trend persisted in leaf nodes 2 and 3, where Super5 consistently showed the highest activities for all three enzymes (β-1,3-glucanase: 1,301.45 and 1,297.32 μmoles/min/mg protein, chitinase: 79.81 and 76.43 μmoles/min/mg protein, PPO: 0.76 and 0.76 μmoles quinone formed/min/mg protein). Similarly, EGMD-6D consistently demonstrated the lowest activities for all three enzymes (β-1,3-glucanase: 427.59 and 401.32 μmoles/min/mg protein, chitinase: 35.83 and 30.32 μmoles/min/mg protein, PPO: 0.53 and 0.50 μmoles quinone formed/min/mg protein).

Significant variations in β-1,3-glucanase activities were observed among mungbean genotypes in infected and distal leaves at different time points (1, 2, 3, and 4 DAI). Most genotypes exhibited significantly different activity levels compared to the initial stage (0 DAI) in infected leaves, while no significant differences were observed in distal leaves. At 1 DAI, most mungbean genotypes, except EGMD-6D, exhibited significantly different β-1,3-glucanase activity levels compared to 0 DAI in infected leaves ($p < 0.05$). Resistant genotypes V4718 and Super5 did not show any disease symptoms, while moderately resistant CN84-1 and susceptible EGMD-6D started to display disease symptoms. In infected leaves, the highest β-1,3-glucanase activity was observed in the resistant genotype Super5 (1,602.13 μmoles/min/mg protein), which was significantly higher than those of the other genotypes. All mungbean genotypes exhibited an increase in β-1,3-glucanase activity in infected leaves (1.23- to 1.50-folds), except for EGMD-6D, while no increase was observed in distal leaves. At 2 DAI, consistent with the findings at 1 DAI, all mungbean genotypes displayed significant increase in β-1,3-glucanase activity levels when compared to the levels at 0 DAI in infected leaves ($p < 0.05$). In infected leaves, mungbean genotypes displayed a 1.34- to 1.77-fold increase in β-1,3-glucanase activities compared to the initial stage (0 DAI), while no discernible increase was observed in distal leaves. The resistant genotype Super5 exhibited the significantly highest β-1,3-glucanase activity in infected leaves (1,863.94 μmoles/min/mg protein) surpassing the other genotypes, followed by V4718, CN84-1 and EGMD-6D (1,621.26, 1,263.84 and 572.13 μmoles/min/mg protein). Interestingly, at 3 DAI, all mungbean genotypes showed significant changes in β-1,3-glucanase activity levels compared to the levels at 0 DAI, particularly in infected leaves ($p < 0.05$). However, no noticeable difference was observed in distal leaves when compared to the activity at 0 DAI. At this stage, the β-1,3-glucanase activity levels reached their highest point in all mungbean genotypes. Among the infected leaves, the resistant Super5 genotype showcased a remarkable elevation in β-1,3-glucanase activity (2,371.83 μmoles/min/mg protein), surpassing all other genotypes with a statistically significant (ranging from 1.08- to 4.00-folds higher). It is noteworthy that mungbean genotypes demonstrated a moderate to substantial augmentation in β-1,3-glucanase activities in infected leaves, ranging from 1.38 to 2.42 times higher, but no significant difference was observed in distal leaves, potentially indicating a localized

defense response. At 4 DAI, there were significant differences in β-1,3-glucanase activities in most mungbean genotypes compared to the levels at 0 DAI in infected leaves excepted for EGMD-6D ($p < 0.05$), but no significant difference was observed in distal leaves. At this stage, all mungbean genotypes exhibited a decrease in β-1,3-glucanase activities compared to those at 3 DAI in infected leaves, but no significant difference was observed in distal leaves. The highest β-1,3-glucanase activities of all mungbean genotypes were found in the resistant genotype Super5 (1,702.26 μmoles/min/mg protein), which were still significantly higher than those of all other genotypes.

In the infected leaves, the β-1,3-glucanase activity levels peaked at 3 DAI in all mungbean genotypes, gradually declining by 4 DAI. During the peak induction stage, the β-1,3-glucanase activity levels of all genotypes were significantly higher than those at 0 DAI ($p < 0.05$). Among the genotypes, the resistant Super5 exhibited the highest activity (2,371.83 μmoles/min/mg protein), followed by V4718 and CN84-1 (2,192.15 and 1,731.62 μmoles/min/mg protein, respectively). The susceptible genotype EGMD-6D showed the lowest β-1,3-glucanase activity (592.11 μmoles/min/mg protein). These activity levels corresponded to the resistance levels of each genotype. Furthermore, the maximum β-1,3-glucanase activities in both resistant genotypes (Super5 and V4718) were significantly ($p < 0.05$) higher than those in the moderately resistant and susceptible genotypes, ranging from 1.36 to 4.00-fold and 1.27 to 3.70-folds higher, respectively. These findings indicate the local induction of β-1,3-glucanase activities in these genotypes, which remained active for up to 3 DAI, while systemic induction was not observed in any of the mungbean genotypes.

The chitinase activities of mungbean exhibited significant variations among genotypes in both infected leaves (leaf node 2) and distal leaves (leaf nodes 1 and 3) at 1, 2, 3 and 4 DAI ($p < 0.05$). No significant chitinase induction was observed in distal leaves of all mungbean genotypes at all stages of inoculation compared to 0 DAI (Figure 4.6). At 1 DAI in infected leaves, except for the mungbean genotype EGMD-6D, all other genotypes displayed significantly different chitinase activity levels compared to 0 DAI ($p < 0.05$). Among the infected leaves, the resistant genotype Super5 exhibited the highest chitinase activities (79.81 μmoles/min/mg protein), significantly higher than all other genotypes, followed by V4718, CN84-1 and EGMD-6D (63.95, 68.57 and 35.85

μmoles/min/mg protein). Chitinase activities in infected leaves of mungbean genotypes increased by 1.07- to 1.30-folds. At 2 DAI, all genotypes exhibited significantly different levels of chitinase activities in infected leaves compared to 0 DAI ($p < 0.05$). Among the genotypes, the resistant genotype Super5 exhibited the highest chitinase activities in infected leaves, measuring 103.62 μmoles/min/mg protein. This value was significantly higher than those observed in all other genotypes. The susceptible genotype EGMD-6D showed the lowest chitinase activities (50.32 μmoles/min/mg protein). Chitinase activities increased by 1.27-, 1.40-, 1.67- and 1.74-folds in infected leaves of mungbean genotypes (CN84-1, EGMD-6D, V4718 and Super5, respectively) compared to 0 DAI. At 3 DAI, all genotypes exhibited distinct levels of chitinase activities compared to the measurement at 0 DAI, indicating significant differences ($p < 0.05$). The chitinase activity levels reached their peak at this stage in most mungbean genotypes, except for the EGMD-6D genotype, which displayed the highest levels at 2 DAI and decreased at this stage. Among the infected leaves, the resistant genotype Super5 displayed the highest chitinase activity (173.02 μmoles/min/mg protein), which was significantly higher than those of all other genotypes (ranging from 1.27- to 2.47-folds higher). Similarly, at 4 DAI, most mungbean genotypes displayed significantly different levels of chitinase activity compared to 0 DAI, excepted for the EGMD-6D genotype ($p < 0.05$). Maximum chitinase activity was observed in the resistant genotype Super5 (102.00 μmoles/min/mg protein), significantly higher than all other genotypes, based on the results at 4 DAI. At this stage, chitinase activity decreased from 3 DAI in all mungbean genotypes in infected leaves, similar to the results of β-1,3-glucanase.

These findings substantiate the local induction of chitinase activities in the examined genotypes, while systemic induction was not observed. During the local induction, the chitinase activity levels reached their peak at 3 DAI in most mungbean genotypes, except for the EGMD-6D genotype, which displayed the highest level at 2 DAI and decreased by 3 DAI. Subsequently, by 4 DAI, these levels gradually declined, Notably, during the peak induction stage, the chitinase activity levels of all genotypes exhibited significant increases compared to those at 0 DAI ($p < 0.05$). Among the genotypes, the resistant genotype Super5 demonstrated the highest chitinase activity (173.02 μmoles/min/mg protein), followed by V4718 and CN84-1 (158.30 and 110.21 μmoles/min/mg protein, respectively). Conversely, the susceptible genotype EGMD-6D

exhibited the lowest chitinase activity (50.32 μmoles/min/mg protein). These observed activity levels aligned with the resistance levels exhibited by each genotype. Furthermore, the maximum chitinase activities in both resistant genotypes (Super5 and V4718) were significantly ($p < 0.05$) higher compared to those in the moderately resistant and susceptible genotypes, with fold differences ranging from 1.60 to 3.44 and 1.44 to 3.15, respectively. This indicates a genotype-specific response to the infection in terms of chitinase activity, with Super5 displaying a particularly robust defense mechanism.

