INVESTIGATION OF MECHANISMS UNDERLYING BLACKLEG MITIGATION IN CANOLA THROUGH THREE NOVEL INTEGRATED PEST MANAGEMENT APPROACHES

BY

KALUHANNADIGE RASANIE ERANKA PADMATHILAKE

A Thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
in partial fulfilment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Department of Plant Science
University of Manitoba
Winnipeg, Manitoba
Canada

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor, Dr. Dilantha Fernando, for believing in me and giving me the opportunity to do a PhD under his supervision. You guided me and took me through this long journey without letting my hand go off from you. You always steered me in the right direction, even when I was so down and feeling lost. You were always there for me in challenges came in not only in academic life but in my personal life. The way you treated me during my pregnancy period and how you react when my Sri Lankan University administration asked me to report for duty when the assigned study leave was over will never ever be forgotten. You are one of the biggest roles who made me what I am today. If it were not you, I would not come this far. I cannot thank you enough sir!

Next, I would also like to thank my PhD committee members Dr. Ayush Kumar and Dr. Curt McCartney for their great advice, guidance, and continuous support for my research and thesis writing from the beginning to the very end. Great thanks to Dr. Mark Belmonte and Dr. Khalid Rashid for their valuable guidance and support they gave me. Thank you, Dr. Claudio Stasolla and Dr. Rob Gulden for the support you gave me to make this a success.

I greatly appreciate Ms. Paula Parks for her great technical assistance and guidance for my research work in the lab and specially in the field at Carman. Without you, my field study won't be that success. Special thanks to all greenhouse staff and technicians in the Department of Plant Science, Cathy Bay, Rob Visser, Jo-Anne Joyce, and Lorne Adam. I wish to acknowledge the support staff in Plant Science, especially Martha Blouw, Martha, you were there for me always when I needed, Christy Doyle, and Bev Godard for their support, advice, and assistance during my time at the department. Next, I sincerely appreciate the financial support given by the University of Manitoba and Syngenta company.

My sincere gratitude goes to Ted Labun and Kurt Anaka from Syngenta to for the great support they gave me on the fungicide study. A special thanks to all my former and current lab members for their support and friendship. Thank you, Harun, Rhodesia, Cunchun, Aria, Liang, Zou, Fei, Abbot, James, Sachithrani and others. Furthermore, I acknowledge the graduate students from other labs Atta, Kenny, Keval, Jonathan, and Hanna.

My thanks must go to all Sri Lankan friends in Winnipeg, specially Ruvin and Saranga for accepting to take care of my baby at their daycare from she was only six months old after my maternity period.

I have to specially thank you Prof. Gamini Seneviratne, National Institute of Fundamental Studies and Dr. Chathuranga Bamunuarachchige, Faculty of Technology, Rajarata University of Sri Lanka for trusting me to sign my very expensive bond document without any hesitation and for being patience until I came back.

A big thank you goes to my workplace, Rajarata University of Sri Lanka. I greatly appreciate the support given by former and current Vice-chancellors, former and current Deans of the Faculty of Agriculture, former and current Heads of the Department of Plant Sciences, and specially the staff at the Department of Plant Sciences for not giving a burden as much as possible during my thesis writing, and all the staff members who helped me even with an encouraging word along this challenging journey.

My sincere gratitude to my parents, "Thaththi" and "Ammi"! Thank you for your unconditional love and all the sacrifices made. Though you two raised me under your eye lashes, you let me go and bared my loss patiently until I made my dream a success. And special thanks to my one and only sister Gayanie Padmathilake. Last not but not least, I thank my husband and my best friend, Wenura Dharmasena for being with me at each and every step I have taken along this challenging journey. You sacrificed your job in Sri Lanka and joined with me to go to Canada to make my dream true. You smiled with me, cried with me at all the ups and downs came along. I would not come this far without having you by my side. Here I cannot forget my little princess, Nichellie, for being such a great kid who came to our lives during the second year of this study. You understood your mom's busy schedule with studies. Though you wanted to play with me, most of the time you satisfied by only holding my hand while watching a cartoon.

This dissertation would not have been possible without the support of you all...!! Thank you very much…!!

I would like to dedicate this thesis

to my husband, Wenura Dharmasena, the best husband a wife could ever wish for

to my princess, Nichellie, the best daughter a mom could ever wish for

to my parents, Thaththi and Ammi, the best parents a daughter could ever wish for

to my supervisor, Dr. Dilantha Fernando, the best supervisor a student could ever wish for..

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Padmathilake, K.R.E., Ph.D. The University of Manitoba, March 2022. Investigation of mechanisms underlying blackleg mitigation in canola through three novel integrated pest management approaches. Ph.D. Supervisor: Dr. W. G. Dilantha Fernando.

Blackleg disease caused by fungus *Leptosphaeria maculans* is one of the most economically significant diseases of canola (*Brassica napus* L). Three approaches that can be used in mitigating of this devastating disease were investigated in this study. Introducing new resistant genotypes is pivotal as host resistance plays the leading role in managing the disease and allows gene rotation. Since *AvrLm7* is one of the most abundant avirulence genes in the pathogen population and *Rlm7* has been reported as a stable host genotype in other countries, this study focused on evaluating the potential of *B. napus* genotype, '01-23-2-1', which carries *Rlm7*. '01-23-2-1'was inoculated with *L. maculans* isolate, carrying *AvrLm7*, and the CRISPR/Cas9 *AvrLm7*-knockout mutant of the same isolate to make incompatible and compatible interactions, respectively. A dual RNA-seq study explored differential gene expressions in both interactions. An upregulation of defense-related genes was observed in the incompatible interaction, and in contrast, the same gene expressions were started late in compatible interactions. High expressions of virulence-related pathogen genes-CAZymes, merops, and effector proteins after 7-dpi in compatible interactions but not in incompatible interaction—confirmed that the pathogen was actively virulent only in compatible interactions. Pydiflumetofen is a broad-spectrum seed treatment fungicide in the succinate dehydrogenase inhibitor (SDHI) group. Pydiflumetofen was evaluated compared to Vibrance® Flexi (current company recommendation) under greenhouse conditions using moderately resistant canola cultivar SY4135, and susceptible cultivar Westar. Both SY4135 and Westar plants developed from pydiflumetofen treated seeds showed significantly lower disease severity in canker formation under controlled conditions. Moreover, both host genotypes exhibited lower disease severity in Green fluorescent protein (GFP) tagged *L. maculans* inoculated seedlings demonstrated control of mycelial growth in planta. Under field conditions, SY4135 and Westar plants treated with pydiflumetofen demonstrated lower disease severity than another three field recommendations, but the difference was not significantly different. However, pydiflumetofen treated SY4135 produced significantly more seed yield than

untreated SY4135. The results revealed pydiflumetofen protects the plant at the critical window for infection. Therefore, pydiflumetofen is a worthy tool that farmers can add to the blackleg control. In addition, co-inoculation and pre-inoculation of less virulent *L. biglobosa* into canola cotyledons showed successful control of the pathogen at morphological, fluorescence microscopic and transcriptomic levels. The observations confirmed the potential of *L. biglobosa* as an effective biological control of the pathogen *L. maculans*. Taken together, the knowledge gained from this dissertation will facilitate to add new tools into the blackleg management toolbox.

FOREWORD

This thesis has been written in manuscript style according to the format outlined by the Faculty of Graduate Studies of the University of Manitoba. This thesis consists of a general introduction followed by a literature review, three chapters, a general discussion, and recommendations for future research. Reference materials, consisting of a list of literature cited and data appendices. The first study has been published in the International journal of molecular sciences under the title "*Leptosphaeria maculans-Brassica napus* Battle: A Comparison of Incompatible vs. Compatible Interactions Using Dual RNASeq". The second study has been submitted to the journal Plant pathology under the title "Pydiflumetofen: a SDHI Seed Applied Fungicide, a Potential Tool for the Canola-Blackleg Management Toolbox". The third study has been published in the journal Plants with the title of "Less Virulent *Leptosphaeria biglobosa* Immunizes the Canola Plant to Resist Highly Virulent *L. maculans*, the Blackleg Pathogen".

CHAPTER 1. GENERAL INTRODUCTION

Canada is the biggest canola producer, accounting for 20–30% of the total world production (Van de Wouw and Howlett 2019), providing around $29.9 billion in income to the Canadian economy and generating more than 207,000 jobs in the country (Canola Council of Canada, 2020). Canada exports 90% of canola seeds, oil, and meals to foreign markets annually (Canola Council of Canada, 2017). Dothideomycete fungus, *Leptosphaeria maculans*, causing blackleg disease is one of the most destructive diseases in canola (*Brassica napus*). This disease is a considerable threat in Canada, Australia, and Europe, which causes more than $900M loss per growing season worldwide (Fitt et al., 2008). Blackleg disease management includes crop rotation, fungicide application, and resistant varieties (Fernando et al., 2007; Kutcher et al., 2011). The utilization of resistant varieties is considered the most effective management strategy (Li and McVetty 2013; Raman et al., 2013). However, the effectiveness of resistant varieties is not long-lasting as the pathogen population is smart enough to overcome these *R*-genes within a short period by the evolution of *Avr* and *avr* genes accordingly. For instance, *Rlm1* and *LepR3* were broken in France and Australia, respectively, within a few years of introduction (Rouxel et al., 2003; Van de Wouw et al., 2010). Zhang et al. (2016) showed *Rlm3*, which was the most frequently used *R*-gene in Canadian canola accessions, has been broken down already. It is essential to diversify the *R*-gene that farmers could use to reduce the selection pressure. Zhang and Fernando (2018) suggested the *R*-gene rotation as another effective strategy to control the ruinous pathogen.

Qualitative resistance or major gene resistance of canola is mediated by single major resistance (*R*) gene. This *R*-gene mediated resistance follows the gene-for-gene theory proposed by Flor (1971). According to Flor, R proteins encoded by *R* genes are recognized as the corresponding avirulence (Avr) proteins encoded by *Avr* genes of the pathogen. The '*R-Avr*' interaction results in a hypersensitive response (HR) in the plant and restricts further pathogen invasion (Flor 1971; Jones and Dang 2006). To date, there are 23 identified, race-specific blackleg R genes as shown in Appendix 3 (Dolatabadian et al., 2021). It is high time to introduce new *R*-genes into the canola seed market to diversify the *R*-genes used by the farmers to avoid a single *R*-gene breakdown. For an effective breeding approach for new canola lines with new *R*-genes, it is crucial to understand the host-pathosystem well. Mainly, the knowledge on host genes important in defense and pathogen genes, which are essential in virulence and pathogenicity. Such

knowledge will lead us to develop more effective genotypes into the *R*-gene pool available for farmers.

According to Liban et al. (2016) and Fernando et al. (2018), *AvrLm7* is one of the abundantly available avirulence genes present in *L. maculans* fungal population in Canada. On the other hand, the corresponding host gene, *Rlm7* has exhibited better performance and slower in breaking down, in countries it has already been introduced. Based on those observations, *Rlm7* can be chosen as the next candidate *R*-gene to be introduced in the Canadian canola seed market (Huang et al., 2018). Understanding of the host-pathosystem in-depth at the transcriptomic level is vital to manage the *R*-gene for a long duration.

As the *Rlm3,* which is used mainly in Canadian prairies, has been broken already, and no introduction of alternative *R*-gene is under proceeding, it is important to implement of an integrated strategic plan to avoid any outbreaks of the pathogen. Using fungicides is one of the backup plans. Typically, foliar fungicides are not cost-effective to use as a control measure of canola blackleg disease as it applies when farmers see clues of severe disease incidence in the crop. It is very tricky to identify the correct timing for fungicide application to manage the pathogen. As the foliar application is expensive for farmers, there is a need for effective seed treatments. Succinate dehydrogenase inhibitors (SDHIs), which belongs to FRAC code 7, is the newest class of fungicide that has entered the fungicide market.

There are two *Leptosphaeria* species that cause the blackleg disease in the canola plant: highly aggressive *L. maculans* and weakly virulent *L. biglobosa*. Several previous studies have shown priming of host resistance caused by weakly virulent *L. biglobosa* infection against any subsequent attacks of aggressive *L. maculans* (Chen and Fernando 2006; Hadrami et al., 2010). Therefore, understanding the effect of the interaction triangle, especially on the host plant, is very important to seek possibilities of using weakly virulent *L. biglobosa* as a biocontrol over disastrous pathogen, *L. maculans.*

Recognizing the importance of seeking for more tools to the blackleg disease management toolbox, the following overarching hypotheses were proposed:

Overarching Hypothesis: Novel *R* gene (host resistance), newly developed seed applied fungicide (chemical control) and weakly virulent *L. biglobosa* (biocontrol) are worth additions to the blackleg management toolbox which can be implemented in controlling the blackleg pathogen

successfully. I propose these to be used in an integrated approach once all possible information on these single management tools are well understood.

Three hypotheses proposed for each study:

1. Hypothesis: Transcriptomic analysis of *Rlm7-AvrLm7* and *Rlm7-avrlm7* interactions lead to understand the incompatible and compatible interactions in-depth specially the defence related host genes and virulence related pathogen genes.
   Objective: Analyze and understand the performance of *Rlm7*, interactions with avirulence *AvrLm7* and virulence *avrLm7* by transcriptomic analysis to learn the defence related host genes and virulence related pathogen genes.
2. Hypothesis: Newly developed SDHI seed treatment Pydiflumetofen can control the blackleg pathogen *L. maculans* successfully.
   Objective: Evaluation of newly developed SDHI seed treatment Pydiflumetofen in controlling fatal pathogen, *L. maculans*.
3. Hypothesis: Weakly virulent *L. biglobosa* has the potential to be used as a biocontrol agent against highly virulent *L. maculans.*
   Objective: Assess the potential of weakly virulent *L. biglobosa* to be used as a biocontrol agent, based on its capability to induce the acquired resistance against highly virulent *L. maculans*.

CHAPTER 2. LITERATURE REVIEW

2.1 Introduction

Canola (*Brassica napus* L.) is the second largest and most crucial oilseed crop, after soybean, in the world. Canada is the largest canola producer globally, making up 20-30% of the total world's production about 18.7 MMT (Van de Wouw and Howlett 2019; Canola Council of Canada, 2020). In the Canadian context, canola is the number one cash crop that significantly impacts the country's economy, with around $29.9 billion of contribution to the annual country economy. Moreover, the crop provides more than 207,000 Canadian jobs and $12 billion in wages. Annually, Canada exports 90% of canola seeds, oil, and meals produced in Canada to foreign markets worldwide (Canola Council of Canada, 2020). Oil for human consumption and meal for livestock feed are the main products of canola. The Canola Council of Canada expects to reach an annual production of 26 million tonnes of canola by 2025 (Zhang and Fernando, 2018).

Blackleg disease caused by Dothideomycete fungus *Leptosphaeria maculans* is one of the most destructive diseases found in canola (West et al., 2001; Howlett, 2004). This devastating pathogen can do significant damage up to 80%, depending on the susceptibility of the cultivar grown (Marcroft et al., 2004). Using resistant cultivars is one of the significant control measures used to keep the disease under control. However, overcoming the resistance genes found in commercially available cultivars is important to seek other controlling measures to avoid disease outbreaks (Zhang et al., 2016). This literature review provides insights into the research progress in understanding this critical crop plant, *B. napus*, this destructive disease, and their interactions.

2.2 The Host

2.2.1 *Brassica napus* L.

The genus *Brassica* comes under the mustard family, *Brassicaceae,* which comprises nearly 3350 species in 340 genera. This genus *Brassica* includes many species of vegetables, ornamentals, oil crops, which are important worldwide. *B. rapa* (Asian cabbage/turnip rape), *B. juncea* (Indian mustard), *B. oleracea* (Mediterranean cabbage, broccoli, and cauliflower), *B. carinata* (Ethiopian mustard *and B. nigra* (black mustard) are cultivated *Brassica* species other than *B. napus* (canola/rapeseed/oilseed rape/colza/Raps) (Hayward et al., 2011). *B. napus* L. is an

allotetraploid carries AACC (2n = 4x = 38), originated from interspecific hybridization between *B. oleracea* (2*n* = 2×9 = 18, genome CC) and *B. rapa* (2*n* = 2×10 = 20, genome AA) (Chalhoub et al., 2014). This herb is an annual or biennial plant. The genome of *B. napus* is 1.13 Gb carrying 90,000-101,000 protein-coding genes (Cheng et al., 2017). The *B. napus* genome comprises nine and ten chromosomes belonging to sub genomes C_n and A_n, respectively.

Though the term rapeseed is used as an alternative for canola, to be accurate, "Canadian oil" or "canola" is the edible member of rapeseed. Seeds contain high oil (40-42%) and protein (43.6%). Protein content in seed meals is rich in amino acids such as lysine, methionine, and cystine. Rapeseed oil contains 20-55% erucic acid despite high protein content. Erucic acid (EA; C22:1ω9) is a long-chain and monounsaturated fatty acid harmful for human consumption. Erucic acid was effectively eliminated through breeding programs in developing canola, which is also known as double low or "00" rapeseed oil in the European Union (McKeon 2016). Canola contains less than 2% erucic acid in oil and less than 30 µmol of glucosinolates per gram of air-dried oil-free meal (Canola Council of Canada 2020). The high level of glucosinolates in the meal led a number of side effects such as goitrogenicity (Pettit et al., 1944).

2.2.2 Importance of Canola

Canola is grown worldwide, in North, Central and South American countries; Asian countries such as China, Japan, Iran, Russia, India; European countries: Denmark, Netherlands, and Norway; Australia. In the Canadian context, canola oil is the most widely used edible oil. Canada is the number one producer and exporter of Canola oil globally, producing 13% of the global supply. Canola oil is rich in omega-3 fatty acids and contains 60-65% monounsaturated fats and 30-35% polyunsaturated fats making it one of the healthiest edible oils (Kostik et al., 2013). Moreover, canola oil carries lower cholesterol levels making it more demanding (Lin et al., 2013). On the other hand, canola meal, a by-product of seed oil extraction, is widely used in livestock, fish, poultry, and swine feed. Rapeseed is still cultivated for industrial purposes. For instance, it has been used as high-quality lubricants, hydraulic fluid, and plastics (Canola Council of Canada 2020).

2.2.3 Diseases in Canola

Several diseases threaten canola worldwide. Fungal pathogens cause the major diseases of canola except a few, including blackleg (caused by *L. maculans* and *L. biglobosa*), sclerotinia stem rot (caused by *Sclerotinia sclerotiorum*), Fusarium wilt (caused by *Fusarium avenaceum* and *F. oxysporum*), white rust (caused by *Albuga candida*), downey mildew (caused by *Peronspora parasitica*), light leaf spot (caused by *Pyrenopeziza brassica*), and Verticillium wilt (caused by *Verticillium longisporum*). Moreover, Clubroot (caused by the obligate protist *Plasmodiophora brassicae*) and aster yellows (caused by a pytoplasma) are exceptions caused by pathogens other than fungi. Among these diseases, the blackleg disease is one of the most devastating and economically significant diseases, which causes >$900 annual loss worldwide (Fernando et al., 2007; Van de Wouw et al., 2016).