Considerable variations were noted in the activities of PPO across different genotypes of mungbean, both in infected leaves (leaf node 2) and distal leaves (leaf nodes 1 and 3), at 1, 2, 3, and 4 DAI ($p < 0.05$). Nevertheless, it is important to mention that no significant induction of PPO activities was observed in distal leaves of any mungbean genotypes during any stage of inoculation, when compared to the PPO activity at 0 DAI (Figure 4.7). At 1 DAI, the resistant genotype Super5 exhibited the highest PPO activities in infected leaves (0.79 μmoles quinone formed/min/mg protein), which were significantly higher than those of all other genotypes. The subsequent genotypes, V4718 and CN84-1, demonstrated PPO activities of 0.74 and 0.67 μmoles quinone formed/min/mg protein, respectively. In contrast, the susceptible genotype EGMD-6D exhibited the lowest chitinase activity of 0.61 μmoles quinone formed/min/mg protein. It is interesting to note that mungbean genotypes demonstrated a subtle 1.04- to 1.22-fold increase in PPO activities in infected leaves. By 2 DAI, when compared to leaves at 0 DAI, all genotypes exhibited a significant increase in PPO activity ($p < 0.05$). At this stage, the PPO activity levels of EGMD-6D and CN84-1 exhibited the peak levels. Among the genotypes, Super5 showcased the highest PPO activity in infected leaves (0.84 μmoles quinone formed/min/mg protein), followed by V4718, CN84-1 and EGMD-6D (0.77, 0.73 and 0.65 μmoles quinone formed/min/mg protein, respectively). Infected leaves of mungbean displayed an increase of 1.01 to 1.23-fold in PPO activity, when compared to the PPO activity at 0 DAI. Upon reaching 3 DAI, each genotype displayed markedly increased levels of PPO activities in comparison to those observed at 0 DAI ($p < 0.05$). The PPO activity levels of V4718 and Super 5 reached their maximum, exhibiting the highest levels among the genotypes at this time. In contrast, the PPO activity levels of EGMD-6D and CN84-1,

which peaked at 2 DAI, gradually declined. Notably, the resistant genotype Super5 demonstrated the most elevated PPO activities in infected leaves, with a significantly higher level of 0.89 μmoles quinone formed/min/mg protein compared to those of all other genotypes. Conversely, the distal leaves did not exhibit a discernible trend of increasing PPO activities. At 4 DAI, among genotypes, the resistant genotype Super5 exhibited the highest levels of PPO activities, measuring at 0.85 μmoles quinone formed/min/mg protein. This observation significantly surpassed the PPO activities recorded in all other genotypes. A decline in PPO activities from those of 3 DAI was evident across all mungbean genotypes, affecting infected leaves. This decline in PPO activities in infected leaves suggests a gradual waning of the defense mechanisms as time progressed. Despite this decline, the disparities in PPO activities among the genotypes remained prominent, with Super5 retaining a significant lead.

During the process of local induction, the mungbean genotypes EGMD-6D and CN84-1 demonstrated their peak PPO activity levels at 2 DAI. However, as time progressed to 3 DAI, these activity levels gradually declined. In contrast, the V4718 and Super5 genotypes showcased a different pattern, reaching their highest PPO activity levels at 3 DAI and then experiencing a decline by 4 DAI. This induction effect was observed to persist longer in resistant genotypes for a period of 3 DAI in infected leaves. It is important to highlight that during the peak induction stage, all genotypes exhibited a significant increase in PPO activities compared to the initial measurement at 0 DAI ($p < 0.05$). This indicates an active response to the pathogen presence. Among the genotypes, the resistant Super5 genotype demonstrated the highest PPO activity, with a substantial amount of 0.89 μmoles quinone formed/min/mg protein. Following Super5, the V4718 and CN84-1 genotypes exhibited relatively high PPO activity levels, measuring 0.82 and 0.73 μmoles quinone formed/min/mg protein, respectively. Conversely, the susceptible EGMD-6D genotype displayed the lowest PPO activity, recording 0.65 μmoles quinone formed/min/mg protein. These observed activity levels closely corresponded to the resistance levels exhibited by each genotype. Similar with β-1,3-glucanase and chitinase activities, the resistant genotypes (Super5 and V4718) displayed significantly higher PPO activity compared to both the moderately resistant and susceptible genotypes ($p < 0.05$). The fold differences in maximum PPO activities between the resistant genotypes and the moderately resistant and susceptible

genotypes ranged from 1.22 to 1.37 and 1.12 to 1.26, respectively. This suggests that the robust activation of PPO activity was associated with higher resistance to the pathogen in mungbean genotypes. Notably, the mungbean genotypes exhibited a local induction of PPO activities, denoting the activation of defense mechanisms specifically in the vicinity of the infection site. Moreover, the findings indicated a parallel trend between β-1,3-glucanase, chitinase and PPO activities.

When we considered the reduction of necrotic lesions in secondary infection of *C. canescens* using detached leaf assay. The results on both upper leaves (node 1) and lower leaves (node 3) showed that mungbean leaves started to show necrotic lesions at 1 DAI in those of moderately resistant and susceptible genotypes, but not appeared in resistant genotypes (V4718 and Super5). At 2 DAI, the disease severity scores continued to slightly increase in those of moderately resistant and susceptible genotypes both in upper and lower leaves. The resistant genotype V4718 started to show necrotic lesions at this time, but the resistant genotype Super5 still did not show the disease symptoms. Nevertheless, all mungbean genotypes showed necrotic lesions at 3 DAI in both upper and lower leaves and the susceptible genotypes EGMD-6D showed the highest disease severity scores of 3.45 to 3.50. While the resistant genotypes Super5 and V4718 showed much lower disease severity scores of 1.50 to 1.75 and 1.75 to 1.80, respectively. At 4 DAI, two mungbean resistant genotypes Super5 and V4718 were potentially resistant to *C. canescens* isolate PAK-1 in both upper and lower leaves because they were developing least symptoms than those of other genotypes, with average severity scores of 2.00 to 2.50 and 2.00 to 2.20, respectively. However, these secondary inoculation severity scores of all mungbean genotypes were not significantly different from those following 0, 1, 2 and 3 days after primary inoculation, suggesting that prior inoculation with *C. canescens* did not induce systemic acquired resistance in mungbean genotypes. The susceptible mungbean genotype EGMD-6D was the most susceptible with average severity scores of 3.85 to 4.00, which was significantly higher than those of other genotypes. These findings were consistent with the observed resistance levels exhibited by each genotype. These results also showed that there was no difference in the disease severity scores between upper leaf node 1 and lower leaf node 3 within mungbean genotypes at every stages of inoculation.

The study observed local induction of β-1,3-glucanase, chitinase, and PPO activities in all mungbean genotypes, but this induction was not observed in distal leaves. During their peak induction stage, which refers to the period when the defense response is most active, the β-1,3-glucanase, chitinase and PPO activity levels in all genotypes showed significant increases compared to the levels at 0 DAI, indicating the activation of the defense mechanisms. Among the genotypes, the resistant genotype Super5 exhibited the highest activities of these three enzymes, followed by resistant genotype V4718 and moderately resistant CN84-1, while the susceptible genotype EGMD-6D displayed the lowest activities. These activity levels also corresponded to the resistance levels observed in each genotype, suggesting that higher β-1,3-glucanase, chitinase and PPO activities were associated with greater resistance to the pathogen. In this study, the resistant genotype Super5 exhibited highest local induction of these defense-related enzyme activities, which could be attributed to the higher accumulation of corresponding mRNAs in specific cells or tissues, potentially resulting from changes in the expression of resistance-related genes. A study by Campbell et al. (2012) also reported higher transcript abundance of four PR genes, including PR1 (antifungal), PR2 (β-1,3-glucanase), PR3 (chitinase), and PR9 (peroxidase), in a resistant wheat mutant compared to a susceptible wheat cultivar. This increased expression of PR genes enhanced the resistance of the wheat mutant against three different types of rust and powdery mildew diseases. β-1,3-glucanase activities, induced in all mungbean genotypes, likely play a significant role in defense mechanisms against *C. canescens*. On the other hand, chitinase and PPO activities, which exhibited lower levels of induction in all *C. canescens* infected mungbean genotypes, may have a supportive role. The study found that both resistant genotypes exhibited lower disease severity scores from secondary infection compared to susceptible and moderately resistant genotypes, both in upper and lower distal leaves, suggesting that the resistant genotypes possessed more effective defense mechanisms against the pathogen. Overall, these results indicate the involvement of different resistance mechanisms in the mungbean genotypes, with the resistant genotype Super5 showing highest local induction of defense-related enzyme activities, potentially contributing to its enhanced resistance against *C. canescens.* However, it is worth considering that mungbean may employ other defense mechanisms against CLS that are not specifically linked to these enzymes. Therefore, conducting further studies in the future is crucial to gain a more comprehensive understanding of the additional defense strategies utilized by mungbean to combat CLS.

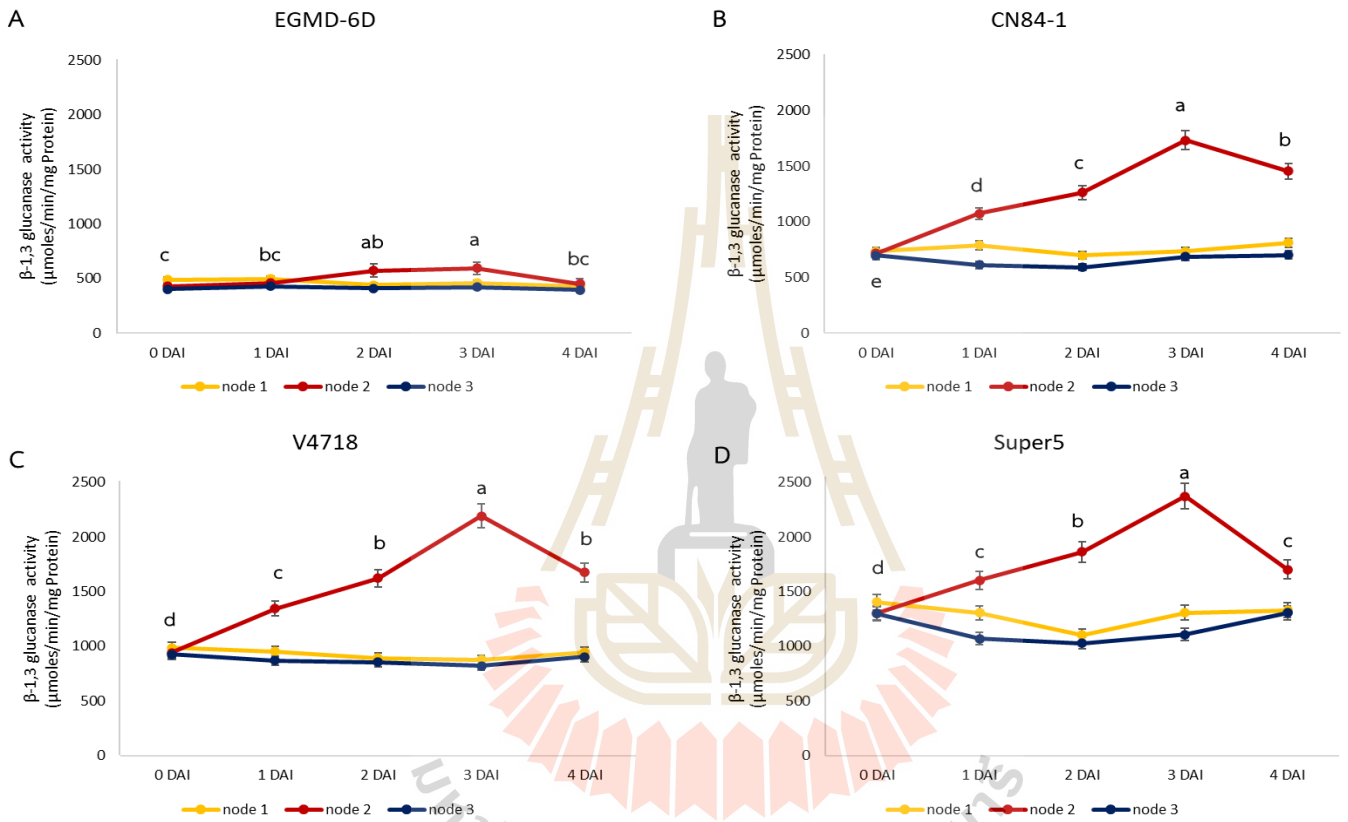

Figure 4.5 Changes in β-1,3-glucanase activities in infected (leaf node 2) and distal (leaf node 1 and 3) leaves of 4 mungbean genotypes (susceptible genotype EGMD-6D (A), moderately resistant genotype CN84-1(B), and resistant genotypes V4718 (C) and Super5 (D)) at 0, 1, 2, 3 and 4 days after inoculation with *C. canescens*. Data followed by different letters in each genotype do not differ significantly ($p < 0.05$) according to Duncan's multiple range test.