2.2.4 Blackleg Disease in Canola

The blackleg disease, also called phoma stem canker, caused by Dothideomycete fungi *L. maculans* and *L. biglobosa*, is a fatal and predominant canola disease (West et al., 2001; Fitt et al., 2006; Fitt et al., 2008). Canadian prairies experienced the first wave of blackleg outbreaks in the 1980s. In 1975, *L. maculans* was first identified in Saskatchewan (McGee and Petrie 1978). Manitoba, Alberta, and British Columbia were preyed on afterward (Gugel and Petrie 1992). Westar was the cultivar, which was primarily grown in the 1980s. Due to tight rotations and accumulation of stubble in the field, the susceptible Westar led to severe outbreaks in Canadian prairies. According to Zhang and Fernando (2018), disease incidence of blackleg reached 52% in Saskatchewan in 1989. In 1991, Westar was abandoned in the early 1990s, and new varieties that were resistant to the disease were introduced (Kutcher et al., 2010). After introducing the resistant varieties in the early 1990s, the disease was under control until the early 2000s (Zhang and Fernando, 2018). However, shortened rotations, no or less diversification due to high market demand for the crop and breakdown of resistance due to less or no crop diversification has led to widespread of the disease by 2012 (Hwang et al., 2016).

The blackleg causal agent was classified into two pathotypes until 2001: highly virulent "A" group strains that cause stem cankers and weakly virulent "B" group strains that do not (Williams and Fitt 1999). Then strains in the "A" group were divided into different pathogenicity

groups (PGs), whereas "B" strains were classified under another species named *L. biglobosa*. The predominant pathogenicity group of *L. maculans* was PG2 (Shoemaker and Brun 2001; Kuusk et al., 2002; Chen and Fernando 2006).

2.3 *Leptosphaeria maculans*

The fungus, *Leptosphaeria maculans* (Desm.) Ces. & de Not. (anamorph: *Phoma lingam* (Tode ex Fr.) Desm.), could infect many cruciferous crops. This fungus was first reported by Tode (1791), who observed the fungus on dried stems of red cabbage. As cited by Henderson (1918), the fungus was named *Sphaeria lingam.* Later, *S. lingam* was renamed as *Phoma lingam* by Desmazières (1849). In 1863, Tulasne and Tulasne suggested the sexual form of the pathogen as *Leptosphaeria maculans* (Desm.) Ces. & de Not. *L. maculans* belong to the kingdom Fungi, phylum Ascomycota, class Dothideomyctes, order pleosporales, family *Leptosphaeria*ceae, genus *Leptosphaeria*, and species *maculans* (Rouxel and Balesdent 2005). The relationship of *L. maculans* with the host plant was experimentally demonstrated in the mid-1900s (Rouxel et al., 2004). The blackleg disease has become a major economic concern since the mid of the 20th century (Roy and Reeves 1975).

The fungus overwinters on infected crop residues for several years (West et al., 2001; Li et al., 2007*)*. Generally, the life cycle commenced by airborne ascospores released from stubble-borne pseudothecia and be transferred over vast distances by the wind (West and Fitt 2005). Ascospores land on cotyledons and leaves and germinate under humid conditions. The emerging hyphae enter the leaves through stomata and wounds. Before entering cells, the fungus colonizes in the apoplast of cotyledon/leaf tissues without exhibiting any symptoms to the outside. Then the hyphae proceed down the petiole, then to the stem, until the stem base via xylem vessels or intercellular spaces of xylem parenchyma and cortical cells (Sexton and Howlett 2001). The darkening of stem bases is the main symptom of this disease (Figure 2.1). Round or irregular, grey color lesions on leaves and cotyledons with black pycnidia release pycnidiospores, which spread by rain splash and led to secondary infection (Travadon et al., 2009, Bousset et al., 2018). Pycnidiospores are the major disease inocula development in Western Canada (Guo et al., 2005; Ghanbarnia et al., 2011), while it was ascospores in Europe and Australia (Marcroft et al., 2004; Fitt et al., 2006). The epidemiology of this pathogen varies from region to region based on the

climate, growing season, cultural practices, cultivars used, and fungal populations (West et al., 2001; Fitt et al., 2006).

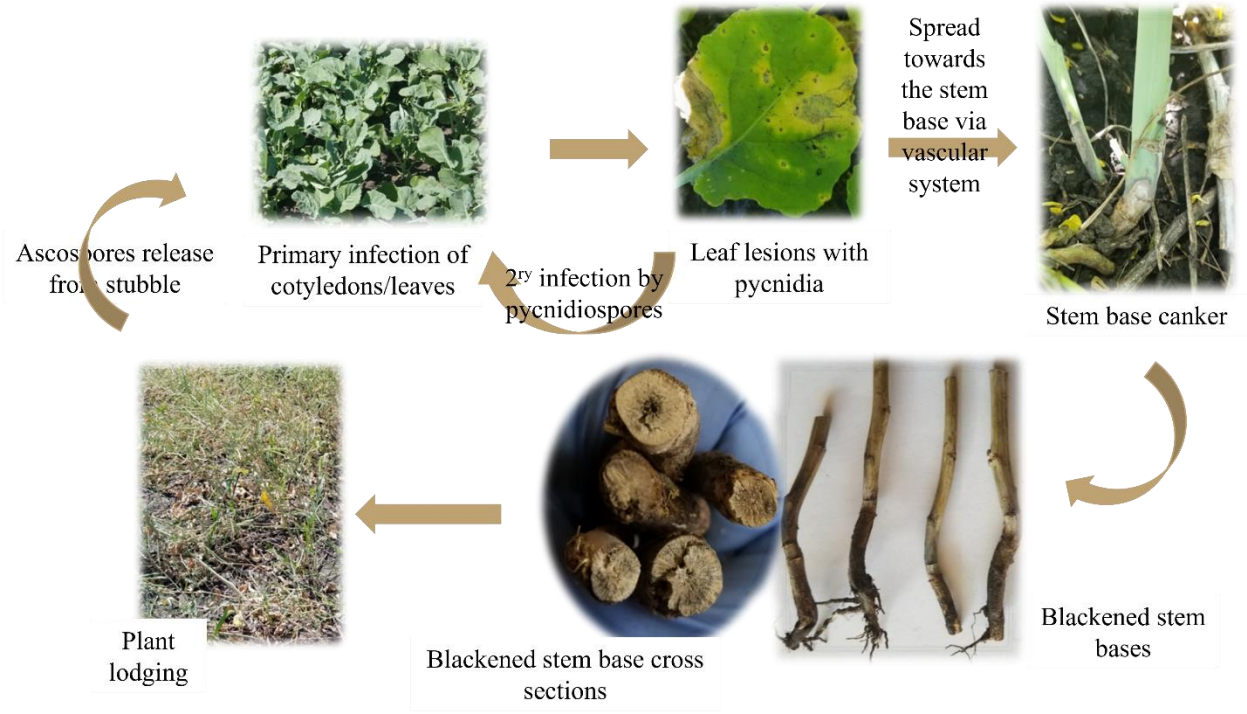


Figure 2. 1 *Leptosphaeria maculans* Life Cycle. The primary inoculation occurs by ascospores released from the stubble from the previous season. Cotyledons or true leaves get infected with ascospores. Pycnidiospores formed in leaf lesions cause the secondary infection. Leaf lesions with black pycnidia, canker at the stem base, and lodging of plants are characteristic symptoms of this disease. The blackening of stem base is why the disease is given the term 'blackleg'. Based on the blackening of the stem base, the disease severity is rated. Pictures were taken in summer, 2019.

L. maculans is a hemibiotrophic fungus. The fungus, which enters the leaf through a stoma or a wound and colonizes the apoplast (as shown in Figure 2.2), starts its life cycle as a biotrophic fungus that obtains nutrients from living cells. The hypha grows endophytic and asymptomatic in planta (Stotz et al., 2014). Later, the fungus transforms into the necrotrophic nutritional strategy in which the fungus secretes toxins, kills the leaf cells, and relies on dead cells for the nutrient requirement. The hyphal front spreads down the petiole in an endophytic and asymptomatic style, finally reaching the stem cortex and causing black/brown blackleg necrotic lesions (West et al., 2001; Stotz et al., 2014).

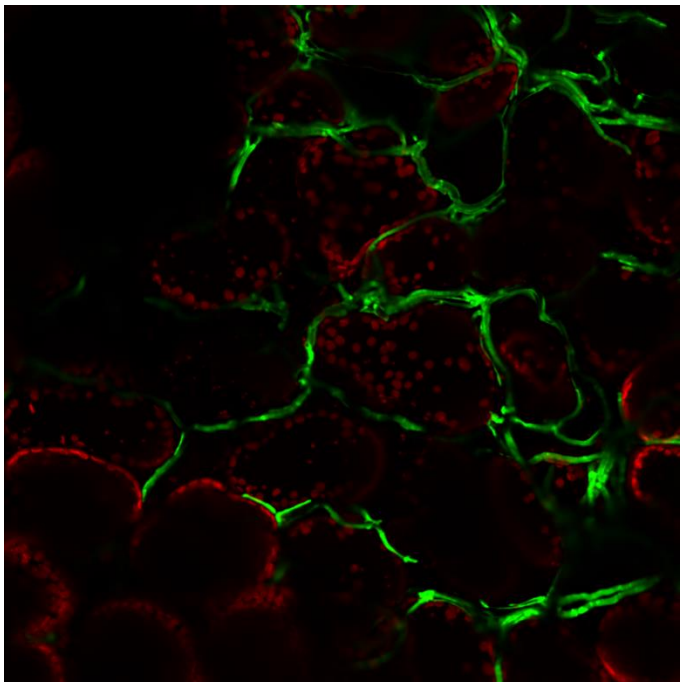


Figure 2. 2 Apoplastic Mycelial Growth of *Leptosphaeria maculans*. After entering through stomate or a wound, the mycelium of *L. maculans* grows apoplastically. The green fluorescence tagged *L. maculans* mycelium observed in intercellular spaces of cotyledons have shown. The red signal represents the Chlorophyll autofluorescence of plant cells (magnification 200x).

2.4 Weakly Aggressive *L. biglobosa*

L. biglobosa is a closely related fungal species different from *L. maculans* based on its genotype and phenotypic performance on the host plant. *L. biglobosa* is explained as a weakly-virulent pathogen based on the damage caused to the host plant (West, 2001). *L. biglobosa* species were divided into six subclades. *L. biglobosa* 'canadensis', *L. biglobosa* 'brassicae', and *L. biglobosa* 'thlaspii' were found in Canada (Mendes-Pereira et al., 2003). Gudelj et al. (2004) suggested *L. maculans* and *L. biglobosa* coexist in European and North American countries, which may have evolved from a common ancestor.

Both *L. maculans* and *L. biglobosa* exhibit a similar life cycle (Rouxel and Balesdent 2005). In contrast to *L. maculans, L. biglobosa* confines its damage to the upper canopy of *B. napus* with upper stem lesions. Also, based on morphological differences of pseudothecia, *L. maculans* and *L. biglobosa* can be further distinguished (Shoemaker and Brun 2001). Furthermore, *L. biglobosa* is a necrotroph, while *L. maculans* is a hemibiotroph (Lowe et al., 2014).

2.5 Qualitative and Quantitative Resistance of the Host Plant Towards the Pathogen

B. napus perform two types of resistance against *L. maculans:* qualitative and quantitative resistance. Single or a few major genes control the qualitative resistance, also called vertical or major gene resistance, and this race-specific resistance activates in the plant particularly at the seedling stage. Multiple genes govern quantitative resistance with minor effects in each. This race non-specific or horizontal resistance is also called minor gene resistance, which is non-race specific and mainly expressed during the adult plant stage called adult plant resistance (Delourme et al., 2004; Raman et al., 2012). Quantitative resistance is not strong enough to prevent pathogen colonization within the host plant but reduces the symptom development, disease epidemics over time, and the impact on yield (Van de Wouw et al., 2016).

Quantitative resistance is the most often and more durable than *R*-gene mediated resistance. Since genes involved in quantitative resistance do not show significant phenotypic effect alone, they cause no strong selective pressure on the pathogen (Delourme et al., 2008; Hayward 2012).

2.6 Gene-for-Gene Interaction

Qualitative resistance incorporated resistance (*R*) genes obey the gene-for-gene interaction model, which explains that for each host *R* gene, there is a corresponding avirulence (*Avr*) gene in the pathogen (Flor 1955). When the R-protein recognizes the corresponding avirulence protein (also known as effector protein), the interaction is called incompatible and causes no disease (Figure 2.3). The disease interaction is called compatible interaction (Fitt et al., 2006). Several major *R* genes are involved in qualitative resistance, and they are called *Rlm* genes or *R* genes. To date, 23 *Rlm* genes of *B. napus* have been identified. They are *Rlm1-Rlm13, RlmS, LepR1-LepR4, BLMR1, BLMR2, LMJR1, LMJR2 and rjlm2* (Dolatabadian et al., 2021). *Rlm2, LepR3, Rlm4, Rlm7* and *Rlm9* are the only ones that have been cloned and characterized. Among 23 *R* genes, some are suspected to be identical or allelic forms (Delourme et al., 2006; Parlange et al., 2009; Larken et al., 2013, 2015; van de Wouw et al., 2014; Ghanbarnia et al., 2015; Dolatabadian et al., 2021, Borhan et al., 2022). *R* gene-mediated qualitative resistance is race-specific. Using the same resistance gene continuously for a long period of time, the selection pressure leads the host resistance less durable due to the rapid evolution of the pathogen virulence over time by sexual reproduction (Zhang et al., 2016; Rouxel and Balesdent 2017). The use of the same resistance gene continuously for a long period of time leads the breakdown of host resistance due to the rapid evolution of the pathogen virulence by sexual reproduction. The evolution of new virulence genes depends mainly on the selection pressure exerts by growing the same ***R*** gene for a long time. There are examples of resistance breakdown happened even within three years after the commercial release of the cultivar (Van de Wouw et al., 2010). On the other hand, the suitability of the climate for sexual reproduction of the pathogen also matters (Zhang et al., 2016).

On the other hand, 14 *Avr* genes have been identified and eight *Avr* genes have been cloned: *AvrLm1, AvrLm2, AvrLm3, AvrLm4-7, AvrLm5-9, AvrLm6, AvrLm10$_{a, b}$ and AvrLm11* (Gout et al., 2006; Fudal et al., 2007; Parlange et al., 2009; Balesdent et al., 2013; Van de Wouw et al., 2014; Ghanbarnia et al., 2015; Plissonneau et al., 2016; Ghanbarnia et al., 2018; Petit-Houdenot et al., 2019). As Flor (1955) explained in *B. napus* and *L. maculans* interaction, each *R* gene in the host responds to an *Avr* gene in the pathogen. However, there are exceptions as well. Two *R* genes detect a single *Avr* gene in some cases. Detection of *AvrLm4-7* by *Rlm4* and *Rlm7* and detection of *AvrLm1* by *Rlm1* and *LepR3* are examples. On the other hand, the *Rlm10* gene in *B. nigra* detects two *Avr* genes, *AvrLm10$_a$* and *AvrLm10$_b$* (Petit-Houdenot et al., 2019).

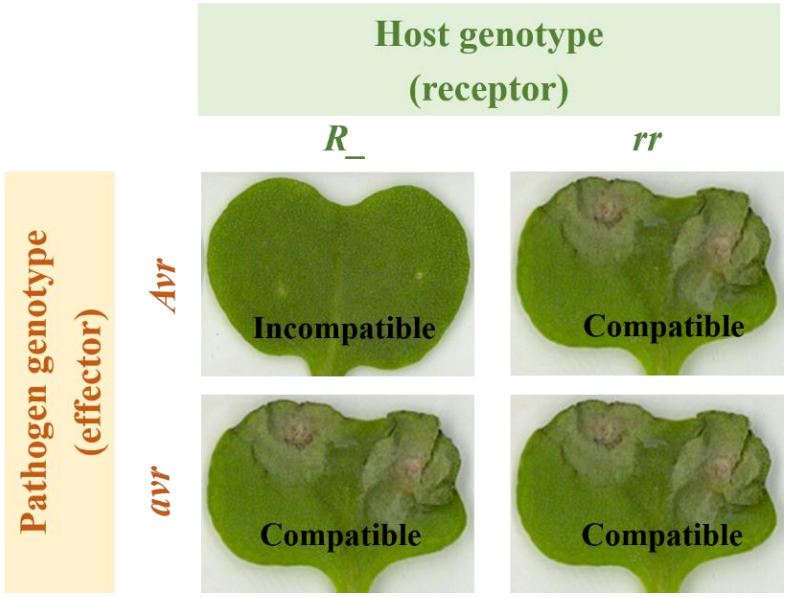


Figure 2. 3 Gene-for-Gene Interaction in the *B. napus-L. maculans* Pathosystem. Avirulence (*Avr*) and virulence (*avr*) genotypes of the pathogen have shown. The *Avr* (avirulence) genotype is capable of expressing effector proteins but not by *avr* (virulence) genotype. Host genotypes have shown as *R_* and *rr*. *R_* expresses the corresponding receptor protein. The incompatible reaction or resistance results due to the recognition of the Avr protein by the corresponding host *R* protein. All the other three interactions showed compatible or diseased reactions. Pictures were taken from cotyledons under controlled conditions.

2.7 Host-Pathogen Interaction

Plants show two-phase immunity responses to pending off pathogen infections. The frontline of plant defense towards the pathogen involves the recognition of pathogen or pathogen features referred to as pathogen-associated molecular patterns (PAMPs) by cell surface-localized pattern recognition receptors (PRRs) (Thomma et al., 2011). Molecules essential to the pathogen, such as bacterial flagellin (Felix et al., 1999) and fungal chitin (Shibuya and Minami 2001), are known as PAMPs recognized by the host plant. This initial recognition leads to the basic immune response of the host plant against the pathogen is called pattern triggered immunity (PTI). Eventually, pathogens evolved to deliver effectors to overcome PTI (Jones and Dangl 2006). Subsequently, host plants possessed the second line of defense referred to as effector-triggered immunity (ETI). ETI is triggered by direct or indirect interaction and recognition of pathogen Avr effector proteins by corresponding plant resistant proteins according to the gene-for-gene interaction model with exceptions as explained above. Although there are exceptions, basically intracellular R proteins are associated with ETI (Thomma et al., 2011).