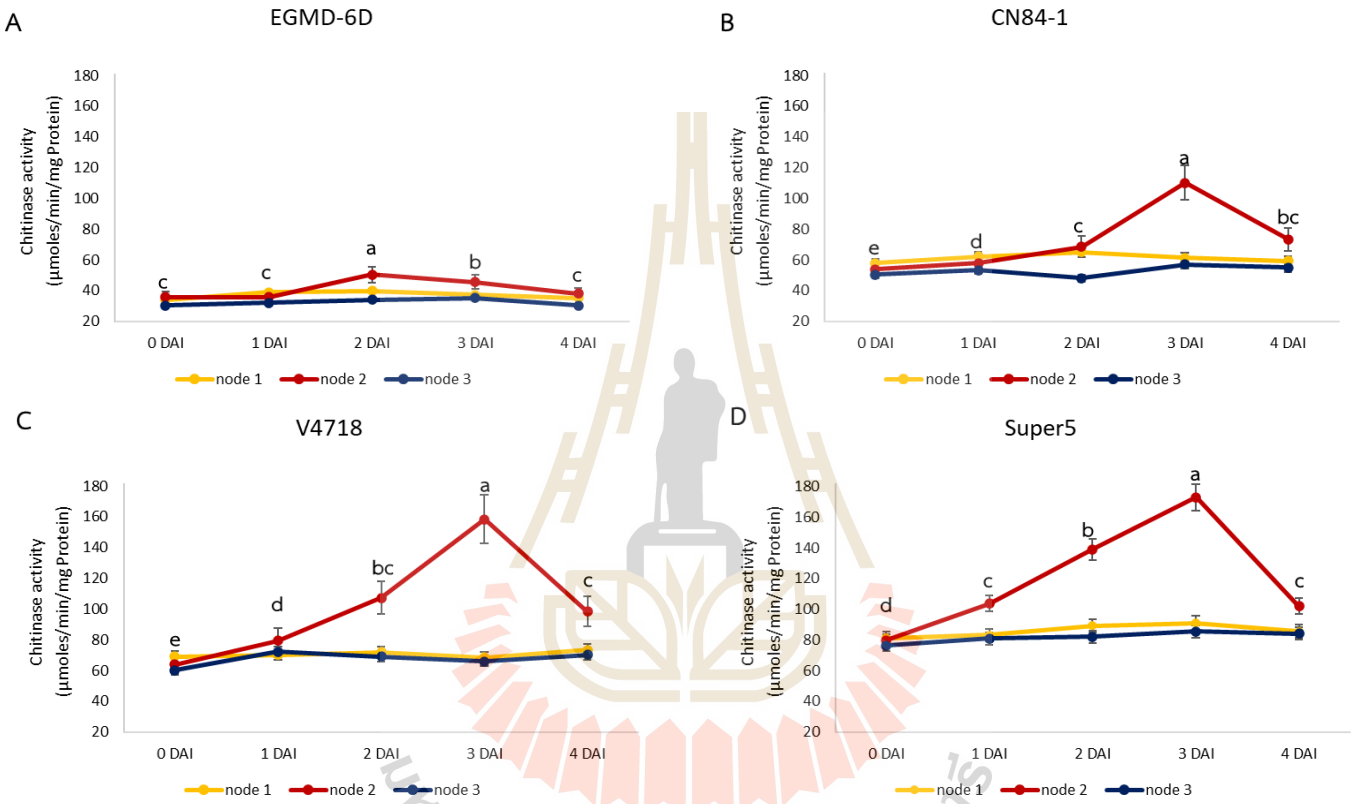

Figure 4.6 Changes in chitinase activities in infected (leaf node 2) and distal (leaf node 1 and 3) leaves of 4 mungbean genotypes (susceptible genotype EGMD-6D (A), moderately resistant genotype CN84-1(B), and resistant genotypes V4718 (C) and Super5 (D)) at 0, 1, 2, 3 and 4 days after inoculation with *C. canescens*. Data followed by different letters in each genotype do not differ significantly ($p < 0.05$) according to Duncan's multiple range test.

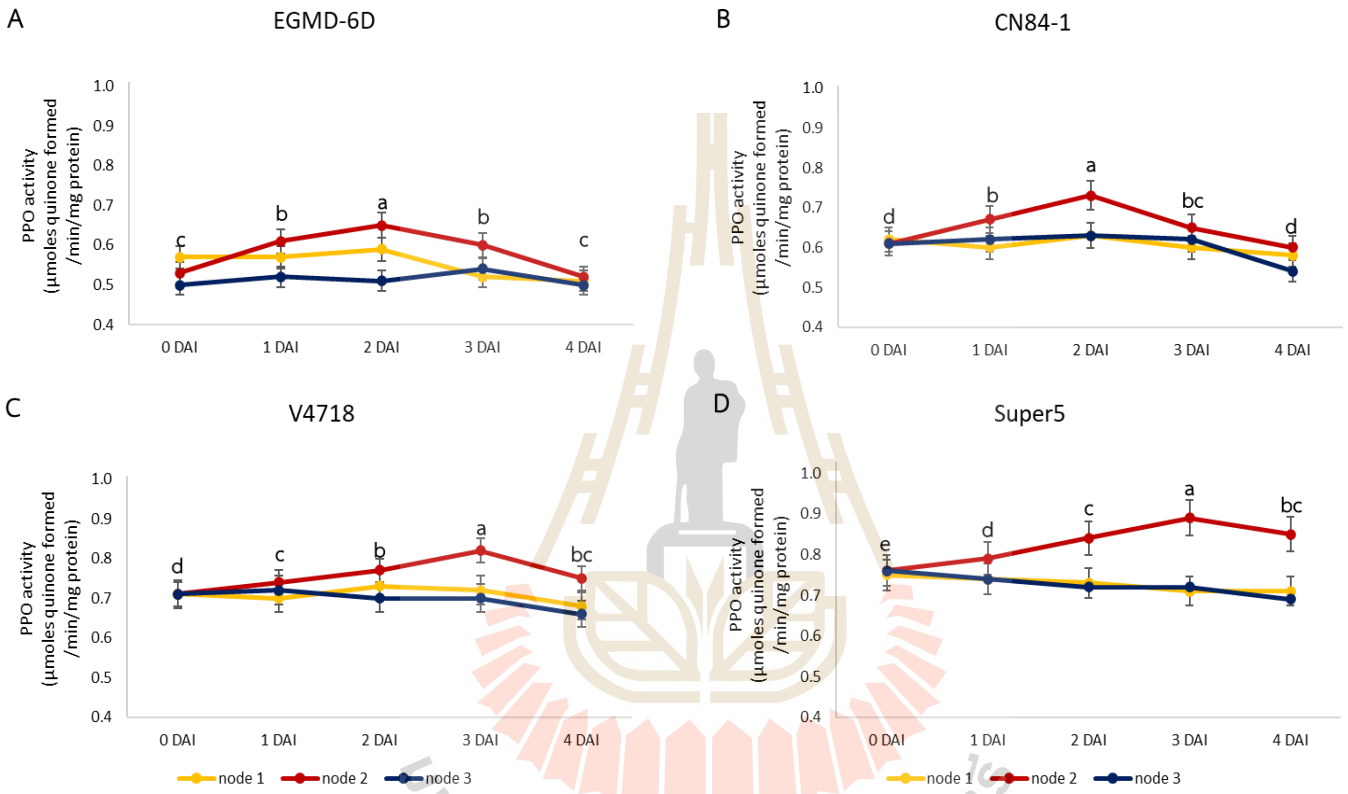

Figure 4.7 Changes in PPO activities in infected (leaf node 2) and distal (leaf node 1 and 3) leaves of 4 mungbean genotypes (susceptible genotype EGMD-6D (A), moderately resistant genotype CN84-1(B), and resistant genotypes V4718 (C) and Super5 (D)) at 0, 1, 2, 3 and 4 days after inoculation with *C. canescens*. Data followed by different letters in each genotype do not differ significantly ($p < 0.05$) according to Duncan's multiple range test.

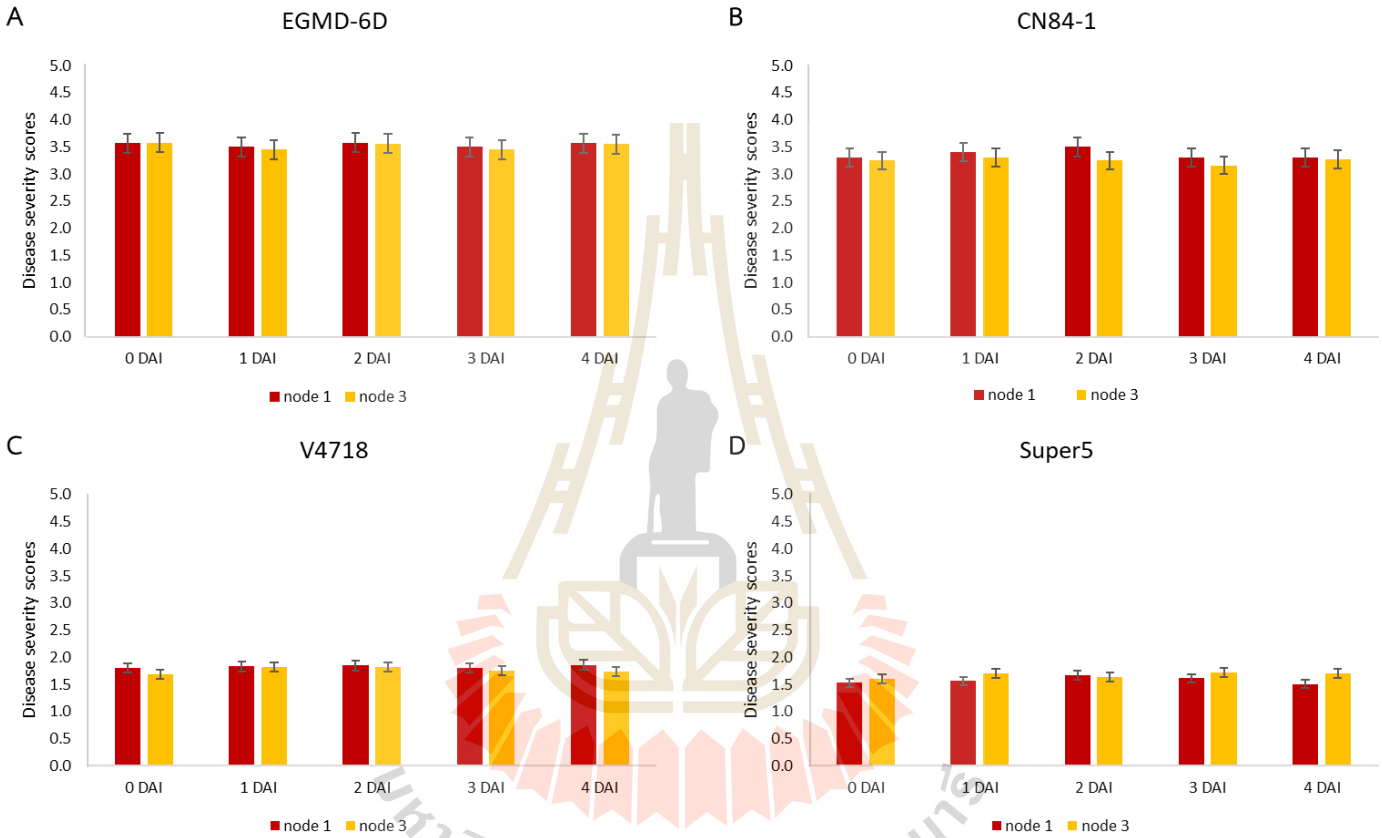

Figure 4.8 Disease severity scores observed at 3 days after secondary inoculation on upper (leaf node 1) and lower (leaf node 2) distal leaves of 4 mungbean genotypes (susceptible genotype EGMD-6D (A), moderately resistant genotype CN84-1(B), and resistant genotypes V4718 (C) and Super5 (D)) at 0, 1, 2, 3 and 4 days after primary inoculation with *C. canescens*.

4.5 Conclusion

Although systemic induction of β-1,3-glucanase, chitinase, and PPO activities was not observed, the local increases in β-1,3-glucanase, chitinase, and PPO activities in all mungbean genotypes and the high activity levels in the resistant genotypes suggest their significant roles in mungbean defense mechanisms against CLS. These enzymes can serve as biochemical markers for disease resistance and have the potential to be used as early screening markers in crop plants. Identifying mungbean genotypes with enhanced resistance to CLS using these markers can contribute to the development of strategies in breeding programs aimed at effectively combating the disease.

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CHAPTER 5
CORRELATION BETWEEN MYCELIUM-INOCULATED DETACHED LEAF AND FIELD ASSESSMENTS OF RESISTANCE TO *Cercospora canescens* IN MUNGBEAN

5.1 Abstract

Cercospora leaf spot (CLS) caused by *Cercospora canescens* is a serious disease of mungbean (*Vigna radiata*) in Thailand. For the selection of resistant varieties in a breeding program, a rapid screening of numerous genotypes for CLS resistance is necessary. Most screening methods use spores as inoculum, but their productivity on culture media is low. The objective of this study was to develop an efficient laboratory inoculation method using mycelium. We compared resistance/susceptibility levels of 19 mungbean genotypes from detached leaf inoculation using mycelium from the most virulent *C. canescens* isolates (SUT1, SUT4, PAK1, and PAK2) with field inoculations. The resistance levels of mungbean genotypes evaluated by the detached leaf inoculations were comparable to the field inoculations when comparing genotypes with a correlation coefficient of 0.822 ($p < 0.01$). Both inoculation methods consistently identified mungbean genotypes V4718, V4785, V4758, and Super5 as resistant to CLS, which will be useful for future breeding programs. These results suggest that the mycelium-inoculated detached leaf assay was as effective as field inoculation for inducing disease symptoms and identifying resistant genotypes. Moreover, mycelium inoculum was much simpler and faster to prepare in large quantities than spore inoculum, allowing effective and economical large-scale screening with multiple isolates to ensure reliability.

5.2 Introduction

Cercospora leaf spot (CLS) disease in mungbean (*Vigna radiata*) is caused by *Cercospora canescens*. This was first reported in 1960 by Munjai et al. from Delhi, India. It is a serious disease that can reduce yield by 50-80% depending on the stage

of infection. It can incur up to 96% yield losses in uncontrolled conditions (Lal et al., 2001; Kaur et al., 2004; Chand et al., 2012). This disease is spread widely in Asia in such countries as India, Bangladesh, the Philippines, Thailand, and in all parts of the world (Pandey et al., 2009). Conidia infection can cause lesions on foliage with an average diameter of 3-15 mm. The symptoms are at first a brown spot which then turns to grey with a reddish-brown margin, with amphigenous fruiting becoming more abundant on the lower surface (Munjal et al., 1960). The disease mostly infects plants at 30-40 days old and causes premature defoliation, which affects the size of pods and grains (Grewal et al., 1980). Although chemical control is the most popular method to control CLS, the use of mungbean resistant varieties is an effective method which is the cheapest, with the most practical and effective features for controlling CLS. The pathogen development for tolerance to resistant varieties is not acute because it takes a long time and may affect their important metabolic processes. However, to develop resistant varieties, a rapid, highly efficient, and reliable laboratory assay for screening a large number of mungbean genotypes for resistance to CLS is required. Most screening methods use conidia as an inoculum, but conidial production on culture media is typically low and may not be produced in some isolates. Moreover, preparing conidial inoculum is a time-consuming process, particularly when large amounts of inoculum must be prepared from numerous culture plates and sporulation capacity varies among fungal isolates. While mycelia grow more quickly and in larger quantities, they have been used as an inoculum in only a few studies (Singh et al., 2002; Chen and Wang, 2005). If mycelia are to be effective in causing symptoms, it would be much simpler to prepare inoculum for these screening assays. Furthermore, a detached leaf assay is an effective method for the rapid screening of large numbers of genotypes as well as for resistance assessment under highly controlled conditions. But the detached leaf assay is only useful if it has a strong correlation with the results of a field evaluation. The objective of this study was to evaluate the effectiveness of the laboratory inoculation method compared with field evaluation by using mycelium of *C. canescens*.

5.3 Materials and methods

5.3.1 Fungal isolation and growth

C. canescens isolation was performed by the following standard tissue isolation technique. The infected mungbean leaves were collected from mungbean fields in 5 locations, including Suranaree University of Technology Farm and Pak Thong Chai district in Nakhon Ratchasima province, as well as Nakhon Sawan, Petchabun, and Phichit provinces. Infected leaves were surface sterilized for 30 seconds in 0.5% (v/v) Clorox before being rinsed 3 times in sterile distilled water. Then, leaf pieces with a 0.5 to 1.0 cm width were cut from the infected area along with some healthy tissues. The leaf pieces were transferred onto potato dextrose agar (PDA) medium (Verma and Agnihotri, 1972) in sterilized petri dishes by an aseptic technique and were incubated at room temperature (27±2°C).

The 10 isolates collected in this study were obtained from SUT-1 and SUT-4 from Suranaree University of Technology Farm, PAK-1 and PAK-2 from Pak Thong Chai district, NW-1 and NW-2 from Nakhon Sawan, PB-1 and PB-2 from Petchabun, and PCH-1 and PCH-2 from Phichit provinces. The morphological characters of the fungus such as mycelia, conidiophores, conidia, and cercosporin production were studied under a compound microscope based on the characteristics of *C. canescens* according to Ellis and Martin (1882). The fungus was sub-cultured on PDA slants and incubated at 27±2°C for 14 days. Then, they were preserved in a refrigerator at 4°C and renewed once every month. The preserved culture was transferred to room temperature before use.

5.3.2 Inoculum preparation

To determine a suitable inoculum concentration for the detached leaf assay, the fungal culture plates were used to prepare mycelium inoculum by adapting the method of Guo et al. (2016). Agar disks were collected from the growing edges of isolate SUT-1 at 14 days old by using a 5 mm cork borer. The different amounts of mycelium-containing agar disks (10, 30, 50, 80, and 100 pieces) were mixed with 5 mL of sterile distilled water in a sterile bottle using a shaker at 2,000 revolutions per minute (rpm) for 2 hours to homogenize the mycelia. The solution was filtered through 2 layers of muslin cloth. These different concentrations of inoculum were tested in a resistant

genotype (V4718) and a moderately resistant to susceptible genotype (CN72). Leaves of similar size (leaves from the second node counted from the top) of 21-day-old plants were collected and used. A completely randomized design (CRD) with 15 replications (1 leaf piece/replication) was used. The leaves were surface-disinfected with 1% (v/v) Clorox and rinsed with sterile distilled water, cut into 1 cm^2 pieces, and placed on water agar (WA; 1.5% (w/v) agar) with 25 mg/L streptomycin in a 90 mm diameter Petri dish. One 5 µL droplet of mycelium inoculum of a fungal isolate was inoculated onto each leaf piece. Sterile distilled water without mycelia was used as a control. The inoculated leaves were incubated at 27°C for a 12-hour photoperiod (1,500 lux) for 3 days to ensure successful infection. The visible lesions appeared 2 DAI but were evaluated after 3 days by rating the symptoms on a 1-5 scale according to Ngegba et al. (2017) (Table 5.1). Disease severity scores of various concentrations indicated that 80 mycelium discs/5 mL of DI water was suitable for evaluation in this assay and so was used for further experiment.