Avirulence effector genes are expressed at specific stages of infection, especially at the necrotrophic stage, to manipulate the host immune response toward the necrotrophic growth and are usually encoded small-secreted proteins (SSPs) (Tyler and Rouxel 2013; Haddadi et al., 2016). Jones and Dangl (2006) proposed zigzag model is insufficient to explain the host-pathogen interaction between *B. napus* and *L. maculans* (Hacquard et al., 2017).

L. maculans overcomes PTI of the host by SSPs at the apoplastic space (Zhou et al., 2019). The ETI of *B. napus* is activated by recognizing pathogen SSPs by RLPs further transmits the defense signals by engaging the surface-localized receptor-like kinases (RLKs) (Larkan et al., 2015; Ma and Borhan 2015). The cloned *Rlm* genes so far: *LepR3* and *Rlm2*, have been characterized as RLPs (Larkan et al., 2015; Ma and Borhan 2015). SOBIR1 is an example of a surface localized RLK (Stotz et al., 2014). The intracellular nucleotide-binding domain and leucine-rich repeat receptors (NLRs) (Dodds and Rathjen 2010) detect pathogen effector proteins within cells. *R* gene mediated ETI causes via the recognition of an *Avr* gene of the pathogen by the corresponding *Rlm* gene in the host leads to HR to restrain the pathogen infection any further (Huang et al., 2006). When the R-protein recognizes the corresponding effector protein, the interaction is incompatible and causes no disease (Fitt et al., 2006).

Incompatible interaction is associated with the massive production of reactive oxygen species. Reactive oxygen species kill affected and surrounding cells to restrict the subsequent spread of the pathogen to the stem and canker development. This defense response is called the hypersensitive response (Fitt et al., 2006; Torres et al., 2006). After the program cell death, signal transducers get activated. Salicylic acid plays a major role in R-gene mediated resistance, and jasmonic acid and ethylene also contribute to it. The signal transduction leads to the induction of pathogenicity-related proteins, leading to defense mechanisms such as cell wall thickening, callose deposition, lignification, and chitinase synthesis to fight against the pathogen (Tsuda et al., 2009). When the pathogen *Avr* gene is not matched with the plant *R* gene, the disease occurs, and interaction is referred to as compatible.

2.8 Management Strategies of the Blackleg Disease

Application of an integrated management system is the best way to control this pathogen. Avoidance techniques, application of approved fungicidal products, and genetic resistance are the three major strategies for successful blackleg management. Crop or *R* gene rotation, crop isolation, and avoiding exposure to the stubble of the previous season are the main avoidance techniques. Three to four-year crop rotation is recommended for effective disease management (Hegewald et al., 2018; Zhang and Fernando 2018). Crop isolation and stubble management can be used to minimize the inoculum pressure and destroy the source of the inoculum (Guo et al., 2008).

2.8.1 Use of Resistant Varieties

Though the use of resistant varieties was a considerable success in controlling the pathogen, continuous utilization of single-gene resistance sources on a large scale exerts intense selection pressure on the pathogen populations, leading to the evolution of pathogen virulence and breaking down of host resistance. Basically, the decrease in the frequency of avirulence alleles takes place by mutants happens in the pathogen genome. Repeat-Induced Point plays a main role here (Van de Wouw et al., 2010). Increased usage of *Rlm1* germplasm resulted in a rapid decrease of the isolates carrying *AvrLm1* in the pathogen population in France (Rouxel et al., 2003). In Australia, canola accession 'sylvestris' was overcome within three years after the commercial release of the cultivar (Sprague et al., 2006; Van de Wouw et al., 2010).

In the Canadian context, resistant cultivars against the blackleg disease were released in the early 1990s, replacing Westar, giving comparatively higher yield at that time (Kutcher et al., 2010a). Quantum, Conquest, Hi-Q, and Q2 were some of the examples. However, today, most commercial cultivars in Canadian prairies carried the same single *R* gene, *Rlm3* (Zhang et al., 2015). Due to the intensive cultivation of the same *R* gene for an extended period, the *Rlm3* gene has been overcome by the pathogen by reducing the *AvrLm3* gene frequency in the pathogen population to the lowest (Zhang et al., 2015). The erosion of *Rlm3* in western Canada has been shown with these observations.

In Manitoba, the assessment carried out based on disease incidence and avirulence allele distribution of *L. maculans* populations from 2010 to 2015 verified the presence of *AvrLm2, AvrLm4, AvrLm5, AvrLm6, AvrLm7, AvrLm11,* and *AvrLmS* alleles in high frequencies and *AvrLm1, AvrLm3, AvrLm9, AvrLepR1,* and *AvrLepR2* alleles were in low frequencies (Fernando et al., 2018). The decrease in the frequency of the last set of avirulence alleles could be explained by the intense selection pressure exerted by the same or limited *R* genes in Canadian canola varieties available in seed markets.

There is, therefore, a definite need to reduce the selection pressure exerted on a canola cultivar with the same *R* gene by growing it continuously for a long time over a large area. Crop rotation plays a major role here by reducing the association between crop and pathogen. The field experiment carried out from 1999 through 2002 at Carman, Manitoba, determined 4-year crop rotation is the most efficient (Guo et al., 2005). However, farmers were unwilling to move away from canola due to a market demanding and financial standpoint. In that setting, plant pathologists brought the concept of *R* gene rotation. Marcroft et al., (2012) demonstrated in Australia that rotation of *R* genes could manipulate the fungal population in the fields to minimize disease pressure. Zhang and Fernando (2018) suggested an *R* gene rotation strategy in the Canadian prairie to control blackleg. Research scientists and the industry are putting more effort into developing varieties with diversified *R* genes to combat virulence genes available in fungal populations in Canadian prairies. Currently, most seed companies use the resistant group on their labels. Integrating R gene rotation and other integrated management practices such as crop rotation, fungicide application, and tillage can substantially reduce the disease in Canadian settings.

Development of genetically equipped canola varieties with diversified resistant genes in combination with quantitative resistance is one of the best approaches should be applied to control

this competitive disease (Zhang and Fernando 2018). According to Liban et al., (2016) and Fernando et al., (2018), *AvrLm7* is one of the avirulence genes present in abundance in the *L. maculans* fungal population. Besides, the *Rlm7* gene has exhibited better performance and quantitative resistance and is slower in breaking down (Huang et al., 2018). Based on the above evidence *Rlm7* was chosen over the other *R* genes as the most suitable candidate for the Canadian canola field. One chapter of this thesis focuses on investigating *Rlm7* and its interactions under virulence and avirulence pathogenicity in-depth at the transcriptional level.

2.8.2 Chemical Control

It is essential to employ an integrated management strategy instead of going by one as mentioned earlier. On the other hand, the new *Rlm* gene diversification of seed germplasm available in the market is still in development. Therefore, it is important to have backup plans for other strategies ready to avoid outbreaks. Using chemical fungicide is one of them. Canada started to use fungicides for blackleg control in 1994 (Juska et al., 1997). Previous studies reveal that *L. maculans* are more sensitive to fungicides than *L. biglobosa* (Eckert et al., 2010; Huang et al., 2011). There are three classes of fungicides commonly used on canola blackleg disease control in the world. They are demethylation inhibitors (DMI), quinone outside inhibitors (QoI), and succinate dehydrogenase inhibitors (SDHI).

DMIs are also known as Sterol Biosynthesis Inhibitors (SBIs) that belong to FRAC code three, including the triazoles, triazolinthiones, and imidazoles. Tilt (propiconazole), Procure (triflumizole), Folicur (tebuconazole), and Rally (myclobutanil) are examples. DMIs inhibit the biosynthesis of ergosterol, which is a significant component of the plasma membrane and essential for fungal growth. They target a membrane-bound P450 monooxygenase, lanosterol 14α-demethylase (erg11, CYP51), a key component of fungi's ergosterol biosynthesis pathway. DMI fungicides inhibit the removal of the C14-methyl group of 24-methylene-dihydro lanosterol and prevent the production of ergosterol (Kelly et al., 1995). Resistance by fungi to the DMI fungicides has been characterized. It is generally controlled by accumulating several independent mutations, or what is known as 'continuous selection' or 'shifting', in the fungus. Accumulation of polymorphisms in CYP51B, which leads to the intensity of azole application, is one mechanism (Cools et al., 2013).

Quinone outside inhibitors (QoIs) are also known as strobilurin fungicides and are given FRAC code 11. Methoxy-acrylates, Dihyrdro-dioxazines, Methoxy-carbamates, and Oximino-acetates are coming under QoIs. QoI fungicides are single-site mitochondrial respiration inhibitors. It binds to the quinol oxidation site of cytochrome b, which is one of three subunits of cytochrome bc1 (ubiquinol oxidase) complex. Due to the bound of QoI compounds, the electron transfer between cytochrome b and cytochrome c1 gets blocked and inhibits the production of ATP in the fungus (Bartlett et al., 2002; Fisher and Meunier 2008). Liu (2014) observed variations in the sensitivity of different *L. maculans* in Canada to QoI fungicides. Fungal resistance against QoIs occurred due to a single common polymorphism in the cytochrome b coding sequence (G143A), which emerged in various pathogen populations.

The results of these fungicides were satisfied with susceptible cultivars. After releasing resistant cultivars with a combination of good rotation practices, farmers did not rely on fungicides much. However, as the blackleg disease raises its head and makes significant issues, growers have begun to accommodate fungicide applications with other measures (Kutcher et al., 2011). As the fungus infection can happen from the cotyledon stage to even bolting, a single fungicide application may not be sufficient to protect the plant. As the fungicides are no more effective enough after the fungus reaches the stem, the correct timing of fungicide application is crucial in blackleg control (Steed et al., 2007; Liu 2014). As Markell et al., (2008) stated, foliar fungicide applications were advised between seedlings' two- and four-leaf growth stages to avoid exposure to the blackleg inoculum which is known as the critical window of the plant life span. Studies in Australia exhibited a reduction of disease severity and yield improvement on susceptible and moderately resistant (MR) cultivars by applying fungicides within two-to-four leaf stage under high disease pressure (Khangura and Barbetti 2004; Marcroft and Potter 2008). However, the performance indicated was depending on the fungicide type (Sprague and Burgess 2001; Sprague et al., 2007).

The next class of fungicides used against the blackleg disease was succinate dehydrogenase inhibitors (SDHIs), which belong to FRAC code 7. Pyridinecarboxamides and Pyrazole-4-carboxamides are SDHIs. SDHIs inhibit mitochondrial respiration of fungi, similar to QoIs (Avenot and Michailides 2010). Succinate dehydrogenase (SDH), a key component in the electron transport chain and plays a role in the tricarboxylic acid cycle, is the target site of SDHI compounds (Horsefield et al., 2006). SDHIs inhibit the SDH by physically blocking the ubiquinone-binding

site. This inhibition leads to inhibiting ATP production and growth of the fungus (Avenot and Michailides 2010; Sierotzki and Scalliet 2013). SDHI fungicides fight against a broad spectrum of fungal diseases. The succinate dehydrogenase inhibitors (SDHI) class was the fastest-growing type in market adaptation due to its high activity level and lack of other effective alternatives (Sierotzki and Scalliet 2013). Based on initial studies using UV-irradiated mutant strains, Georgopoulos and Ziogas (1977) demonstrated the capability of resistance development in fungal strains by mutations in the SDH genes. ILeVO™ is an SDHI fungicide that has been registered in Canada but only for controlling *F. virguliforme* in soybeans in the early season.

The thesis chapter on chemical control set out to assess newly developed SDHI seed treatment on the blackleg disease. Pydiflumetofen is a newly developed broad-spectrum seed-applied fungicide developed by Syngenta and is branded as Adepidyn™. This product targets explicitly Soybean sudden death syndrome caused by *F. virguliforme*, Bakanae on rice caused by *Gibberella fujikuroi*, and blackleg disease in canola.

To date, there are no reports on the efficacy of any SDHI seed treatment against foliar infection of blackleg disease in canola except fluopyram (Peng et al., 2020). As Pydiflumetofen is a seed care product, it can successfully target early infections more efficiently than a foliar fungicide (Peng et al., 2012). Cotyledon and early-leaf infection during the 2-4 leaf stage is the critical period of the plant life span that can cause a significant yield reduction by a disease infection. Besides, the seed could be a suitable vector for carrying out many fungal pathogens within and on the surface. Furthermore, when the seed is sown, the coming seedlings are attacked by many soil-borne pathogens (Gugel and Petrie 1992; Mancini and Romanazzi 2014; Hubbard & Peng 2018). Moreover, seed treatment is a more effective and economic measure of blackleg control, which can be easily manipulated in integrated management practices.

2.8.3 Biological Control

Highly virulent *L. maculans* and weakly virulent *L. biglobosa* are the two different species coming under the same genus and cause blackleg disease in canola. They spend a similar life cycle, and they coexist on canola in Europe, North America, and Australia (West et al., 2001; West et al., 2002). However, the interaction between these two species and the host plant is not well understood yet.

Plants have evolved a wide variety of responses towards fungal attacks to defend themselves. Apart from the gene-for-gene relationship and quantitative resistance deployed by minor genes, plants also possess inducible defenses or resistance mechanisms. Once induced in the host plant, acquired resistance operates locally at the site of infection and systemically all over the plant. The systemic acquired resistance (SAR), associated with defense priming, can protect the whole plant from subsequent fungal attacks (Lucas 1999; Jung et al., 2009).

The accumulation of salicylic acid (SA) in local and systemic tissues plays a major role in the operation of systemic acquired resistance (SAR), which is required for signal transduction and then leads to the expression of pathogenesis-related proteins (PR) such as PR1 (Durrant and Dong 2004). This SA-dependent SAR induction has been shown in many studies by using synthetic analogs of SA, such as benzo (1, 2, 3) thiadiazole-7-carboxylic acid and 2, 6-dichloroisonicotinic acid (Latunde-Dada and Lucas 2001; Cools and Ishii 2002). Interestingly going further, scientists were succeeded in detecting pre-inoculation with avirulent fungal pathogens that can induce resistance against virulent pathogens (Sequiera 1983). Moreover, several studies have shown the scenario of SAR building up in the canola plant against the devastating pathogen *L. maculans* by weakly virulent *L. biglobosa* in the same genus (Mahuku et al., 1996; Chen and Fernando 2006; Hadrami et al., 2010).

Furthermore, Liu and group (2007) demonstrated that SAR induced by *L. biglobosa* pre-treatment was mediated predominantly through the JA/ET-dependent pathway rather than the SA pathway. They detected the enhancement of the JA/ET-dependent pathway based on the enhanced expression of *PDF1.2*, which is commonly used as the marker for the above-said pathway. Liu et al., (2007) further explained SAR induced hemibiotrophic pathogen *L. biglobosa* is capable of a prolonged, active, but symptomless colonization of the natural host before the necrotrophic stem canker phase of *L. maculans*.

The last chapter of this thesis reports work done to investigate resistance induced against *L. maculans* by pre-inoculation of *L. biglobosa*, with a focus on providing cellular and molecular evidence involving microscopic observation of GFP- and DsRed- tagged *L. maculans* and *L. biglobosa* isolates, respectively and gene expression work in both host and the pathogen sides. Insight into the host-pathogen interaction is crucial in managing the disease efficiently and effectively. RNA-seq technique was used for this purpose.

2.9 RNA-seq

2.9.1 RNA-seq Technique and its Development

RNA-sequencing (RNA-seq) has become a ubiquitous tool used in understanding genomic function. The workflow of the procedure begins with RNA extraction and rRNA depletion or mRNA enrichment. Then it is followed by cDNA synthesis and library preparation with adaptor ligation, which leads to sequencing. The analysis starts with quality assessment of sequenced reads, aligning and/or assembling the sequencing reads to a transcriptome, filtering and normalizing between samples, and analyzing expressions of individual genes or comparisons of differential expressions among genes according to the biological question of the study. Differential gene expression analysis remains the primary application of RNA- seq, which is used as a standard research tool (Stark et al., 2019). RNA-seq had developed and improved over the last decade from when it was first introduced. There are approximately around 100 distinct methods that have been derived from the standard protocol. Initially, the RNA-seq technique was carried out based on Illumina short-read sequencing. However, recent advances in long-read RNA sequencing and direct RNA sequencing have been led to new openings (Byrne et al., 2017; Garalde et al., 2018).

2.9.2 The Application of RNA Sequencing in Exploring Host-Pathogen Interactions

The high resolution of RNA-seq allows parallel analysis of different organisms interacting. Transcriptomic analysis of counterparts of the host-pathogen interaction is one of the examples. Studying host-pathogen interactions started focusing only on one side at a single attempt once (Tremblay et al., 2012; Becker et al., 2017). Then these transcriptomic studies were improved as simultaneous RNA-seq analysis of both host and pathogen transcriptomes simultaneously, so-called 'dual RNA-seq' (Westermann et al., 2012). Dual RNA-seq allows comparatively more precise gene expression analysis of plant pathogens and their respective hosts simultaneously. This approach has become very popular due to many reasons. The technique allows detecting the whole set of in-depth knowledge on a particular host and a pathogen with a minute amount of RNA from both sides. RNA-seq does not need predesigned species-specific probes and is more sensitive than previous microarray and northern blotting (Westermann et al., 2012; Hayden et al., 2014). The pathogen's transcriptomic data leads to mine clues of pathogenicity and virulence factors, which

will be important in the fight against the pathogen. Transcriptomic knowledge gained by the host will favor fortifying the host plant to face any hostile pathogenic environment (Westermann et al., 2017). However, the reproducibility of the technique can be reduced due to lack of standardization between sequencing platforms and read depth, equivalent to the percentage of total transcripts sequenced. The high cost of RNA-seq facility is not affordable for many laboratories.

Lowe et al. (2014) were the first to investigate gene expression of the *L. maculans* – *B. napus* pathosystem by RNA-seq. They investigated gene expression in susceptible cotyledons at 7 and 14 dpi exposed to *L. maculans* or *L. biglobosa*. Moreover, Sonah et al. (2016) conducted a comparative transcriptomic analysis of *L. maculans* virulence factors expressed during compatible and incompatible interactions with *B. napus*. The dual RNA-seq technique also has been used to gain insight into the *B. napus*-*L. maculans* pathosystem. For instance, Haddadi et al. (2016) have used this tool to mine into the lifestyle transition from biotrophy to necrotrophy and effector repertoire of *L. maculans* during the colonization in *B. napus* seedlings. Furthermore, Zhou et al. (2019) proceeded with a dual RNA-seq analysis to study *B. napus R*-genes *LepR3* and *Rlm2*-mediated defense response against the pathogen's infection, *L. maculans*.

It is always advisable to control this devastating pathogen by using integrated pest management approach instead of using one single method. Though there are multiple number of methods that can be used to control this pathogen, genetic host resistance plays a fundamental role (Zhang et al. 2018). Under a condition in which the pathogen has overcome the current host resistance, it is crucial to introduce new *R* genes and understand how to maintain that resistance for a more extended period without breaking down.

Leptosphaeria maculans-Brassica napus battle: A comparative study of incompatible Vs compatible interactions using the same host and the 'same pathogen'

K. Rasanie E. Padmathilake[1] and W. G. Dilantha Fernando[1,*]

[1]Department of Plant Science, University of Manitoba, Winnipeg, MB R3T2N2, Canada 1; Dilantha.Fernando@umanitoba.ca
*Correspondence: Dilantha.Fernando@umanitoba.ca

This manuscript was published in International Journal of Molecular Sciences: Padmathilake KRE, Fernando WGD. *Leptosphaeria maculans-Brassica napus* Battle: A Comparison of Incompatible vs. Compatible Interactions Using Dual RNASeq. International Journal of Molecular Sciences. 2022; 23(7):3964. https://doi.org/10.3390/ijms23073964.

Author Contributions: W.G.D.F. and K.R.E.P. designed the experiments. K.R.E.P. performed the experiments and analyzed the data. K.R.E.P. wrote the manuscript and W.G.D.F. revised the manuscript. Funding acquisition was done by W.G.D.F. All authors have read and agreed to the published version of the manuscript.

CHAPTER 3. *LEPTOSPHAERIA MACULANS-BRASSICA NAPUS* PATHOSYSTEM: INTRINSIC GENE EXPRESSION OF *RLM7-AVRLM7* PATHOSYSTEM UNDER INCOMPATIBLE AND COMPETIBLE INTERACTIONS

3.1 Abstract

Leptosphaeria maculans causes blackleg disease, which is one of the most destructive diseases of canola (*Brassica napus* L.*)*. Due to the erosion of the current resistance in *B. napus*, it is pivotal to introduce new resistant genotypes to the growers. This study evaluated the potential of *Rlm7* gene as resistance to its corresponding avirulence *AvrLm7* gene is abundant. The *Rlm7* line was inoculated with *L. maculans* isolate with *AvrLm7*; UMAvr7; and the CRISPR/Cas9 knockout *AvrLm7* mutant, umavr7, of the same isolate to cause incompatible and compatible interactions, respectively. Dual RNA-seq showed differential gene expressions in both interactions. High expressions of virulence-related pathogen genes-CAZymes, merops, and effector proteins after 7-dpi in compatible interactions but not in incompatible interaction—confirmed that the pathogen was actively virulent only in compatible interactions. In the host, salicyclic and jasmonic acid biosynthesis and signaling-related genes, defense-related *PR1* gene (GSBRNA2T00150001001), and GSBRNA2T00068522001 in the NLR gene family were upregulated starting as early as 1- and 3-dpi in the incompatible interaction and the high upregulation of those genes after 7-dpi in compatible interactions confirmed the early recognition of the pathogen by the host and control it by early activation of host defense mechanisms in the incompatible interaction.

3.2 Introduction

Canola (*Brassica napus* L.) is the number one cash crop in Canada that contributes CAD $29.9 billion to the annual country economy, including more than 207,000 Canadian jobs and CAD $12 billion in wages (Canola Council of Canada, 2020). Annual global and Canadian canola production is estimated as 70 MMT and 18.7 MMT, respectively (Canola Council of Canada, 2020; Zhai et al., 2021). Blackleg disease, caused by the actinomycete fungus *Leptosphaeria*

maculans (Desm.) Ces. & de Not. (anamorph: Phoma lingam (Tode ex Fr.) Desm.) is one of the predominant fatal disease in canola, which causes losses worth more than $900 million per growing season worldwide and trade conflicts (West et al., 2001; Fitt et al., 2006 and 2008; Zhang and Fernando 2018).

Host genetic resistance is a major tool in the fight against blackleg disease. Canola exhibits two types of resistance: major gene (qualitative) resistance and minor gene (quantitative) resistance. Canola expresses the gene-for-gene interaction model explained by Flor (1971) but with some exceptions. If the host resistance gene matches the pathogen's avirulence gene, the host will initiate a hypersensitive interaction, killing the cells surrounding the infected site and stopping further spreading of the pathogen, which is called an incompatible interaction (Rimmer 2006). The diseased interaction is called compatible interaction (Becker et al., 2017). To date, 18 major *R* genes have been identified in *Brassica* species: *Rlm1-11, RlmS, LepR1-4, BLMR1, and BLMR2* (Yu et al., 2005, 2008, 2013; Delourme et al., 2006; Long et al., 2011; Larkan et al., 2013, 2015), but only two (*LepR3 and Rlm2*) have been cloned (Larkan et al., 2013, 2015; Yu et al., 2013). Genes *LepR3* and *Rlm2* are the alleles of the same gene and encode leucine-rich repeat receptor-like proteins (LRR-RLP) (Larkan et al., 2013, 2015). In *L. maculans,* 16 *Avr* genes have been identified and the genes *AvrLm1, AvrLm2, AvrLm3, AvrLm4-7, AvrLm5-9, AvrLm6, AvrLm10, AvrLm11* have been cloned (Gout et al., 2006; Fudal et al., 2007; Parlange et al., 2009; Balesdent et al., 2013; Van de Wouw et al., 2014; Ghanbarnia et al., 2015, 2018; Plissonneau et al., 2016, 2017b; Petit-Houdent et al., 2019).

R gene resistance is not a consistent resistance response over the years and is constantly challenged with the new evolution of sexually reproducing diverse races of *L. maculans.* There are several examples of *R* gene breakdown of canola species due to selection pressure exerted by continuous use of a single *R* gene over time. The breakdown of *Rlm1* in France and 'sylvestris' in Australia can be shown as examples (Rouxel et al., 2003; Van de Wouw et al., 2010).

The major *R*-gene used by Canadian farmers, *Rlm3*, has been overcome by the pathogen and corresponding pathogen avirulence genotype (Zhang et al., 2016). *Rlm3* was reported in Canadian canola varieties released from the 1990s onwards. Though Rlm1 and 'sylvestris' resistance breakdown happened within a few years, Rlm3 resistance was very effective until 2005 (Zhang et al., 2016). By 2012, the frequency of *AvrLm3* in *L. maculans* population had dropped to 2.7% and reached the lowest level of frequency in the pathogen population in Western Canada

(Liban et al, 2016). Zhang et al., (2016) explained that asexual pycnidiospores becoming the major source of inoculum in Western Canada might have played a major role due to the climate. This explains the necessity of introducing new host R genes into the seed market to reduce the selection pressure exerted on the host genotypes. Based on the high frequency of *AvrLm7* genotype presence in the pathogen population and stability of the corresponding host genotype *Rlm7* in countries where it has been already introduced, *Rlm7* is the next best candidate host genotype to be introduced into the canola host gene pool (Fernando et al., 2018; Mitrousia et al, 2018).

L. maculans is a hemibiotrophic pathogen (Fitt et al., 2006). Biotrophs assimilate nutrients directly from host cells without killing them by being in the apoplast. On the other hand, necrotrophs break into the host cell and cause cell death before nutrient take-in. Among all, hemibiotrophs are the most exciting group that exhibits the biotrophic nature at the initial phase and emulates the latter's necrotrophic characteristics (Chowdhury et al., 2017). The underlying mechanisms of the above switching nature during plant hemibiotrophic interactions remains elusive.

Plants use phytohormones for signaling to distinguish among the above-mentioned different types of pathogens and activate appropriate responses (Pieterse et al., 2009). Phytohormones are multifaceted signal molecules, which play a significant role in plant immunity programs. Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play major roles among them (Tsuda et al., 2009, 2013; Huang et al., 2020). In addition, phytohormones strongly link with the pathogens' trophic nature in the plant system (Huang et al., 2020). Studies conducted using transgenic and mutant lines revealed that responses against biotrophic pathogens and hemibiotrophic pathogens are in general regulated by SA (Tsuda et al., 2009; Vlot et al., 2009). In contrast, JA and ET replace that role when it comes against necrotrophs (Farmer et al., 2003; Tsuda et al., 2009). That explains that there is reciprocal inhibition between SA and JA. Previous studies exhibited SA-JA antagonism supports plant defense strategies based on the pathogen trophic nature encountered (Tsuda et al., 2009, 2013; Van der Does et al., 2013). Despite that, the SA-JA relationship is not always antagonistic. There is research evidence for positive regulating of SA and JA. Tamaoki et al. (2013) explained that the defense system activated by both SA and JA signaling interaction during the induction of defense response. Agrawal et al. (2000) observed that disease-resistant marker gene rice PR1b gene is induced by both SA and JA and suggested there should be at least a partly shared signal transduction pathway used for signaling of both JA and

SA. Tsuda et al., (2009) revealed that synergistic and compensatory relationships exist among SA and JA pathways and are important for optimal resistance to different pathogens.

The basic immune system of plants is triggered by pathogens and related molecules, which are detected by extracellular membrane receptors such as receptor-like kinases (RLKs) and receptor-like proteins (RLPs), called as the pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) (Dangl et al., 2013). Pathogens evolved to implement the next level of virulence by possessing effector proteins to defeat basic plant immunity, PTI. Effectors are pathogen proteins that modulate the plant's innate immunity and facilitate infection (Oliva et al., 2010). Consequently, some plants evolved intracellular resistant proteins to recognize some pathogen effectors and are called avirulence proteins at the onset of infection and lead to the next level of immunity called effector-triggered immunity (ETI) (Petit-Houdenot and Fudal 2017). Avirulence proteins are pathogenicity proteins that are specifically 'recognized' by genotypes of the host plant carrying the corresponding resistance proteins mainly under the nucleotide-binding leucine-rich repeat (NLR) receptor family (Rouxel and Balesdent 2010; Zhen et al., 2019). ETI triggers hypersensitive reaction (HR), causing programed cell death (PCD) surrounding the infected site, limiting further pathogen spread. ETI triggered resistant interaction is known as incompatible interaction whereas, the interaction causing disease is called compatible interaction in a plant system with lacking resistant proteins of and/or with a pathogen lacking specific avirulence effectors. During ETI induction of plants, both SA and JA accumulation increases simultaneously (Mur et al., 2006). Though the past studies showed the crosstalk between SA and JA, the cooperative interplay between SA and JA exhibited by Spoel et al. (2007) explains the ETI-associated PCD does not trigger the susceptibility to necrotrophs. In addition, phytohormones showed a higher correlation with the trophic switch of a hemibiotrophic pathogen in the plant system, which is still not fully revealed.

In understanding host-pathogen interactions from both contexts simultaneously, scientists have used dual RNA-seq, which is the most promising high-throughput and highly sensitive technique for genome-wide transcriptional study. Past several years, the RNA-seq technique has been widely used to investigate plant pathosystems, including the canola-blackleg system. Becker et al. (2017) and Zhai et al., (2021) investigated canola as the host system with *Rlm3* and *Rlm1*, respectively. Haddadi et al., (2016) plant explored *L. maculans* pathogen system, Becker et al., 2019) studied the host pathosystem with *Rlm2-AvrLm2* by a dual RNA-seq study.

This study was conducted to follow disease progression through a time-course in the *Rlm7* host genotype, which is the next candidate genotype being introduced into the market. A *L. maculans* isolate with *AvrLm7* was used to create an incompatible interaction with the host, in contrast, the *AvrLm7* gene mutated with the same isolate was used to form a compatible interaction. Both isolates were inoculated in Westar genotype to see their performances in a no-*R*-gene background. Genome-wide transcriptomic studies are important to understanding the molecular and genetic background of a host pathosystem. However, this study focused on understanding the *Rlm7-AvrLm7* pathosystem at a genome-wide transcriptomic level. The most exciting point here is using the *Rlm7* genotype to study both incompatible and compatible interactions, both with and muting *AvrLm7* of the same *L. maculans* isolate, with the host and pathogen backgrounds made the same except the candidate avirulence gene, *AvrLm7*. *AvrLm7* gene mutation was carried out using the CRISPR/Cas9 technique in our lab (Zou et al., 2020). The main objective of this detailed transcriptomic study was to have a deeper view of the transcriptional responses mediated by canola *Rlm7* genotype as the host, which would be the next potential candidate to be introduced in the Canadian canola market upon facing challenges with the pathogen *L. maculans* and pathogen virulence genes that involve beating the host defense under incompatible and compatible interactions. That knowledge can be successfully employed in management strategies to maintain the host genotype without being overcoming by the pathogen. In addition, an analysis of the trophic characteristics of *L. maculans* was conducted.

3.3 Materials and Methods

3.3.1 Plant Material

B. napus 01-23-2-1 line (Dilmaghani et al. 2009) carrying *Rlm7* and Westar (no *R* genes) were used as host germplasm. Three replicates were used for each treatment. Seeds were grown in Sunshine mix #4 (SunGro Horticulture, Canada Ltd., Vancouver, BC) RNeasy Plant Mini Kit in a growth chamber under 16 h photoperiod (18℃ dark and 21℃ light).

3.3.2 Wild-type *L. maculans* Isolate UMAvr7 and Mutant Isolate umavr7

L. maculans, isolate UMAvr7 (with *AvrLm5-6-7*) and *AvrLm7* mutant of the same isolate umavr7 (with eight base pair deletions in coding region) generated by using CRISPR/Cas9 system (Zou et al., 2020) were grown on V8 juice agar (20% (v/v) V8 vegetable juice, 0.075% (w/v) $CaCO_3$, 1.5% (w/v) Agar and 100 µg mL^{-1} of Streptomycin) at 22˚C under room temperature. One single pycnidiospore isolated from each culture was used to obtain pure cultures of each isolate for further steps. After sporulation pycnidiospores were collected in sterilized dH_2O. Pycnidiospore suspensions were diluted to final concentration of 2 x 10^7 spore mL^{-1}. Seven-day-old seedlings of line 01-23-2-1 (with *Rlm7*) and Westar were punctured with a modified tweezer as one per each lobe and were inoculated with a 10 µL droplet of inoculum (four inoculation sites per seedling). Both host genotypes were inoculated with UMAvr7 and umavr7). Inoculated cotyledons will be air dried for at least 12 h before watering. The mock control seedlings were treated the same but with sterilized dH_2O instead of the inoculum.

3.3.3 Sample Preparation for RNA-seq

B. napus cotyledons were collected at 0, 1-, 3-, 7-, and 11- days post inoculation (dpi) with three biological replicates from each treatment at each time point. Then the samples were frozen with liquid nitrogen and were stored at -80°C. RNA was extracted using 100 mg of ground tissue using RNeasy Plant Mini Kit (Qiagen Cat. No. 74904) according to the manufacturer's instructions. Three biological samples from each treatment at each time point were used. The quantity and quality of each sample were analyzed by using NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and the 2100 Bioanalyzer (Serial no. DE13806078, Agilent Technologies, Palo Alto, CA). RNA integrity and purity were analyzed further by running in 2% agarose electrophoresis gels. RNA samples (samples with RIN >7) were sent for Genome Quebec, Quebec, Canada for RNA-seq library preparation and sequencing. The sequencing was done using Illumina 4000 HiSeq PE100 method.

3.3.4 RNA-seq Analysis

The quality of RNA-seq reads were checked with Fastqc. Read processing by clipping of barcode adapters from RNA-seq reads and removing of low-quality reads were done (read quality <30) using Trimmomatic software (Bolger et al., 2014). Reads were trimmed from both ends until the average of all 5 bp sliding windows reached a Phred score of 25 or higher and all the sequences shorter than 30 bases were discarded. Processed quality reads were aligned to the *L. maculans* (https://fungi.ensembl.org/*Leptosphaeria_maculans*/Info/Annotation/) and *B. napus* (http://www.genoscope.cns.fr/brassicanapus/) genomes and transcriptomes with Tophat2 (Trapnell et al., 2009). Two-bases mismatch was allowed for the alignment. The minimum and maximum intron length was set to 50 and 500,000, respectively. Reads aligned to multiple sites were removed prior to further analysis.

Identification of unannotated transcripts was done using Cufflink tools. The gene expression level for each annotated and unannotated transcripts were estimated as the number of Fragments (reads) per kilobase of transcript per million mapped reads (FPKM) HTseq tool was used to count reads. The differentially expressed genes (DEGs) were identified using Omicsbox software (https://www.biobam.com/omicsbox. Genes were considered as significantly differentially expressed with false discovery rate (FDR) ≤0.05 and the absolute value of log2 (Fold-change) ≥2. The quartile normalization that excludes the top 25% of expressed genes to improve detection of less abundant genes. The -M option to mask rRNA, -b, and -u option for bias correction and option to normalize for aligned tags instead of total tags were used with the Cufflink software. HTseq tool was used to count reads prior to DEGs identification. Sample quality and expression levels of transcripts were estimated using DESeq2 package.