Table 5.1 Disease severity scores for Cercospora leaf spot of mungbean.

Scales	Rating	Description
1	No infection	No visible symptoms
2	Low infection	1-25% of total leaf area is covered by lesions
3	Moderate infection	26-50% of the total leaf area is covered by lesions
4	Severe infection	51-75% of the total leaf area is covered by lesions
5	Very severe infection	76-100% of the total leaf area is covered by lesions

Source: Modified from Ngegba et al. (2017).

5.3.3 Fungal pathogenicity analysis

The 10 isolates from 5 locations were evaluated for pathogenicity to select the most virulent isolates. Two, which were moderately resistant to susceptible mungbean genotypes (CN72 and CN84-1), and 3 resistant mungbean lines (V4718, V4758, and V4785) were grown in pots under greenhouse conditions. Leaves from the second node (counting from the top) of 21-day-old plants were collected and used for the detached leaf assay. The leaf pieces were placed on water agar (WA; 1.5% (w/v) agar) with 25 mg/L streptomycin in a 90 mm diameter petri dish and inoculated with

one droplet of 5 µL of *C. canescens* inoculum (80 mycelium disks/5 mL), while the control leaves were inoculated with DI water. A CRD with 9 replications (1 leaf piece/ replication) were used. The inoculated leaves were kept for 3 days at 27°C in a 12-hour photoperiod (1,500 lux). Disease severity was rated at 3 DAI as previously described. The most virulent four isolates of *C. canescens* (SUT-1, SUT-4, PAK-1, and PAK-2) were selected for further use in the experiment.

To compare the effects of inoculating detached leaves at different plant develop-mental stages, leaves of 21-day-old plants (vegetative stage) and 45-day-old plants (reproductive stage) of 5 mungbean genotypes (CN72, CN84-1, V4718, V4785, and V4758) grown under greenhouse condition were inoculated with mycelium inoculum. Inoculum from each of 4 *C. canescens* isolates (SUT-1, SUT-4, PAK-1, and PAK-2) was prepared separately at 80 mycelium discs/5 mL of DI water according to the methods previously described. Equal amounts of solution from each of the 4 isolates were mixed together well before being used as an inoculum. The leaf pieces were inoculated with the inoculum as previously described. A CRD with 9 replications (1 leaf piece/replication) was used. Disease severity was assessed 3 DAI. The results demonstrated that the resistance status within the genotype was mainly identical at both developmental stages and the 45-day- old plants (reproductive stage) were therefore used for the next experiment.

5.3.4 Comparison of detached leaf and field assessments

The 19 mungbean genotypes with varying resistance/susceptibility levels to *C. canescens* (4 resistant genotypes; V4718, V4758, V4785, and Super5, 1 susceptible genotype; EGMD-6D, 3 moderately resistant genotypes; CN72, CN84-1, and KING, and 11 breeding lines; O2-31, O2-37, O2-39, P01, P05, H3, H4, B1, B2, D5, and G1) and 4 virulent isolates of *C. canescens* (SUT-1, SUT-4, PAK-1, and PAK-2) were used in this study. All plants were grown during August to October in an experimental field in Pak Thong Chai district, Nakhon Ratchasima province, from August to October 2021. There were 3 rows per genotype, 50 cm apart, with 20 cm between plants. Each row consisted of 10 plants. The genotype CN72 was planted as a guard row around the field.

The 45-day-old plants were used for both detached leaf and field assessments. For both detached leaf and field assessments, mixed inoculum from 4 isolates with the optimal mycelium concentration (80 mycelium discs/5 mL of DI water) was used. One day prior to inoculation, we watered the plants to increase the moisture content of the experimental field. A CRD with 9 replications (1 plant/replication) was used for both detached leaf and field assessments. Nine plants per genotype were randomly selected (3 plants from each row) and tagged for use in both detached leaf and field assessments. One leaf per plant of the selected plants (9 plants/genotype) were collected and kept for detached leaf assessment. The inoculation method for the detached leaf assay was carried out according to the method described previously. Disease severity was assessed at 3 DAI by rating on the same scale as in Table 5.1.

For the field assessment, mycelium inoculum (same preparation as in the previous detached leaf assay) was used. The guard row was inoculated by spraying the mycelium inoculum on the leaf surface with a mist sprayer until it drained to ensure that there was a uniform infection spread to the genotypes being tested. Disease severity was rated from the sampled plants at 15 DAI.

5.3.5 Statistical methods

All experiments were conducted in a CRD. The mungbean genotypes were classified as resistant (scores 1.0-2.4), moderately resistant (scores 2.5-3.4), and susceptible (scores 3.5-5.0). The disease severity scores from all experiments were analyzed by using analysis of variance (ANOVA) by SPSS software (version 16; SPSS Inc.; Chicago, IL, USA). The Pearson's correlation coefficient was used to analyze the relationships between the detached leaf assay and field evaluations by SPSS software (version 16; SPSS Inc.; Chicago, IL, USA).

5.4 Results and discussion

In the laboratory, a detached leaf evaluation was conducted using mycelium, a suitable concentration of which evaluated by comparing various concentrations (10, 30, 50, 80, and 100 mycelium discs/5 mL of DI water) in the resistant genotype (V4718) and the moderately resistant to susceptible genotype (CN72). It was found that 80 mycelium discs/5 mL of DI water was the most suitable for the evaluation because

this concentration can clearly identify the different levels of resistance between the 2 mungbean genotypes. At the lower and higher concentrations, the disease severity scores were not well differentiated between the 2 genotypes (Table 5.2). Therefore, the 80 mycelium discs/5 mL of DI water were used for a pathogenicity analysis as well as detached leaf and field assessments of the 19 mungbean genotypes.

In this study, 10 isolates of *C. canescens* belonging to different locations (isolates SUT-1 and SUT-4 from Suranaree University of Technology Farm, PAK-1 and PAK-2 from Pak Thong Chai district, NW-1 and NW-2 from Nakhon Sawan, PB-1 and PB-2 from Petchabun, and PCH-1 and PCH-2 from Phichit provinces) were evaluated for pathogenicity to select the most virulent isolates for further use in the experiment. When inoculated on 5 mungbean genotypes varying in resistance levels (CN72, CN84-1, V4718, V4758, and V4785), isolates SUT-1, SUT-2, PAK-1, and PAK-2 were found to be highly virulent, while the remaining isolates were moderately virulent (Table 5.3). These results suggest that there were variations in the pathogenicity of *C. canescens*. Similarly, Iqbal and Mukhtar (2014) reported that *Macrophomina phaseolina*, a fungus causing charcoal rot in mungbean, had pathogenic variation and a broad host range, which enabled it to survive better.

Table 5.2 Differential responses of mungbean genotypes on detached leaf assay against *C. canescens* isolate SUT1 at different concentrations of mycelium inoculum.

Inoculum concentrations (pieces/5mL)	Genotypes			
	CN72		V4718	
	Severity scores	Resistance levels[1]	Severity scores	Resistance levels
10	1.00 ± 0.00	R	1.00 ± 0.00	R
30	2.00 ± 0.00	R	1.36 ± 0.11	R
50	2.40 ± 0.05	R	1.53 ± 0.13	R
80	3.23 ± 0.07	MR	2.00 ± 0.07	R
100	3.73 ± 0.07	S	3.23 ± 0.07	MR

[1] Cercospora leaf spot resistance levels based on disease severity scores as follows: 1.0-2.4 = resistant (R), 2.5-3.4 = moderately resistant (MR), and 3.5-5.0 = susceptible (S).