3.3.5 RNA-seq Data Validation by RT-qPCR

Frozen cotyledons (collected at 0-, 1-, 3-, 7-, and 11- dpi) were ground in liquid nitrogen with a sterilized pestle and mortar. Total RNA was extracted PureLink® Plant RNA Reagent (Ambion, https://www.thermofisher.com) and treated with a TURBO DNA-free™ Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Reverse transcription of the first-strand cDNA was performed using the 1st strand cDNA Synthesis Kit (Thermo Fisher) with 1 µg total RNA. Next, 4.2 µL of 100-fold diluted cDNA, 5 µL of PowerUp™ SYBR™ Green Master

Mix (Applied Biosystems™), and 0.4 µL of each primer (10 mM) were used for PCR reaction. Primer sequences have shown in Table 3.1 (based on Zhang 2016; Yang 2021). Real time qPCR reactions were performed on a CFX96 Real-Time Instrument (Bio-Rad, USA) with the amplification program of 50°C for 2 min, 95°C for 2 min, 40 cycles of 95°C for 15 s, 60°C for 1 min. Melting curve analysis was performed by increasing 0.5°C at 5 s/step from 65 to 95°C. Actin was used as the reference gene in the analysis. The relative gene expression level was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Table 3. 1 List of Selected Defense Related Genes in *Brassica napus* and their Forward and Reverse Primers Used in RT-PCR

Gene	Full Name	Defense Signaling Pathway	Forward Primer (5′3′)	Reverse Primer (5′3′)
WRKY70	WRKY transcription factor 70	SA signaling pathway	ACATACATAGGAAAC CACACG	ACTTGGACTATCTTCAGAA TGC
PR1	Pathogenesis-related protein 1	SA pathway	GGCTAACTATAACCAC GATTC	GTTCCACCATTGTTACACC
WRKY33	WRKY transcription factor 33	JA signaling pathway	TGTCGGACAGCTTGGG AAAG	AGAGGACGGTTACAACTG GAGAAA
PDF1.2	Plant defensin 1.2	Ethylene and JA pathway	AAATGCTTCCTGCGAC AACG	AGTCCACGTCTCCGATCT CT
RbohD	Respiratory burst oxidase homolog protein D	ROS production	TATCCTCAAGGACATC ATCAG	TATCCTCAAGGACATCATCA G
APX6	Ascorbate peroxidase	ROS scavenging	AGTTCGTAGCTGCTAA ATATT	GGAGTTGTTATTACCAAGAA A
CHI	Chitinase	Pathogen chitin degradation	TGCTACATAGAAGAA ATAAACGG	TTCCATGATAGTTGAATC GG
COMT	Caffeic acid O-methyltransferase	Lignin biosynthesis	TCTTCAAGAATTTCAC GCAGTG	CGTCCCTAAAGGTGATGCTA TT
AOS	Allene oxide synthase	Involved in JA biosynthesis	CGCCACCAAAACAA CAAA	GGGAGGAAGGAGAGAGG TTG
ICS1	Isochorismate synthase 1	Involved in SA biosynthesis	AGCGTGACTTACTAAC CAG	CAAACTCATCATCTTCCCTC
Act	Actin	Reference gene	CTGGAATTGCTGACCG TATGAG	GTTGGAAAGTGCTGAGG GATG

3.4 Results

3.4.1 Response of 01-23-2-1 and Westar to Avirulent UMAvr7 and Virulent umavr7 Isolates

B. napus line (01-23-2-1) showed no disease lesion development with the infection of UMAvr7 even after 11 dpi except a thin layer of dead tissues surrounding the inoculated sites. On the other hand, 01-23-2-1 showed a lesion development with the inoculation of isolate umavr7. Westar showed lesion development with both isolates. Lesions were started to show up from seven dpi onwards among the days considered (Figure 3.1). Mock samples showed only the marks of pinched sites of cotyledons (Figure 3.1. B.).

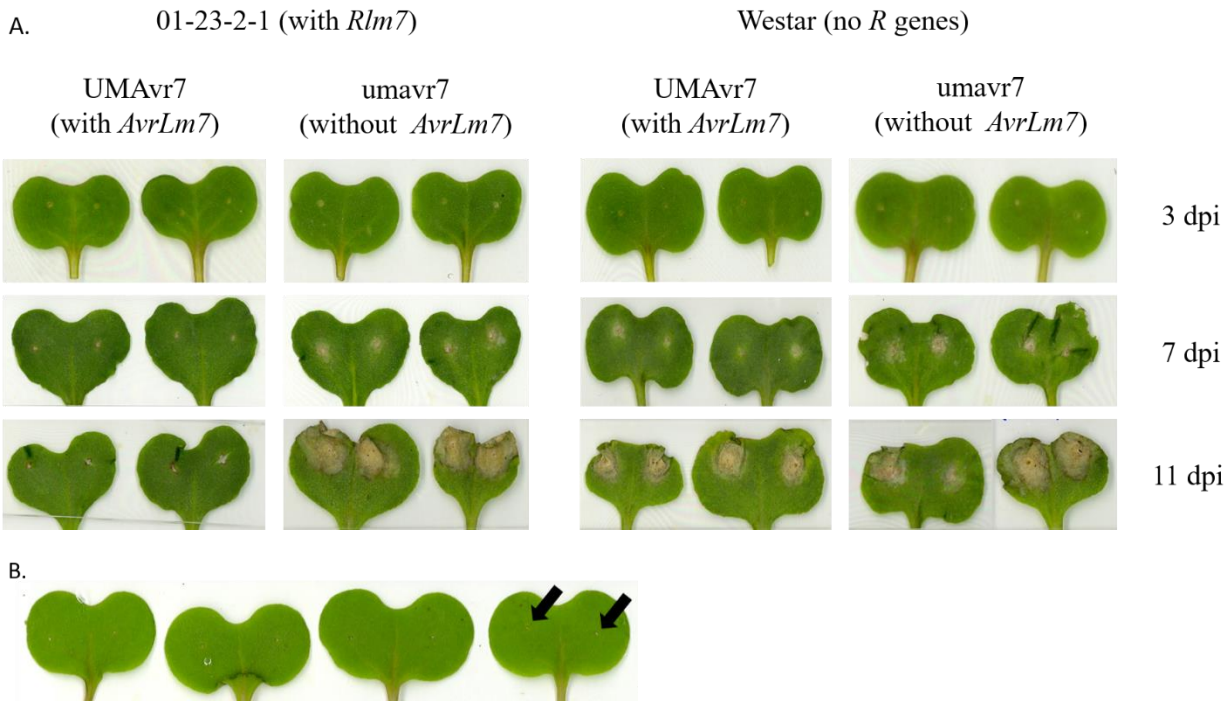


Figure 3. 1 Phenotype Response of 01-23-2-1 (with *Rlm7*) and Westar to the Infection of UMAvr7 and umavr7 at 3-, 7-, and 11-Days Post Inoculation under Controlled Conditions. A: UMAvr7 isolate with *AvrLm7* and umavr7 isolate without *AvrLm7* were inoculated to both 01-23-2-1 and Westar. The lesion development observed has shown at 3-, 7-, 11- dpi. B: Mock experiment. The arrows show the mock-inoculated sites. Seedling were grown under 16 h photoperiod (18 ℃ dark and 21 ℃ light) in a growth chamber.

3.4.2 Transcriptomic Study Results

The dual deep RNA-seq approach produced approximately 3,427 million raw reads generated from a total of 72 samples and average of 48 million reads were obtained per single sample.

3.4.2.1 Expression of Avirulence Genes of *L. maculans in Planta*

In planta expression of avirulence gene *AvrLm7* (Lema_P086290) was observed only in UMAvr7 isolate but not in umavr7 at any time point with none of the hosts (Figure 3.2). *In planta* expression of *AvrLm7* of UMAvr7 was the highest at three dpi with both 01-23-2-1 and Westar. The expression was least at one dpi and then the expression getting lowered at 7- and 11- dpi. The expression of *AvrLm7* was comparatively higher in Westar than in 01-23-2-1 line. In axenic cultures, both isolates did not express *AvrLm7* nor *AvrLm6* (Lema_P049940*). AvrLm5* (Lema_P070880) was observed at a low expression level (data not shown).

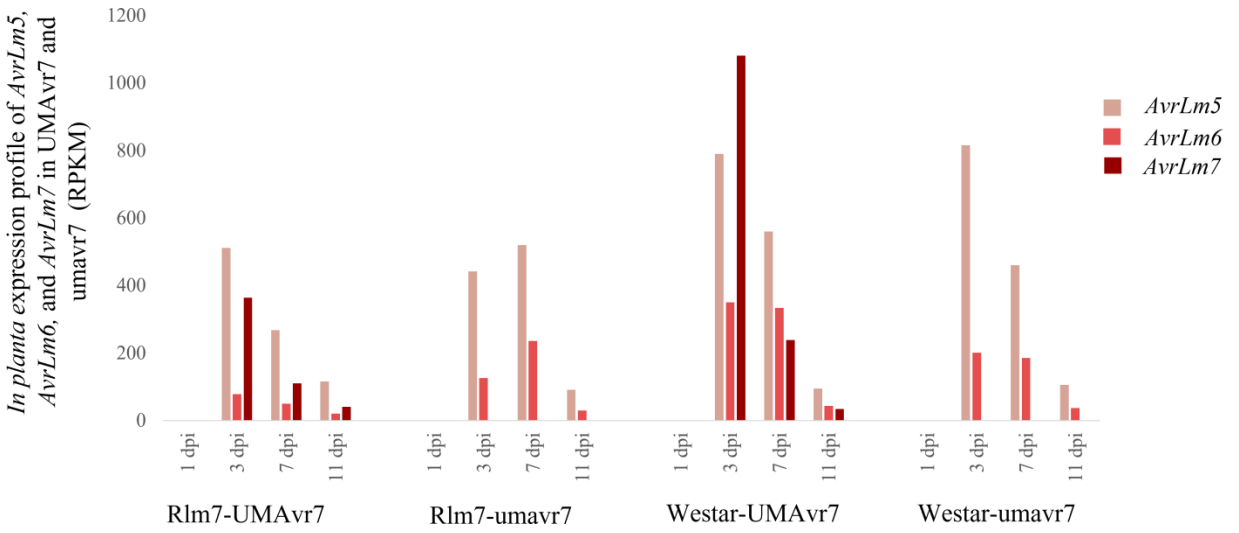


Figure 3. 2 *In planta* Expression of *AvrLm5, AvrLm6* and *AvrLm7* of *Leptosphaeria maculans* UMAvr7 and umavr7 Isolates in Infected Host Plants. Dual RNA-seq results exhibited *in planta* expression of *AvrLm5, AvrLm6* and *AvrLm7* in UMAvr7 and umavr7 isolates was observed with both infected 01-23-2-1 (with *Rlm7*) and Westar plants. The expression of *AvrLm5* and *AvrLm6* were observed in both isolates, while the expression of *AvrLm7* was shown only in UMAvr7 isolate in both hosts. Results are representative of three biological replicates.

3.4.2.2 Differentially Expressed Genes (DEGs) at 1-, 3-, 7-, and 11- dpi

UMAvr7 showed 114 Differentially expressed genes (DEGs) compared to umavr7 in axenic culture with expression log of absolute value of fold change cutoff (logFC) ≥ 2 and false discovery rate (FDR) ≤ 0.05 were considered (Appendix 2).

The DEGs of two hosts and two isolates in all four host–pathogen interactions at 1-, 3-, 7, and 11-dpi were assessed compared to the initial time point 0-dpi. Rlm7 line showed DEGs of 7,713 and 12,785 at 7- and 11-dpi in incompatible interaction, and 15203 and 18596 in compatible interaction which is nearly two-times higher ($p < 0.05$). Pathogen isolates UMAvr7 demonstrated 326, 491, 871, 9,050 number of DEGs at 1-, 3-, 7-, and 11-dpi, whereas umavr7 isolate exhibited 464, 1,109, 3,402, and 3,970 number of DEGs in interactions with the Rlm7 line ($p < 0.05$). The expression patterns of DEGs were similar in all three compatible interactions (Figure 3.3). Unique and shared DEGs in two canola genotypes and two *L. maculans* isolates under four different host–pathogen interactions were analyzed as shown in Figure 3.4. The analysis of unique and shared DEGs in two *L. maculans* isolates in interaction with Rlm7 line is shown in Figure 3.5. The DEGs at 1-, 3-, 7-, and 11- dpi were higher in umavr7 isolate which makes a compatible interaction with the 01-23-2-1 compared to UMAvr7 which makes an incompatible interaction (Figure 3.3, 3.4, and 3.5). The difference increased with the time as clearly shown in Figure 3.5.

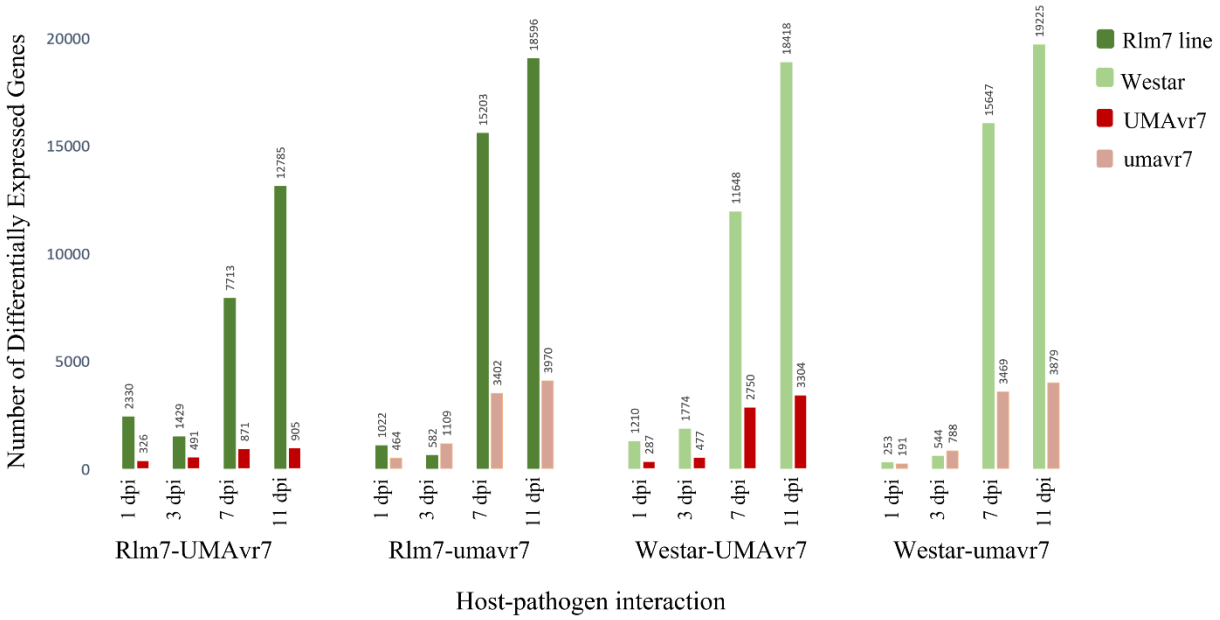


Figure 3. 3 Differentially Expressed Genes (DEGs) in Two Canola Genotypes and Two *L. maculans* Isolates under Four Different Host-Pathogen Interactions. Number of DEGs in two hosts, 01-23-2-1 (with *Rlm7*) and Westar (no *R* genes), and in two *L. maculans* isolates UMAvr7 (with *AvrLm7*) and umavr7 (mutant of *AvrLm7*) under each host-pathogen interaction at 1-, 3-, 7-, and 11- days post inoculation compared to 0 dpi analyzed by dual RNA-seq. Results are representative of three biological replicates. The fold change cutoff (logFC) ≥ 1 and ≤ -1 and false discovery rate (FDR) were taken as ≤ 0.05.

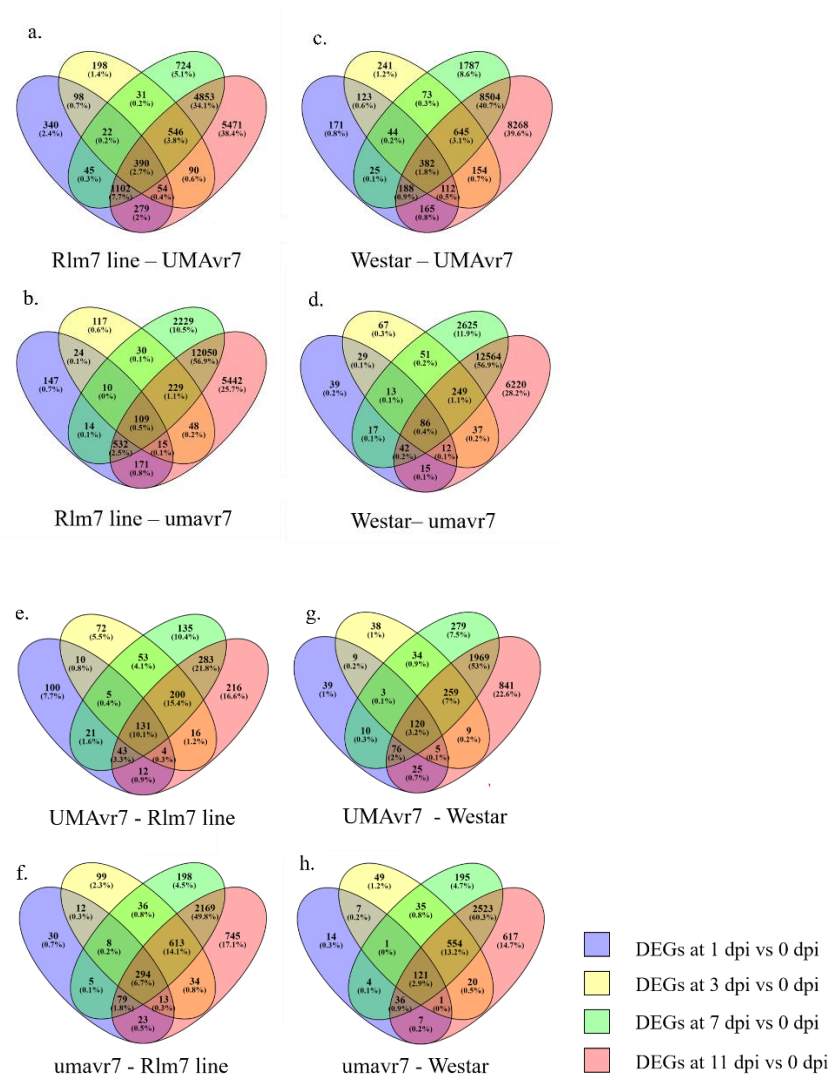


Figure 3. 4 Unique and Shared Differentially Expressed Genes (DEGs) in Two Canola Genotypes and Two *L. maculans* Isolates under Four Different Host-Pathogen Interactions. Unique and shared DEGs in two hosts, 01-23-2-1 (with *Rlm7*) and Westar, and in two *L. maculans* isolates UMAvr7 (with *AvrLm7*) and umavr7 (mutant of *AvrLm7*) under each host-pathogen interaction at 1-, 3-, 7-, and 11- days post inoculation compared to 0 dpi analyzed by dual RNA-seq. Results are representative of three biological replicates. The fold change cutoff (logFC) ≥ 1 and ≤ -1 and false discovery rate (FDR) were taken as ≤ 0.05. Unique and shared DEGs in 01-23-2-1 (a and b), in Westar (c and d), in UMAvr7 (e and f), in umavr7 (g and h) in incompatible and compatible interactions, respectively.

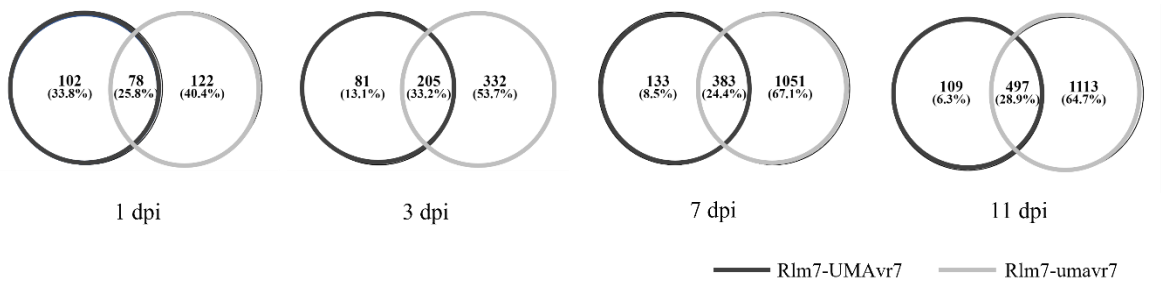


Figure 3. 5 Unique and Shared Differentially Expressed Genes (DEGs) in Two *Leptosphaeria maculans* Isolates in Interaction with 01-23-2-1 (with *Rlm7*). Unique and shared DEGs in two *L. maculans* isolates UMAvr7 (with *AvrLm7*) and umavr7 (mutant of *AvrLm7*) in incompatible (Rlm7-UMAvr7) and compatible (Rlm7-umavr7) interactions with 01-23-2-1 at 1-, 3-, 7-, and 11-days post inoculation compared to 0 dpi analyzed by dual RNA-seq. Results are representative of three biological replicates. The fold change cutoff (logFC) ≥ 1 and ≤ -1 and false discovery rate (FDR) were taken as ≤ 0.05.

3.4.2.3 Differentially Expressed Virulence Genes in the Pathogen Isolates

In addition to *AvrLm7* gene, there were number of other effector genes differentially expressed at different timepoints in both isolates in the interactions with 01-23-2-1 (Figure 4.6). The upregulation of effector genes in three compatible interactions were obvious compared to the incompatible interaction, *AvrLm7-Rlm7*. There was a list of necrotrophic effectors which were expressed only at 11-dpi.

Table 3. 2 *Leptosphaeria maculans* Effector Genes Differentially Expressed only at 11 dpi (Necrotrophic Stage) Compared to 0 dpi. Name of effector gene, and log fold change expression of each gene under each interaction have been shown. Results are representative of three biological replicates. The fold change cutoff (logFC) ≥ 2 and false discovery rate (FDR) were taken as ≤ 0.05. UMAvr7: wild type isolate with *AvrLm7*; umavr7: mutant of *AvrLm7*; Rlm7: *Brassica napus* genotype 01-23-2-1 with *Rlm7*; Westar: susceptible check with no *R* genes.

Leptosphaeria maculans locus	Annotation	Interaction (pathogen-host)			
		UMAvr7		umavr7	
		Rlm7	Westar	Rlm7	Westar
Lema_P023410	predicted protein	4.38	15.83	6.47	15.77
Lema_P038230	predicted protein	7.22	15.15	13.09	13.66
Lema_P101380	predicted protein	6.07	14.91	3.31	
Lema_P081900	hypothetical protein LEMA_P081900.1	6	14.57	14.69	5.72
Lema_P103890	hypothetical protein LEMA_P103890.1	6.87	14.31	11.24	13.83
Lema_P065720	hypothetical protein LEMA_P065720.1	5.83	14.05	3.36	13.46
Lema_P007760	predicted protein	3.23	13.47		
Lema_P007750	hypothetical protein LEMA_P007750.1	7.32	12.81	11.79	12.74
Lema_P006460	similar to secreted protein	3.66	12.77		
Lema_P108620	hypothetical protein LEMA_P108620.1	7.04	12.44		
Lema_P100530	predicted protein	3.02	4.28		
Lema_P054290	hypothetical protein LEMA_P054290.1	3.65	3.11		
Lema_P073150	hypothetical protein LEMA_P073150.1	6.17	2.95		
Lema_P105100	hypothetical protein LEMA_P105100.1	3.76	2.31		
Lema_P002430	hypothetical protein LEMA_P002430.1	2.87		2.47	2.69
Lema_P023340	hypothetical protein LEMA_P023340.1			15.05	3.61
Lema_P048250	predicted protein			14.24	14.39
Lema_P102730	hypothetical protein LEMA_P102730.1			12.58	2.6
Lema_P056750	hypothetical protein LEMA_P056750.1			11.99	12.26
Lema_P121470	predicted protein			11.67	12.25
Lema_P062600	hypothetical protein LEMA_P062600.1			11.37	11.83
Lema_P086760	predicted protein			11.34	12.15
Lema_P058240	predicted protein			11.27	12.23
Lema_P055050	hypothetical protein LEMA_P055050.1			10.96	11.3
Lema_P103880	hypothetical protein LEMA_P103880.1			10.73	12.9
Lema_P119660	hypothetical protein LEMA_P119660.1			10.59	10.74
Lema_P053640	predicted protein			10.39	10.51
Lema_P111950	hypothetical protein LEMA_P111950.1			10.17	10.51
Lema_P092370	predicted protein			10.17	10.35
Lema_P109050	predicted protein			10.11	10.94
Lema_P086880	hypothetical protein LEMA_P086880.1			10.05	10.53
Lema_P055250	hypothetical protein LEMA_P055250.1			9.9	10.3
Lema_P084030	predicted protein			9.74	10.29
Lema_P038270	predicted protein			9.38	10.25
Lema_P003630	predicted protein			4.89	4.91
Lema_P041540	hypothetical protein LEMA_P041540.1			3.46	2.95
Lema_P103680	hypothetical protein LEMA_P103680.1			3.07	12.91
Lema_P018540	hypothetical protein LEMA_P018540.1				12.49
Lema_P109600	predicted protein				10.15
Lema_P079720	similar to female reproductive tract protease GLEANR_897			13.95	3.47

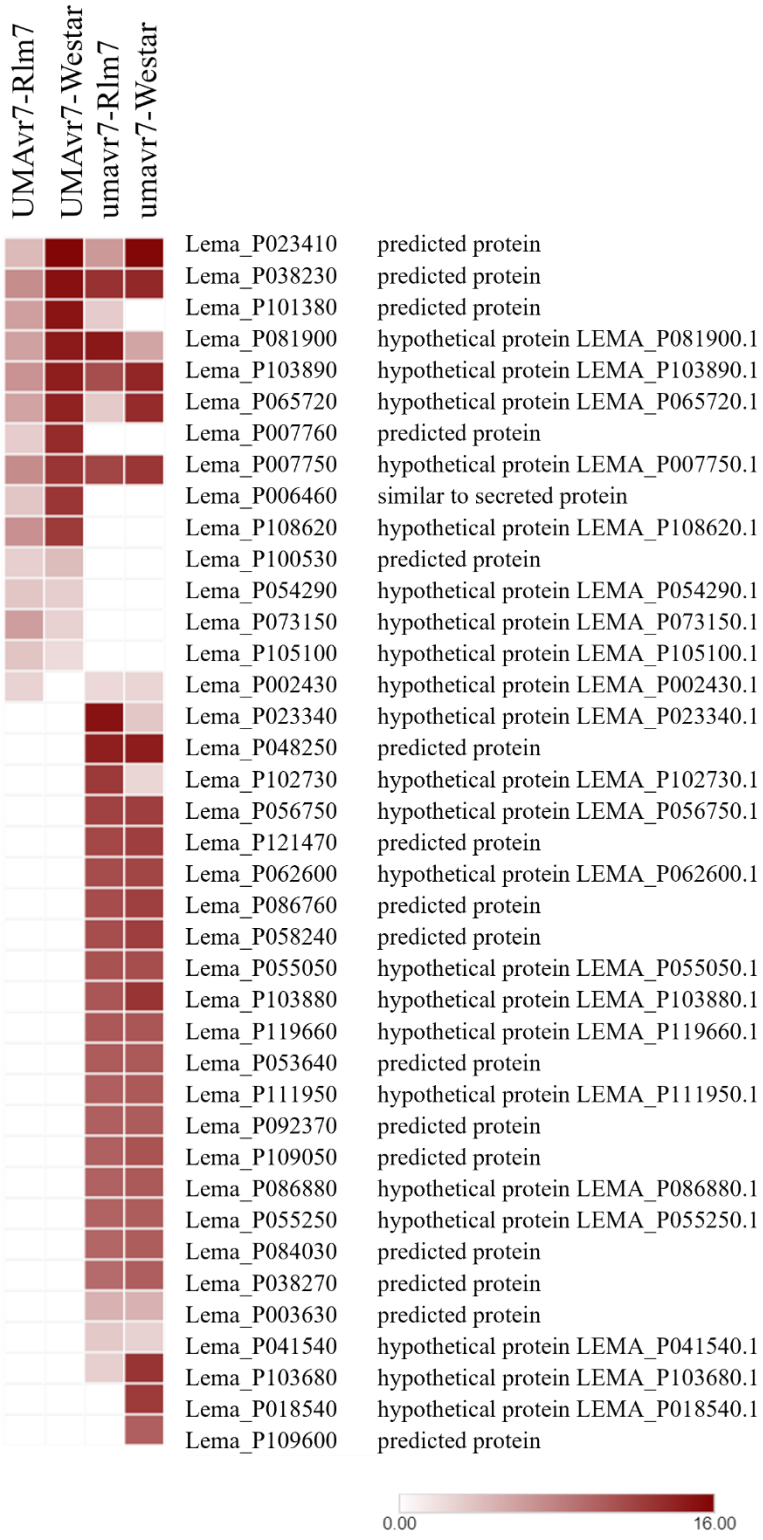


Figure 3. 6 Differentially Expressed Effector Genes of *Leptosphaeria maculans* Isolates, UMAvr7 and umavr7 in *Brassica napus* Genotypes, Rlm7 and Westar. Heatmap shows the differentially expressed effector genes of UMAvr7 and umavr7 *in planta* compared to zero dpi.

Logarithm of absolute value of fold change cutoff (logFC) ≥ 2 and false discovery rate (FDR) ≤ 0.05 were considered. Locus and putative annotation of *L. maculans* effector genes, and log fold change expression of each gene under each interaction have been shown. UMAvr7: wild type isolate with *AvrLm7*; umavr7: mutant of *AvrLm7*; Rlm7: *Brassica napus* genotype 01-23-2-1 with *Rlm7*; Westar: susceptible check with no *R* genes. Results are representative of three biological replicates.

A high number of genes encoding carbohydrate-active enzymes (CAZymes) of UMAvr7 and umavr7 were differentially expressed at latter timepoints, 7- and 11- dpi. The expressions were higher in compatible interactions compared to those in the incompatible interaction (Figure 4.7 and Appendix 1).

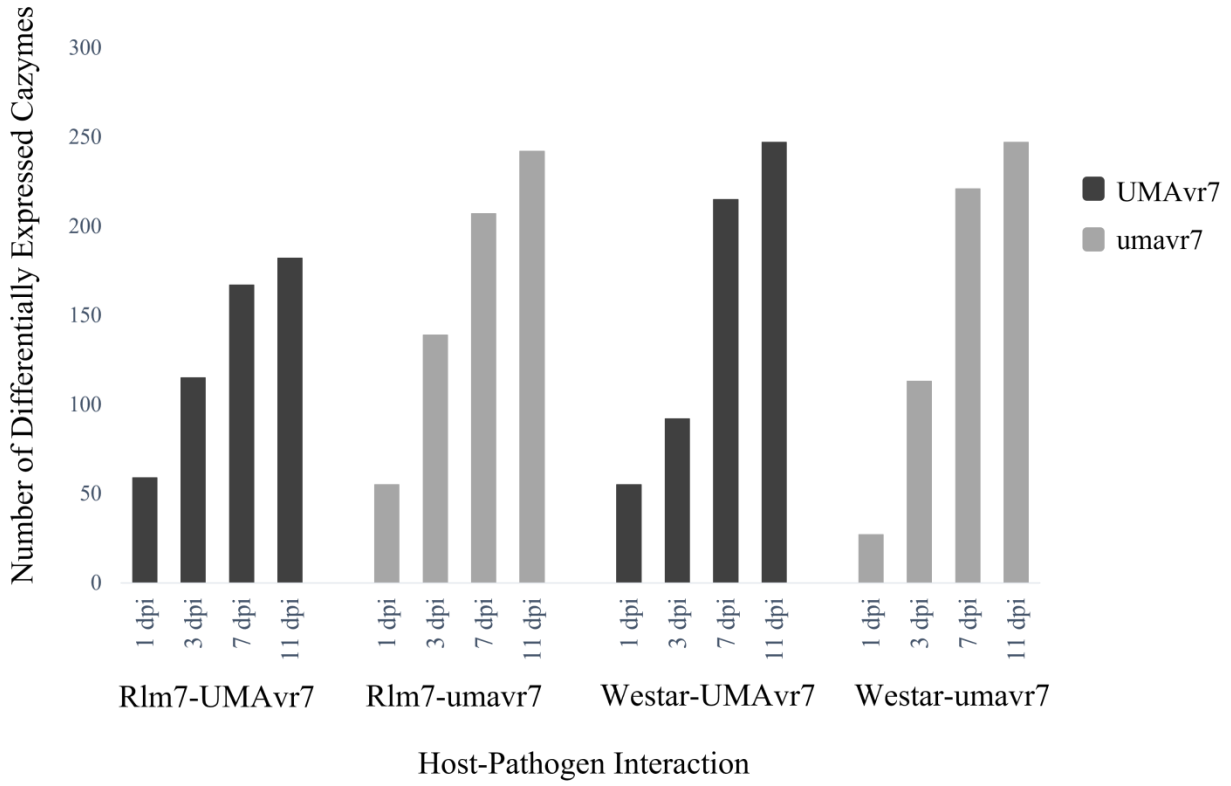


Figure 3. 7 Differentially Expressed CAZymes of *Leptosphaeria maculans* UMAvr7 and umavr7 Isolates in Infected Host Plants. Dual RNA-seq results exhibited differentially expressed CAZymes of UMAvr7 and umavr7 isolates in both infected 01-23-2-1 (01-23-2-1) and Westar plants. The differential expression of CAZymes were higher in compatible interactions than in the incompatible interaction. The expression level was increased with time from 1- to 11-dpi in each interaction. Results are representative of three biological replicates. The fold change cutoff (logFC) ≥ 1 and ≤ -1 and false discovery rate (FDR) ≤ 0.05 were considered.

Furthermore, the differentially expressed Merops, which are peptidases (also termed proteases, proteinases, and proteolytic enzymes) in both isolates were higher in compatible interactions compared to the incompatible interaction (Figure 3.8).

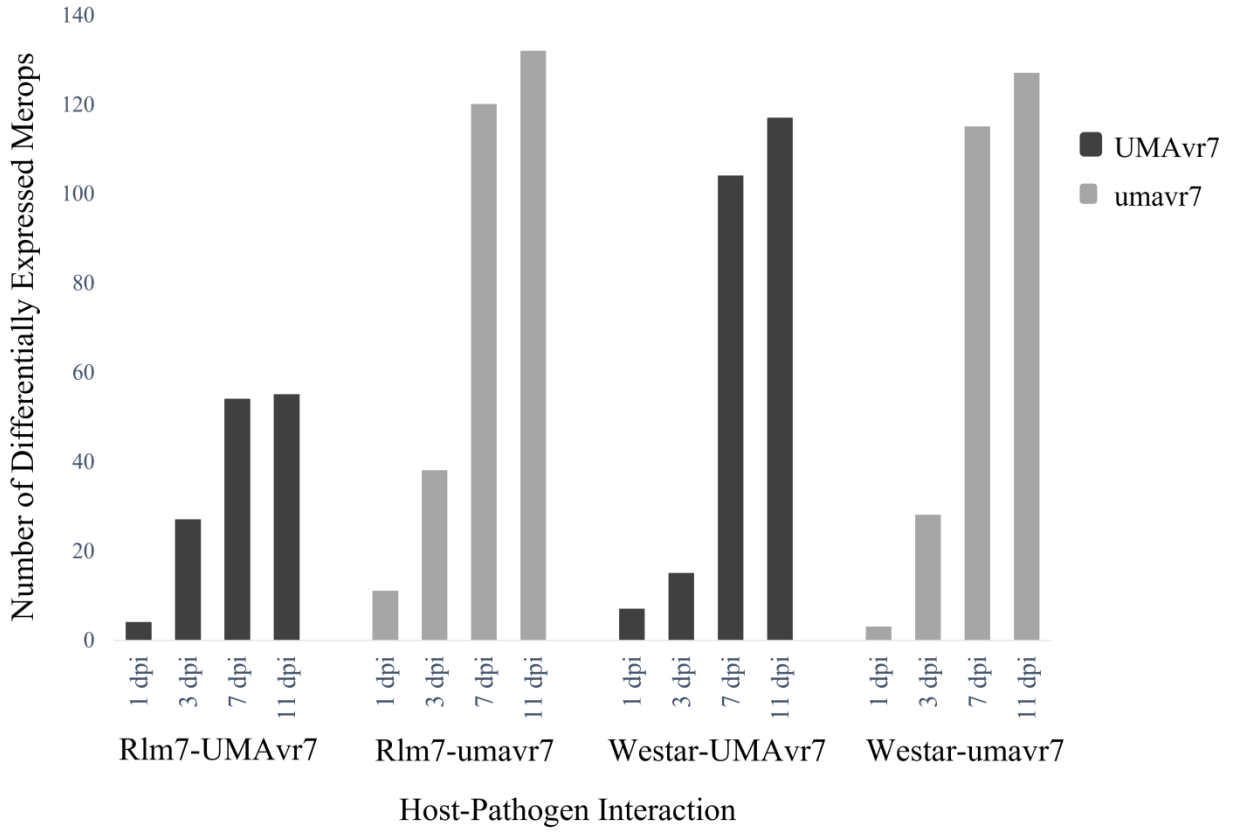


Figure 3. 8 Differentially Expressed Merops of *Leptosphaeria maculans* UMAvr7 and umavr7 Isolates in Infected Host Plants. Dual RNA-seq results exhibited differentially expressed Merops (peptidases) of UMAvr7 and umavr7 isolates in both infected 01-23-2-1 (with *Rlm7*) and Westar plants. The differential expression of peptidases was higher in compatible interactions than in the incompatible interaction. The expression level was increased with time from 1- to 11- dpi in each interaction. Results are representative of three biological replicates. The fold change cut off (logFC) ≥ 1 and ≤ -1 and false discovery rate (FDR) ≤ 0.05 were considered.

3.4.2.4 Differentially Expressed Defense Related Genes in the Host Plants

The differential expression of NLRs in two host plants under incompatible and compatible interactions was clearly observed at fold change cut off (logFC) ≥ 2 and false discovery rate (FDR) ≤ 0.05 (Figure 3.9). In all three compatible interactions, the upregulation of NLRs was clearly observed from 7-dpi onwards. In contrast, upregulation of one NLR gene (GSBRNA2T00068522001) was observed at early as 3-dpi onwards. The upregulation of NLRs: GSBRNA2T00035409001, GSBRNA2T00046655001, GSBRNA2T00049133001, GSBRNA2T00056878001, GSBRNA2T00065542001, GSBRNA2T00068521001, GSBRNA2T00072854001 were seen in all four interactions. There was a set of NLRs specifically upregulated only in incompatible interaction (Figure 4.9). In addition, differential expression of RLPs and SPs were also upregulated at later parts, i.e., 7- and 11- dpi in each interaction. GSBRNA2T00025294001 was upregulated in Rlm7 plant under incompatible interaction. On the other hand, Westar demonstrated upregulation of GSBRNA2T00006438001 under its compatible interactions with both isolates (data not shown).