To evaluate the effects of the plant, developmental stages on the detached leaf assay, 5 mungbean genotypes (CN72, CN84-1, V4718, V4785, and V4758) were inoculated with mycelium inoculum at 2 different stages (21 days after sowing (DAS) (vegetative stage) and 45 DAS (reproductive stage)). It was demonstrated that resistance within the genotype was mainly identical at both developmental stages (Table 5.4). These results indicate that relative disease resistance is consistent with genotypes regardless of the stages of plant development. Guo et al. (2016) also found that the resistance of boxwood plants inoculated with *Calonectria pseudonaviculata* mycelium at various developmental stages remained stable over time. Therefore, mycelium screening assays can be performed in the laboratory at any stage of plant development with accurate results. The early laboratory screening assay using the leaves at the vegetative stage will be helpful to reduce the time required for a resistance evaluation in the mungbean breeding programs.

Table 5.3 Differential responses of mungbean genotypes on detached leaf assay against 10 isolates of *C. canescens*

Genotypes	Isolates																			
	SUT-1[1]		SUT-4		PAK-1		PAK-2		NW-1		NW-2		PB-1		PB-2		PCH-1		PCH-2	
	Scores	Rl[2]	Scores	Rl	Scores	Rl	Scores	Rl	Scores	Rl	Scores	Rl	Scores	Rl	Scores	Rl	Scores	Rl	Scores	Rl
V4718	1.76 a[3]	R	2.00 a	R	1.81 a	R	1.24 a	R	1.17 a	R	1.28 a	R	1.56 a	R	1.17 a	R	1.28 a	R	1.44 a	R
V4758	2.22 b	R	2.36 b	R	2.26 b	R	2.07 b	R	1.95 b	R	2.00 b	R	2.05 b	R	1.90 b	R	1.83 b	R	1.35 a	R
V4785	2.28 b	R	2.37 b	R	2.22 b	R	2.09 b	R	1.97 b	R	2.13 bc	R	1.58 a	R	1.65 b	R	1.76 b	R	1.81 b	R
CN72	3.26 c	MR	3.19 c	MR	3.33 c	MR	3.31 c	MR	2.24 bc	R	2.31 c	R	2.33 b	R	2.28 c	R	2.34 c	R	2.40 c	R
CN84-1	3.37 c	MR	3.32 c	MR	3.35 c	MR	3.28 c	MR	2.42 c	R	2.33 c	R	2.38 b	R	2.35 c	R	2.23 c	R	2.24 c	R

[1] Isolates of *C. canescens* were obtained from infected mungbean leaves in the fields at Suranaree University of Technology Farm (SUT-1 and SUT-4), Pak Thong Chai district (PAK-1 and PAK-2), Nakhon Sawan (NW-1 and NW-2), Petchabun (PB-1 and PB-2), and Phichit (PCH-1 and PCH-2) provinces.

[2] Rl = resistance levels.

[3] Data followed by different letters in each column differ significantly ($p < 0.05$) according to Duncan's multiple range test.

Table 5.4 Disease severity of *C. canescens* on detached leaf assay in the laboratory with isolates SUT-1, SUT-4, PAK-1, and PAK-2 inoculation of 5 mungbean genotypes on leaves of 21- and 45-day-old plants.

Genotypes	Detached leaf assay using 21-day-old plants		Detached leaf assay using 45-day-old plants	
	Severity scores	Resistance levels[1]	Severity scores	Resistance levels
V4718	1.70 ± 0.16 a[2]	R	2.34 ± 0.06 a	R
V4758	2.23 ± 0.06 b	R	2.48 ± 0.07 a	R
V4785	2.24 ± 0.06 b	R	2.35 ± 0.05 a	R
CN72	3.27 ± 0.03 c	MR	3.16 ± 0.09 b	MR
CN84-1	3.33 ± 0.02 c	MR	3.09 ± 0.05 b	MR

[1] Cercospora leaf spot resistance levels based on disease severity scores are as follows: 1.0-2.4 = resistant (R), 2.5-3.4 = moderately resistant (MR), and 3.5-5.0 = susceptible (S).

[2] Data followed by different letters in each column differ significantly ($p < 0.05$) according to Duncan's multiple range test.

When the disease severity of 19 mungbean genotypes was evaluated at 45 DAS using mycelium inoculum detached leaf assay with 4 virulent *C. canescens* isolates, the average disease severity scores of the mungbean genotypes was significantly different ($p < 0.01$), varying from 2.34 (resistant) to 4.36 (susceptible) (Table 5.5). The susceptible genotype (EGMD-6D) was found to have the highest disease severity score (4.36). While all resistant genotypes (V4718, V4785, V4758, and Super5) possessed low disease severity scores (2.34, 2.35, 2.48, and 2.48, respectively), all the moderately resistant genotypes (CN72, CN84-1, and KING) and most of the breeding lines were moderately resistant to CLS (Table 5.5). Nevertheless, 4 breeding lines (H3, H4, D5, and G1) were found to be susceptible. These findings suggest that *C. canescens* mycelium can be used as an inoculum to determine mungbean resistance to CLS. Our results are similar to those reported by Guo et al. (2016), which suggested that mycelium inoculum can determine the resistance of boxwood plants to blight (*C. pseudonaviculata*) under laboratory conditions, with a correlation coefficient of 0.91 ($p < 0.01$) between the detached leaf and whole plant assays.

In the field experiment, disease symptoms first appeared at 10 DAI on the susceptible genotype (EGMD-6D). At 15 DAI, average disease severity scores varied significantly ($p < 0.01$) among 19 mungbean genotypes. The severity of the

disease ranged from 1.00 (resistant) to 3.67 (susceptible) (Table 5.5). It was found that the field results were consistent with the laboratory results from the detached leaf evaluation using mycelium. The severity scores of the disease were mostly higher on detached leaves, possibly due to higher disease pressure on detached leaves than on field plants, but both methods reliably identified the levels of disease of most mungbean genotypes, especially those that were resistant or susceptible. Moreover, the disease symptoms from Table 5.6 confirm that both methods show consistent disease lesions, which can be visually observed and easily rated to identify the severity of the symptoms. These results indicate that the mycelium inoculation enabled us to evaluate the CLS resistance of plants under field or laboratory conditions.

Four resistant genotypes (V4718, V4785, V4758, and Super5) were classified as resistant, while EGMD-6D and 2 breeding lines (H4 and D5) were classified as susceptible using both laboratory and field disease assessments. Two moderately resistant genotypes (CN84-1 and KING) and 6 breeding lines (O2-31, O2-37, O2-39, P01, B1, and B2) were classified as moderately resistant by both methods. However, 1 moderately resistant genotype (CN72) and 3 breeding lines (P05, H3, and G1) were found to have minimal variation between both methods. Different rates of disease development may have been observed in different genotypes due to the genetic background of mungbean genotypes (Chueakhunthod et al., 2020). Iqbal and Mukhtar (2014) also reported that there was pathogenic variability among *M. phaseolina* isolates associated with mungbean genotypes. The existence of genetic diversity in mungbean and pathogenic variability of pathogens suggests that resistant varieties should be developed by pyramiding several resistance genes.

Pearson's correlation was used to assess the relationship between laboratory and field evaluations. The correlation between laboratory and field results was highly significant ($p < 0.01$), with a Pearson's correlation coefficient of 0.822 (Figure 5.1). Significant correlations between laboratory and field evaluations revealed that resistance to *C. canescens* in the laboratory was comparable to that observed in the field. Furthermore, the laboratory evaluation was sufficient for determining resistant and susceptible responses. The coefficients of variation (CV) in the field test were higher (16.38%) than in the laboratory evaluation (9.16%). This indicates that the laboratory screening assay is more precise in determining disease responses. Similar

to the study of Poolsawat et al. (2012), higher CV was observed in the field test compared to the laboratory evaluation of resistance to *Sphaceloma ampelinum* causing anthracnose in grapevine.

Table 5.5 Disease severity of *C. canescens* on detached leaf assay in the laboratory and field inoculations of 19 mungbean genotypes.