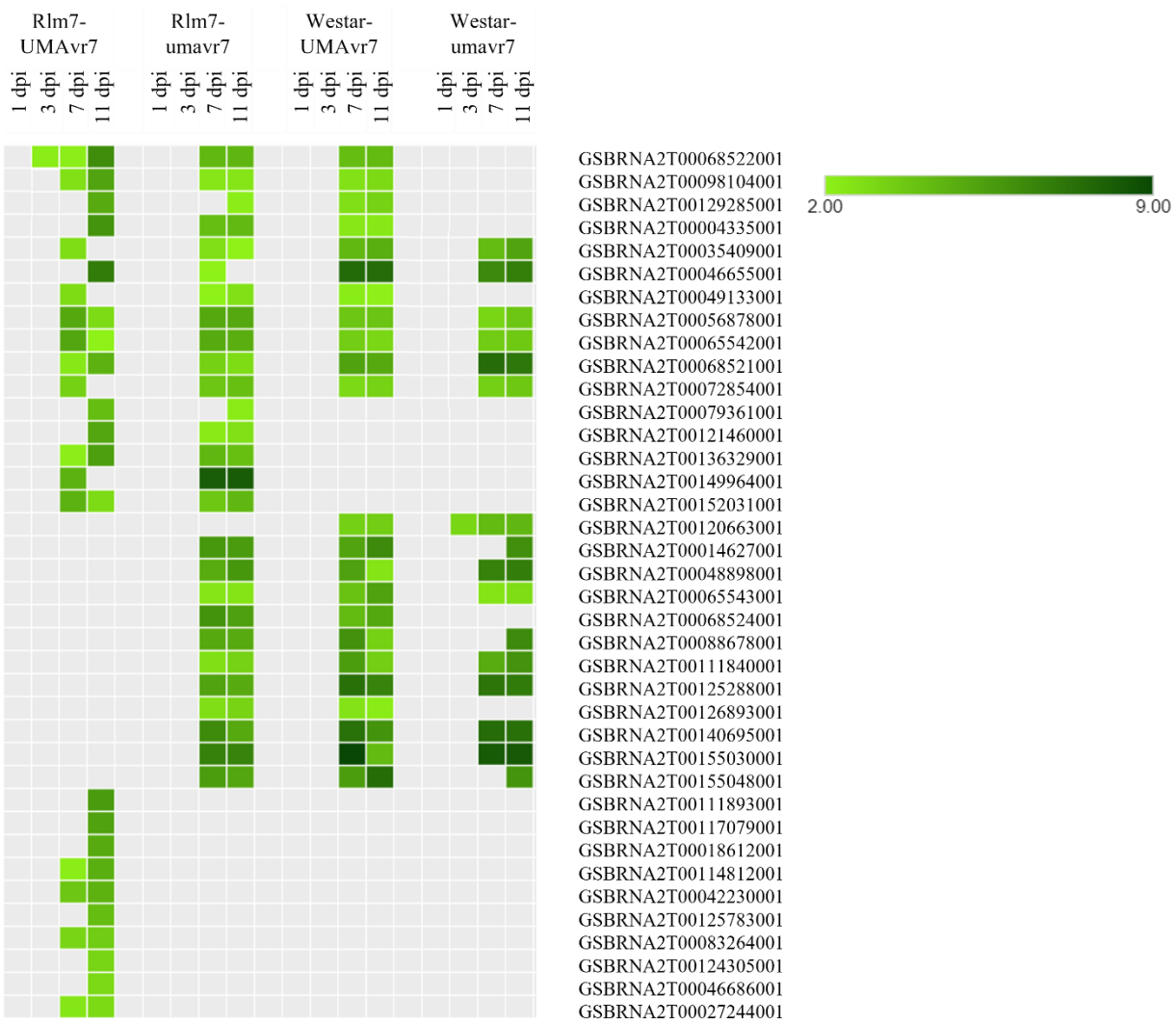


Figure 3. 9 Differential Expression of Host NLR Genes of Host Plants. Differential expression of NLR gene in Rlm7 and Westar plants under Rlm7-UMAvr7, Rlm7-umavr7, Westar-UMAvr7, Westar-umaver7 interactions have shown. Logarithm of absolute value of fold change cutoff (logFC) ≥ 2, false discovery rate (FDR) ≤ 0.05.

Differentially expressed genes related to SA biosynthesis and signaling in the host genotypes was observed at 1-dpi in all three interactions considered. However, PR1 (GSBRNA2T00150001001) gene upregulation was demonstrated in the incompatible interaction at 3-dpi, which was absent in other two compatible interactions shown. The genes related to SA biosynthesis and signaling were highly expressed in all three interactions at later stages as shown in Figure 3.10. A higher number of genes related to JA biosynthesis and signaling pathway were differentially expressed starting earlier (1- and 3-dpi) in the incompatible interaction compared to compatible interactions. How ever, compatible interactions demonstrated higher upregulation of those genes compared to the incompatible interaction (Figure 3.11).

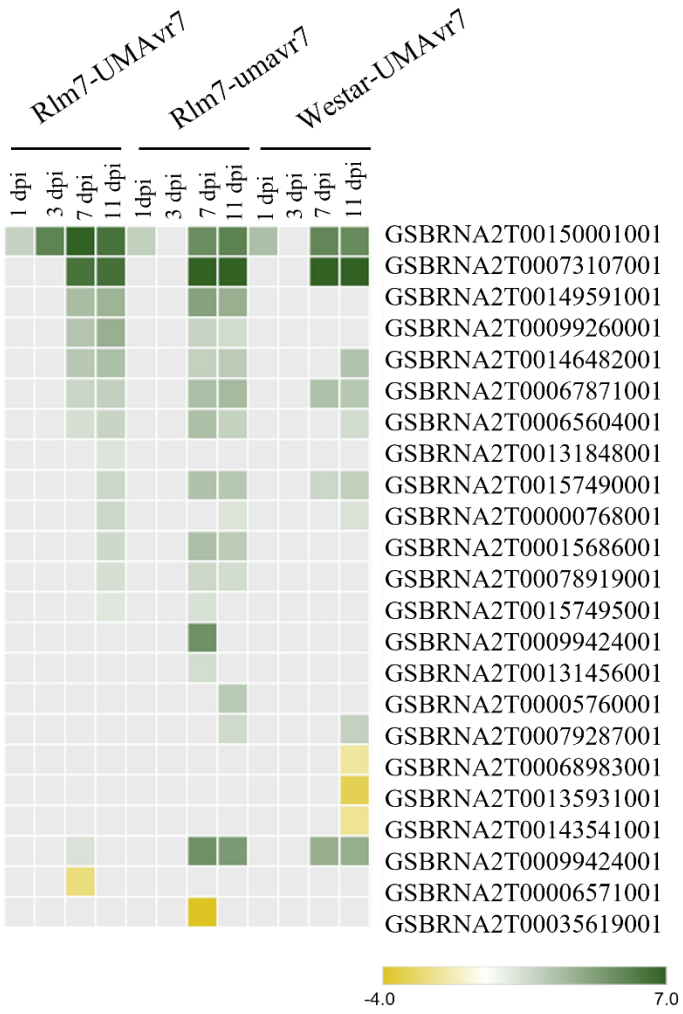
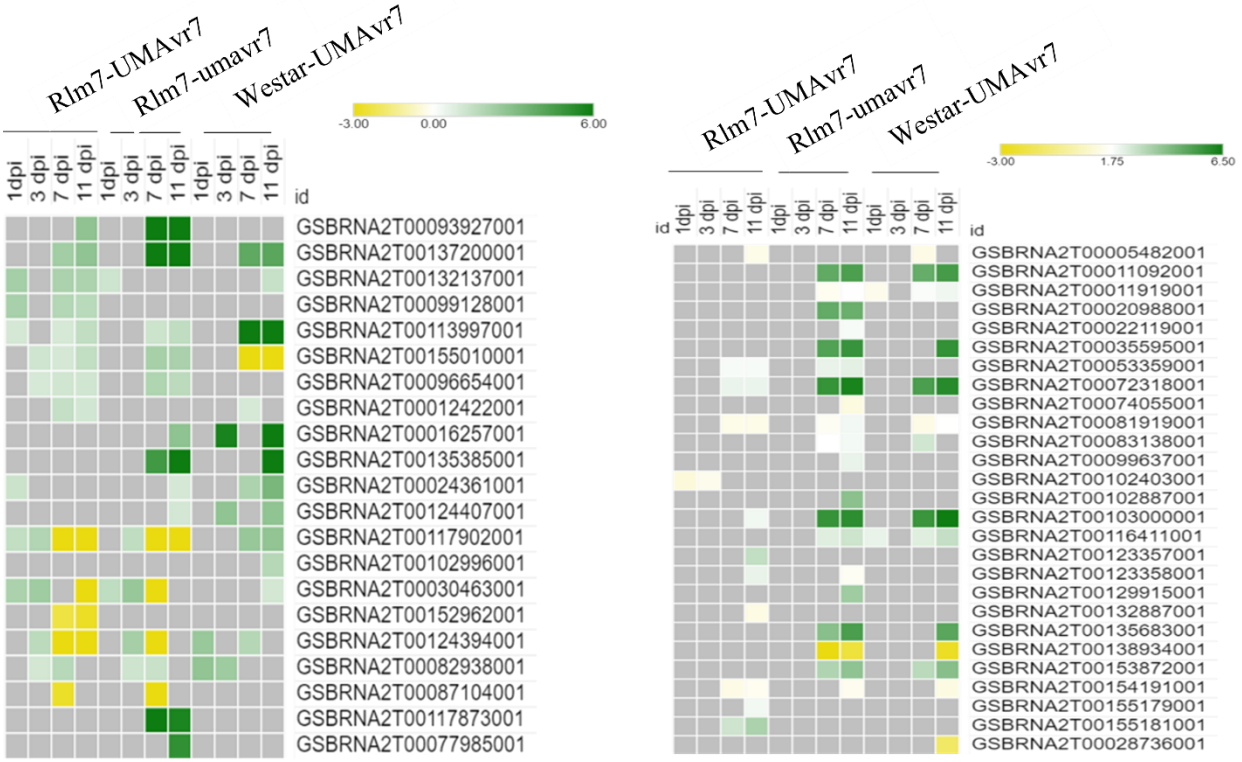


Figure 3. 10 Differential Expression of Host Genes Related to Salicylic Acid Biosynthesis and Signaling Pathways. Rlm7-UMAvr7, Rlm7-umavr7 and Westar-UMAvr7 interactions have shown. Logarithm of absolute value of fold change cutoff (logFC) ≥ 2 and false discovery rate (FDR) ≤ 0.05 were considered.

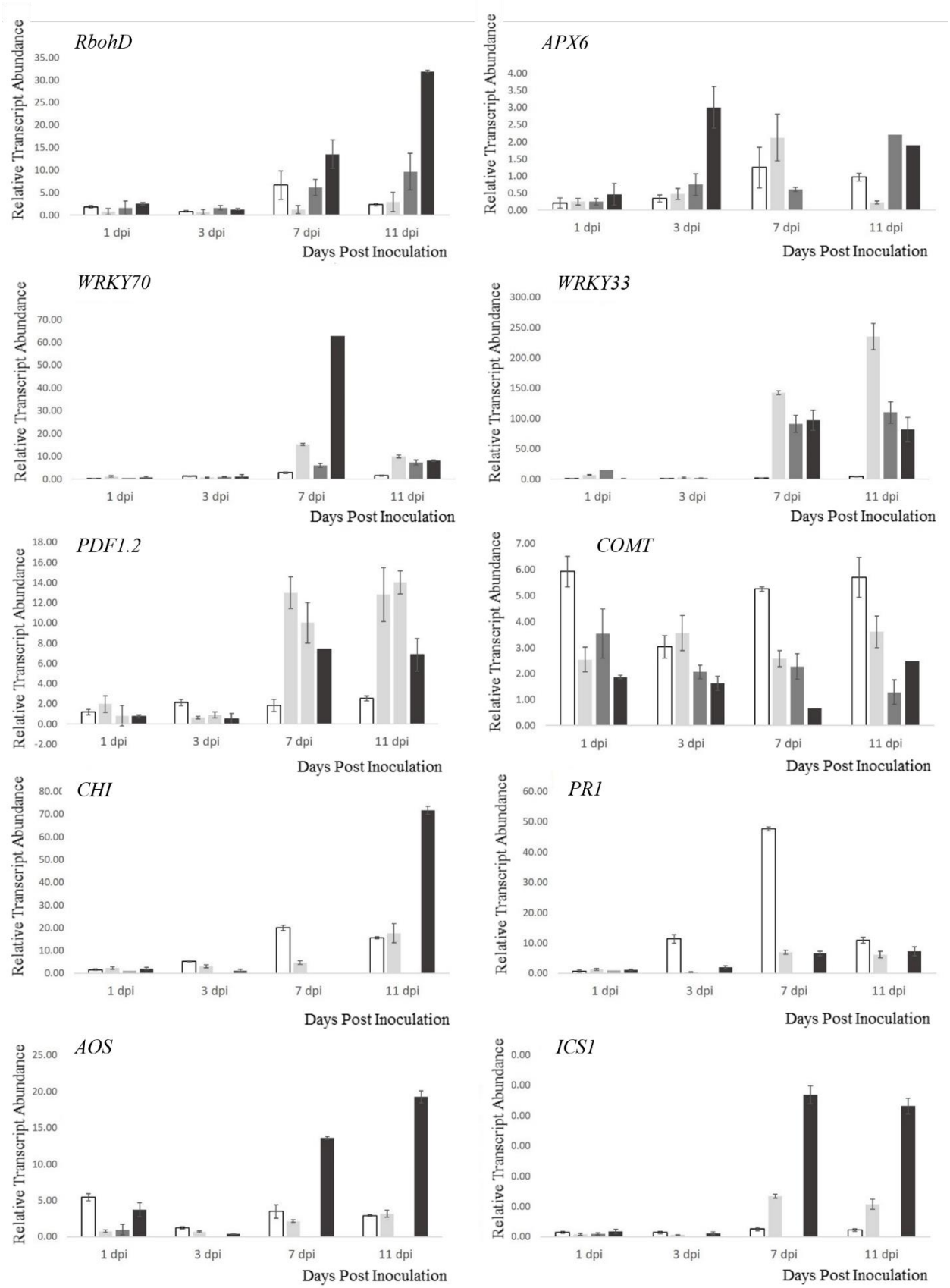


JA biosynthesis JA signaling

Figure 3. 11 Differential Expression of Host Genes Related to Jasmonic Acid Biosynthesis and Signaling Pathways. Rlm7-UMAvr7, Rlm7-umavr7 and Westar-UMAvr7 interactions have shown. Logarithm of absolute value of fold change cutoff (logFC) ≥ 2, false discovery rate (FDR) ≤ 0.05.

3.4.2.5 RNA-seq Data Validation by RT-qPCR

RBOHD, WRKY33, PDF1.2, AOS, and *ICS1,* genes involving in ROS production and JA and SA biosynthesis, expressions upregulated starting from early stages (1- and 3- dpi onwards) in Rlm7-UMAvr7 incompatible interaction. These genes expression started later in compatible interactions and expressions were highly upregulated from seven dpi onwards (Figure 3.12).


RbohD
Relative Transcript Abundance
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
1 dpi
3 dpi
7 dpi
11 dpi
Days Post Inoculation
APX6
Relative Transcript Abundance
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
1 dpi
3 dpi
7 dpi
11 dpi
Days Post Inoculation
WRKY70
Relative Transcript Abundance
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
1 dpi
3 dpi
7 dpi
11 dpi
Days Post Inoculation
WRKY33
Relative Transcript Abundance
300.00
250.00
200.00
150.00
100.00
50.00
0.00
1 dpi
3 dpi
7 dpi
11 dpi
Days Post Inoculation
PDF1.2
Relative Transcript Abundance
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
-2.00
1 dpi
3 dpi
7 dpi
11 dpi
Days Post Inoculation
COMT
Relative Transcript Abundance
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
1 dpi
3 dpi
7 dpi
11 dpi
Days Post Inoculation
CHI
Relative Transcript Abundance
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
1 dpi
3 dpi
7 dpi
11 dpi
Days Post Inoculation
PR1
Relative Transcript Abundance
60.00
50.00
40.00
30.00
20.00
10.00
0.00
1 dpi
3 dpi
7 dpi
11 dpi
Days Post Inoculation
AOS
Relative Transcript Abundance
25.00
20.00
15.00
10.00
5.00
0.00
1 dpi
3 dpi
7 dpi
11 dpi
Days Post Inoculation
ICS1
Relative Transcript Abundance
0.00
0.00
0.00
0.00
0.00
0.00
0.00
1 dpi
3 dpi
7 dpi
11 dpi
Days Post Inoculation

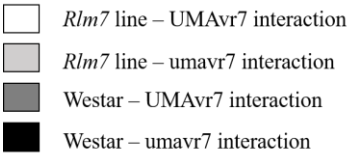


Figure 3. 12 *Brassica napus* Gene Expression Following Inoculation with *Leptosphaeria maculans.* Relative transcript abundance of *RBOHD, APX6, WRKY70, WRKY33, PDF1.2, COMT, CHI, PR1, AOS* and *ICS1* in susceptible (S) and resistant (R) cotyledons as measured at 1-, 3-, 7-, and 11- dpi. The interactions of Westar with both isolates have been also analyzed. Actin (GenBank accession number AF111812.1) was used as the internal control and to normalize expression data. Relative transcript abundance is normalized relative to 0 dpi in each treatment. Error bars represent standard deviation of the mean. The results are based on three replicates in three independent experiments.

3.5 Discussion

The incompatible interaction clearly exhibited an early onset of defense-related genes, further confirming the involvement of gene-for-gene interaction (Flor, 1971) in canola blackleg pathosystem. In compatible interaction, the abovementioned gene expressions commenced at later points, such as 7-dpi, proving the absence of early recognition of the pathogen.

In this study, UMAvr7 (with *AvrLm7*) and canola genotype ’01-23-2-1′ with *Rlm7* exhibited incompatible interaction where ETI led PCD due to early recognition of avirulence protein AvrLm7 by corresponding host Rlm7 protein. The Rlm7 line showed no lesion development over time, but only a thin ring of dead tissue surrounding the wound of inoculated cotyledons (Figure 3.1). That layer of dead tissue was distinct from what was seen in mock samples as shown in Figure 3.1B, further confirming the layer observed resulted from PCD (Becker et al., 2017). This thin layer of dead tissue and no further lesion development confirmed that PCD prevented further colonization of the pathogen in cotyledon under incompatible interaction. In contrast, both Rlm7 line and Westar showed compatible interactions with virulent isolate, umavr7.