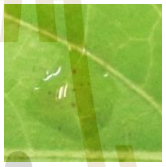
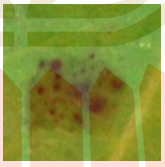
Genotypes	Detached leaf assay		Field	
	Severity scores	Resistance levels[1]	Severity scores	Resistance levels
V4718	2.34 ± 0.06 a[2]	R	1.00 ± 0.00 a	R
V4758	2.48 ± 0.07 a	R	2.33 ± 0.08 d	R
V4785	2.35 ± 0.05 a	R	1.83 ± 0.22 c	R
Super5	2.48 ± 0.06 a	R	1.33 ± 0.08 b	R
EGMD-6D	4.36 ± 0.06 h	S	3.50 ± 0.14g h	S
CN72	3.16 ± 0.09 de	MR	2.33 ± 0.17 d	R
CN84-1	3.09 ± 0.05 cde	MR	2.67 ± 0.08 de	MR
KING	3.28 ± 0.03 e	MR	3.33 ± 0.08 gh	MR
O2-31	2.94 ± 0.01 bc	MR	2.67 ± 0.08 de	MR
O2-37	2.80 ± 0.04 b	MR	2.67 ± 0.08 de	MR
O2-39	2.97 ± 0.03 bcd	MR	3.17 ± 0.08 fg	MR
P01	2.87 ± 0.02 b	MR	2.83 ± 0.08 ef	MR
P05	2.82 ± 0.01 b	MR	2.33 ± 0.08 d	R
H3	3.68 ± 0.03 fg	S	3.17 ± 0.08 fg	MR
H4	3.57 ± 0.05 f	S	3.67 ± 0.08 h	S
B1	3.29 ± 0.03 e	MR	3.33 ± 0.08 gh	MR
B2	2.86 ± 0.05 b	MR	2.83 ± 0.22 ef	MR
D5	3.81 ± 0.06 g	S	3.67 ± 0.08 h	S
G1	3.82 ± 0.04 g	S	3.33 ± 0.08 gh	MR
CV (%) [3]	9.16		16.38	

[1] Cercospora leaf spot resistance levels based on disease severity scores are as follows: 1.0-2.4 = resistant (R), 2.5-3.4 = moderately resistant (MR), and 3.5-5.0 = susceptible (S).

[2] Data followed by different letters in each column differ significantly ($p < 0.05$) according to Duncan's multiple range test.

[3] CV = Coefficient of variation.

Table 5.6 Disease severity of *C. canescens* in detached leaf assay and field conditions.

Resistance levels	Genotypes	Methods	
		Detached leaf assay	Field
Resistant	V4718		
	Super5		
Moderately resistant	CN84-1		
	KING		
Susceptible	EGMD-6D		
	D5		

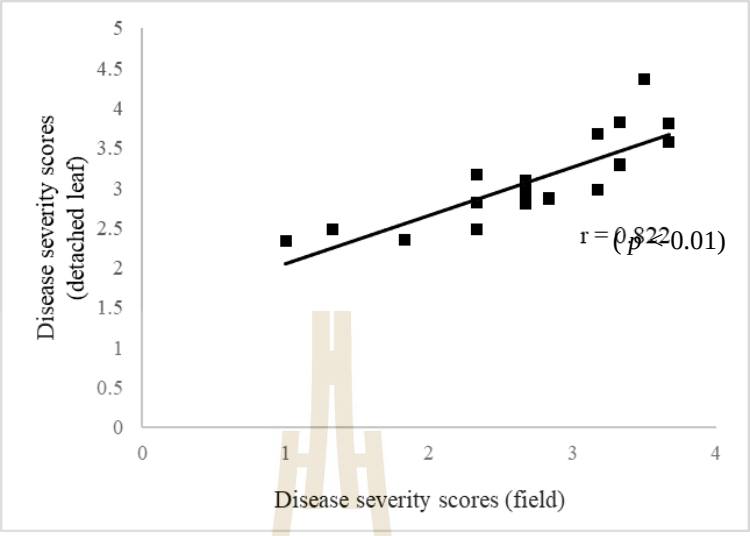


Figure 5.1 Correlation between disease severity scores of mycelium-inoculated detached leaf and field assessments of mungbean genotypes.

5.5 Conclusion

We have successfully developed an efficient screening assay for CLS resistance in mungbean, which could screen a large number of genotypes in the laboratory and is simple and easy to prepare, while also taking less time. In less than a week, the disease status of numerous mungbean genotypes can be identified using this laboratory screening assay. These results suggest that mycelium can be used as an inoculum in the laboratory and field disease assessments. The method is effective and reliable for the screening of CLS resistance in mungbean varieties/lines and their breeding lines, which will be a useful method for developing resistant mungbean varieties in breeding programs

5.6 References

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CHAPTER 6
CONCLUSION

Cercospora leaf spot, a significant disease, poses a major threat to mungbean (*Vigna radiata* (L.) Wilczek) cultivars in Thailand. Evaluating the resistance levels of various mungbean genotypes to CLS and developing effective disease management strategies are essential. Our study found that the best culture media were mungbean leaf agar (MLA) and lettuce leaf agar (LLA) for *C. canescens* growth in terms of colony diameter. When grown on various culture media, the isolates showed variations in their morphological characteristics. The twenty isolates in this study had genetic similarity ranging from 0.84 to 1.00, according to an analysis using RAPD. These isolates were divided into four different groups according to their geographic origins using cluster analysis, which used the unweighted paired grouped mean arithmetic average. Six different mungbean genotypes and 20 *C. canescens* isolates were used in the pathogenicity assessment, and it was found that genotypes V4718 and Super5 were resistant to the majority of the isolates. According to the results, *C. canescens* isolates from different regions differ in terms of their growth, morphology, genetics, and virulence. From the study of mungbean genotypes in response to CLS, it was discovered that the two susceptible genotypes, EGMD-6D and SUT1, had lower concentrations of the β-1,3-glucanase, chitinase, and PPO activities in infected leaves. On the other hand, the β-1,3-glucanase, chitinase, and PPO activities were higher in the resistant genotypes V4718 and Super5. The HR symptoms varied in intensity across mungbean genotypes, which was consistent with the enzyme activities. The resistant genotypes, V4718 and Super5, showed signs of HR, but the susceptible or moderately resistant genotypes did not. It is significant to note that this study did not find any evidence of systemic induction. Two up-regulated protein bands, one at 17 kDa and the other at 39 kDa, were discovered by SDS-PAGE on infected leaves. Only the susceptible mungbean genotypes EGMD-6D and SUT-1 contained the 17 kDa band. With the exception of Super5, a resistant genotype that displayed the 39 kDa band at

the pre-inoculation stage (0 DAI), the majority of mungbean genotypes expressed this band after inoculation. The differential expression of these proteins suggests that different mungbean genotypes may have different resistance mechanisms, and suggests that β-1,3-glucanase, chitinase, and PPO are critical parts of the defense system of mungbean against CLS. Detached leaf inoculations were performed using mycelium obtained from the most virulent *C. canescens* isolates, SUT1, SUT4, PAK1, and PAK2, to develop an efficient laboratory inoculation technique for evaluating the resistance/susceptibility levels of 19 mungbean genotypes. When compared to field inoculations, the disease severity of mungbean genotypes evaluated by detached leaf inoculations were comparable, with a correlation coefficient of 0.822 ($p < 0.01$). CLS-resistant mungbean genotypes V4718, V4785, V4758, and Super5 were consistently identified as useful for future breeding programs using both inoculation methods. The study highlights the potential of the resistant genotypes V4718 and Super5 as valuable sources of resistance in future breeding programs.

VITAE

Ms. Tana Jaichopsanthia was born on August 12, 1996 at Nakhon Ratchasima, Thailand. She graduated with the Bachelor of Science Degree Program in Crop Production Technology, Suranaree University of Technology in 2019. Then, in the same year, she decided to further study for master degree in School of Crop Production Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. During her studies, she received a scholarship from SUT, which provided support for her tuition fees and living costs. Her research topic was diversity of *Cercospora canescens* and resistance mechanisms of mungbean to cercospora leaf spot under supervision of Professor Dr. Piyada Alisha Tantasawat. The results from some parts of this study have been presented in the 4th International Conference on Agriculture and Agro-Industry 2022 (ICAAI2022), November 10-11, 2022, Chiang Rai, Thailand (Correlation between mycelium-inoculated detached leaf and field assessments of resistance to *Cercospora canescens* in mungbean).