Though the Rlm7–umavr7 interaction was compatible, the lesion development became evident only from 7-dpi. The observations explain the hemibiotrophic nature of the pathogen, which initially survives as a biotroph in the cotyledon apoplast without making any symptoms, then switching into its necrotrophic phase later in the lifecycle. There was no activation of ETI in this system due to the absence of initial recognition of pathogen avirulence protein by the host. Therefore, no activation of PCD to limit the pathogen spread throughout the cotyledon. After colonization within the cotyledon apoplast, the pathogen transferred into its necrotrophic stage, making necrotic lesions on cotyledons. The number of pathogen DEGs in incompatible and compatible interactions clearly expressed the higher numbers in compatible interactions, verifying that the pathogen was under control in the incompatible interaction.

Pathogen avirulence proteins are effectors that manipulate host cell structure and function, thereby facilitating infection of plant pathogens (Oliva et al., 2017). Transcriptomic results confirmed that umavr7 isolate was a knockout mutant by not expressing the *AvrLm7* in either of the hosts at any timepoint used (Figure 3.2) (Zou et al., 2020). The higher expressions of AvrLm7, *AvrLm6*, and *AvrLm5* at 3-dpi confirmed the higher avirulence gene expression with the biotrophic pathogens (Kloppholz et al. 2011). The *AvrLm7* gene expression was lower at the necrotrophic stage. The expression was comparatively higher in Westar than that in 01-23-2-1 line. This

observation can be explained by the case that, in Westar, the pathogen was in an environment with no *R* genes. Therefore, no barriers were exerted at the initial stage from the host due to lack of early recognition of the pathogen. Once the host defense mechanisms are activated against the pathogen at the latter stage, the pathogen would now have colonized, established, and become robust to overcome the plant defense as it was too late to be defended against. Low expression of all three avirulence genes at 1-dpi could be explained in that the pathogen was not established yet in planta at that time point. In axenic cultures, even UMAvr7 did not exhibit the expression of the gene *AvrLm7* nor *AvrLm6* and exhibited shallow expression of *AvrLm5* (data not shown). The performances explained above indicate that *AvrLm7* was not a housekeeping gene, and it commences its expression during the infection procedure within a host plant. In addition, the results further confirmed umavr7 is a knockout mutant by not expressing the *AvrLm7* at any of the time points considered. Though Zou et al. (2020) generated the umavr7 by knocking out only the *AvrLm7* gene by CRISPR/Cas9 genome editing system and confirmed the mutant does not have any off targets in the genome by subsequent sequencing studies, UMAvr7 showed 114 DEGs compared to umavr7 under in vitro conditions (Appendix 2). This observation could be explained in that the *AvrLm7* gene could be interconnected with the expression of other genes in the pathogen metabolism. Therefore, the *AvrLm7* gene might have other important roles as well in the pathogen system except its role as an effector protein.

In addition, the results further confirmed umavr7 is a knockout mutant by not expressing the *AvrLm7* at any of the time points considered. Though, Zou et al., (2020) generated the umavr7 by knocking out only the *AvrLm7* gene by CRISPR/Cas9 genome editing system and confirmed the mutant not to have any off targets in the genome by subsequent sequencing studies, UMAvr7 showed 114 DEGs compared to umavr7 under in vitro conditions (Appendix 2). This observation could be explained as *AvrLm7* gene could be interconnected with the expression of other genes in the pathogen. Therefore, *AvrLm7* gene might have other important roles as well in the pathogen system except the role of effector protein.

There were a number of effector genes differentially expressed at 3-, 7-, and 11-dpi compared to 0-dpi other than *AvrLm5, AvrLm6,* and *AvrLm7* in isolates in the interactions with the hosts (Appendix 1). Effectors involve the reprogramming of host plant cells and modulating of plant immunity to facilitate the infection by overcoming PTI (Stergiopoulos and Wit 2009). The presence of highly expressed different effector proteins in umavr7 at 7- and 11-dpi in the

compatible interaction with the Rlm7 line explains the involvement of different effector proteins in the pathogenicity after inoculation. In addition, both isolates exhibited several effector genes differentially expressed only at 11-dpi, i.e., in the necrotrophic stage of the pathogen (The fold-change cutoff (logFC) ≥1 and ≤-1 and false discovery rate (FDR) ≤ 0.05). Necrotrophs use an arsenal of effectors to disable susceptible hosts prior to colonization (Tan et al., 2010). They explained that necrotrophic effector function acts in an 'inverse' manner of gene-for-gene interaction. The interaction between a necrotrophic effector and corresponding protein produced by a host dominant sensitivity gene causes the disease. All the necrotrophic effectors shown in Table 4.2 are simple sequence repeats (Stotz et al., 2018). The necrotrophic effectors demonstrated comparatively low expressions in UMAvr7 isolate in its incompatible interaction with the 01-23-2-1 line, confirming the pathogen was under control in that interaction.

The genes encoding CAZymes are important in modifying fungal cell walls and degrading plant cell walls (O'Connell et al., 2012). Enzymes such as cellulases, hemicellulases, and pectinases are prominent in degrading host cell walls. Although, the majority of CAZymes were highly and differentially expressed during the necrotrophic phase (Appendix 1). According to O'Connell et al. (2012), pectin degrading enzymes become upregulated to facilitate the pathogen entering the plant system. In this study, this might not occur in the real situation as pathogen inoculation was facilitated by wounding the cotyledons. However, there were still differentially expressed CAZymes as they were required for the pathogen for getting into the host system. Differentially expressed CAZymes were higher in compatible interactions, where the pathogen colonizes and infects the host successfully compared to the resistant incompatible interaction in which the pathogen was controlled by the host.

Moreover, peptidases (also termed proteases, proteinases, and proteolytic enzymes) were highly expressed in compatible interactions compared to the incompatible interaction. Upon host penetration, fungal pathogens use proteases that degrade plant antimicrobial proteins, such as pathogenesis-related proteins, including plant chitinases and protease inhibitors that support plant immunity (Jashni et al. 2015). Generally, hemibiotrophs and necrotrophs exhibit a higher number of secreted proteases than biotrophs (Ohm et al., 2012). Jashni et al. (2015) explained that in incompatible interaction, the host plant activates its proteases and protease inhibitors, modifying or damaging pathogen proteases. The study results demonstrated lower expression of peptidases

in the incompatible interaction, and their higher expressions in compatible interactions confirm the pathogen control by the host under the incompatible interaction.

Host gene expressions related to plant defense exhibited differences between compatible and incompatible interactions. With logFC ≥ 2 as a cut-off point, an upregulated NLR gene (BnaA04g11170D protein [Source: UniProtKB/TrEMBL; Acc: A0A078HQ40]) was observed as early as 3-dpi only in the incompatible interaction, suggesting the possibility of the gene being involved in gene-for-gene interaction.

In hemibiotrophic pathogens, expression of phytohormones, especially SA, JA, and ET signaling vary between incompatible and compatible interactions (Garg et al., 2013). As Chowdury et al., (2017) the most exciting observation regarding host-hemibiotroph interaction was that the host plant could tune its defense strategy accordingly as the pathogen changes its trophic state. Tissues at and distal to the inoculation site exhibited accumulation of defense signals including phytohormones such as SA (An and Mou 2011; Fu and Dong 2013) and subsequently systemic production of pathogen-related antimicrobial proteins (PRs) such as Pathogenesis-Related Protein 1 (PR1) (Durrant and Dong 2004). As shown in Figure 3.10, PR1 (GSBRNA2T00150001001) gene expression was upregulated in all three interactions at even 1-dpi around a logFC value of two. This observation could be due to the initial stress caused by the abiotic stress caused in the cotyledons due to wounding (Akbudak et al., 2020). However, the differential expression of the gene was raised more than 10 times at 3-dpi in the Rlm7 line in the incompatible interaction, while all other compatible interactions did not show a differential expression of *PR1* gene at 3-dpi.

All most all NLR genes were considered as demonstrating relatively late expressions, suggesting it less likely that NLR genes are involved in regulation of the biotrophic phase of the pathogen. Interestingly, the *B. napus* gene GSBRNA2T00068522001 (BnaA04g11170D) was expressed early as 3-dpi of pathogen colonization (Figure 3.12) in the incompatible interaction. BnaA04g11170D as a crucial drought responsive gene (Wang et al., 2017) and a disease resistance gene (Summanwar et al., 2021).

The outstanding high expression of *PR1* in the incompatible interaction, 01-23-2-1-UMAvr7, could prove the early recognition and activation of plant defense due to gene-for-gene interaction. Moreover, in the compatible interactions, the pathogen might be colonizing in the host apoplast without harming the plant cells during its biotrophic phase so that the host cannot identify

the threat within. Subsequently, the pathogenic fungus becomes virulent once it reaches the required quorum sensing (QS). QS is a density-dependent signaling mechanism of microbes. When their signaling compounds reach the threshold level, it synchronizes the expression of virulence factors as a function of the fungal density to overcome the host defense (Padder et al., 2018). Therefore, it could be suggested that the pathogen reached its QS and started establishing the infection so the host plant could recognize its rival. As the pathogen switches into its necrotrophic phase, the killing of host tissues for survival could be observed by lesion development. Based on the lesion sizes seen at 7-dpi, it was clear lesion development had been started between 3- and 7-dpi. Based on previous observations, it is possible to mark this point at 5- or 6-dpi (Yang and Fernando 2021). Unfortunately, we did not set a timepoint within that period to confirm this.

SA plays a vital role in PTI, ETI, and systemic acquired resistance (SAR) (Pieterse et al., 1998; Tsuda et al., 2009). SA is involved in establishing and maintaining SAR (Vlot et al., 2008). SA is also responsible for signal transduction. Reduced SA accumulation relates to lowering basal defense gene expression leading to susceptibility (Salzmanetal.,2005) and can explain disease development in the compatible interactions. SA impacts other hormonal pathways, such as JA and ET, through antagonistic and synergistic interactions (Mohr and Cahill, 2007). Incompatible interaction led by ETI causes PCD to control the further spreading of biotrophs as they cannot survive on dead tissues. Interestingly, Spoel et al. (2007) suggested that ETI does not suppress JA-dependent defense, which results in the plant being protected by necrotrophs even with PCD condition as JA is the key phytohormone in defense against necrotrophs. Differential expression levels of JA and ET of the host plant under the incompatible interaction were lower than the compatible interactions. The compatible interactions exhibited clear raised expressions of JA biosynthesis, JA signaling, ET biosynthesis, and ET signaling genes at the latter part, 7- and 11-dpi, in which the pathogen survived as a necrotroph (Figure 3.11). JA is one of the significant phytohormones which plays a vital role in plant defense against necrotrophic pathogens (Berens et al., 2017). Our observations can further confirm this as it did not show upregulation in gene expression related to JA in the incompatible interaction. In contrast, SA levels remain upregulated at later stages studied: 7- and 11-dpi. This continued upregulation of SA levels in Rlm7–umavr7 interaction could be due to the SAR developed in the plant system. On the other hand, it can suggest that the pathogen is under control in the system and the remaining population might last as

biotrophs at later time points studied. Chowdhury et al. (2017) also explained that the biotrophy-to-necrotrophy switch was delayed in incompatible interactions.

Therefore, these results suggest that in the canola blackleg pathosystem, the host plant regulates the pathogen under incompatible interaction by limiting it at its initial infectious site, and the residual pathogen remains in a biotrophic phase without switching into the necrotrophic phase until dies without required resources. In addition, this observation verifies what we suggested regarding reaching the QS to start its mission as a necrotroph, being the stage, which is the real threat to the host. In addition, host defense-related genes, especially SA and JA biosynthesis and signaling-related genes, GSBR-NA2T00150001001 (*PR1*) and GSBRNA2T00068522001 in NLR gene family started to upregulate early in the incompatible interaction, confirming the early recognition of the pathogen. The high upregulations of the afore mentioned genes after 7-dpi only in the compatible interactions confirmed the active pathogen virulence in compatible interactions, but not in the incompatible interaction in which the pathogen was under control.

3.6 Conclusion

The findings of this study will help the canola blackleg research and breeding programs to identify genes controlling host defense against *L. maculans* and pathogen genes related to its virulence in disease development. The thorough understanding of Rlm7-AvrLm7 pathosystem will be significant to maintain the Rlm7 genotype last with-out breaking down.

Enrichment of the blackleg management toolbox is significant as an integrated management strategy is the best solution to mitigate this devastating disease. Despite the previous records of the high cost of using foliar fungicides in canola production, seed treatment fungicides have the potential to avoid outbreaks of blackleg disease under the breakdown *Rlm3* resistance due to extensive usage.

Pydiflumetofen: a SDHI Seed Applied Fungicide, a Potential Tool for the Canola-Blackleg Management Toolbox

K. Rasanie E. Padmathilake, Paula S. Parks, Robert H. Gulden, Jonathan Rosset, Liang Zhao, W. G. Dilantha Fernando[1]*

[1]Department of Plant Science, University of Manitoba, Winnipeg, MB R3T2N2, Canada 1; Dilantha.Fernando@umanitoba.ca
*Correspondence: Dilantha.Fernando@umanitoba.ca

This manuscript is in press.

Author Contributions: W.G.D.F. and K.R.E.P. designed the experiments. K.R.E.P. performed the experiments and analyzed the data. P.S.P. supported in field experiments. R.H.G. and J.R. helped in statistical analysis. L.Z. conducted a disease incidence experiments under controlled conditions. K.R.E.P. wrote the manuscript and W.G.D.F. revised the manuscript. Funding acquisition was done by W.G.D.F. All authors have read and agreed to the published version of the manuscript.

.

CHAPTER 4. EVALUATION OF NOVEL SDHI SEED APPLIED FUNGICIDE, PYDIFLUMETOFEN ON BLACKLEG DISEASE MANAGEMENT IN CANOLA

4.1 Abstract

Blackleg is a major fungal disease of canola caused by *Leptosphaeria maculans*. Due to the breakdown of current host resistance, having an effective fungicide to avoid seedling infection is important to avoid disease. Pydiflumetofen is a newly developed broad-spectrum seed treatment fungicide in the succinate dehydrogenase inhibitor (SDHI) group. We evaluated the separate and combined application of pydiflumetofen and Vibrance Flexi on early-season control of blackleg using moderately resistant canola cultivar SY4135 and susceptible cultivar Westar. In the greenhouse, seedlings inoculated with green fluorescent protein (GFP)-tagged *L. maculans* showed lesion development and apoplastic mycelial growth in cotyledons in control and Vibrance Flexi treatments but no lesion development or in planta mycelial growth was observed in pydiflumetofen-treated seedlings, in Westar or SY4135, 14 days postinoculation. SY4135 and Westar plants with pydiflumetofen-treated seeds inoculated with *L. maculans* isolate D3 had significantly lower disease severity. Both host genotypes treated with pydiflumetofen showed lower disease severity estimates than the other treatments. Statistically significant disease severity reduction was cultivar-dependent in the field. Canola is most susceptible to blackleg as a seedling, from the cotyledon to two-leaf stage. Pydiflumetofen protects the plant at the critical window for infection. Therefore, pydiflumetofen could be a potential tool that farmers can add to their blackleg management toolbox.

4.2 Introduction

Canola (*Brassica napus* L.), as the world's second most important oilseed crop, is Canadian farmers' most profitable farm crop, which provides more than 207,000 Canadian jobs and $12 billion in wages Canada-wide. Canada exports 90% of canola seeds, grain oil, and meal produced annually to foreign markets (Canola Council of Canada, 2020). Blackleg disease caused by actinomycete fungus, *Leptosphaeria maculans* (Desm.) Ces. & de Not. (anamorph: *Phoma lingam* (Tode ex Fr.) Desm.), is one of the most destructive pathogens found in canola in North America,

Australia, Europe, and many other regions around the world (Zhang and Fernando 2017). This fatal disease can be managed by an integrated approach using crop rotation, resistant cultivars, resistant (*R)*-gene rotation, and fungicide application, while genetic resistance of the host is the mainstay (West et al., 2001; Fitt et al., 2006). However, the host *R*-genes can break down in short order after introduction due to the rapid evolution of the pathogen with the consequence of high selection pressure. For instance, increased *Rlm1* and *LepR3* germplasm usage led to a rapid decrease of isolates carrying corresponding avirulence alleles in the pathogen populations in France and Australia, respectively (Rouxel et al., 2003; Van de Wouw et al., 2010). Zhang et al. (2016) has shown that the most used *R*-gene in Canada, *Rlm3*, has been overcome by the pathogen as the *AvrLm3* allele has reduced to the lowest frequency in the *L. maculans* population in Western Canada. Therefore, it is a definite necessity to reduce the selection pressure exerted on a canola cultivar by the pathogen, mainly when growing high canola frequency rotations for an extended period over a large area (Guo et al., 2008).

An integrated management approach is essential to mitigate *L. maculans* instead of a sole management strategy. *R* gene rotation is the newly introduced strategy, which can be deployed successfully to reduce the selection pressure of the pathogen in addition to crop rotation (Fernando and Zhang 2018). However, diversification of *R* genes is still in progress under breeding programs in seed companies. New tools in mitigating the disease under these prevailing conditions in Canada will help the industry. Since the blackleg disease is increasing in the prairies and making significant issues, especially in the seed trade, growers have begun to accommodate fungicide applications together with other measures (Peng et al., 2021). Canola growers readily use fungicides to control blackleg disease in other countries such as Australia (Marcroft and Potter 2008). As the fungus infection can happen from the cotyledon stage to even bolting, a single application of a fungicide may not be enough to protect the plant. Furthermore, the correct timing of fungicide application is crucial to control this disease (Peng et al., 2021). Moreover, Canada has a vast acreages of canola production, leading to enormous costs for farmers if they go with foliar fungicides. Based on the factors mentioned above, foliar fungicide application would not be the best strategy (Liu 2014). On the other hand, early infections that happen on young seedlings until the first three to four-leaf stage could boost the stem canker to girdle the crown severely, causing plant lodging and resulting in significant yield loss (Guo et al., 2008). Studies in Australia exhibited a reduction of disease severity and yield improvement on susceptible and moderately resistant (MR) cultivars under high

disease pressure by fungicide applications at the two- to four-leaf stage, further confirming the significance of early protection of plants from this disease (Marcroft and Potter 2008). Moreover, the above observations on the effect of early infection control, difficulties caused by timing and cost of foliar fungicides led to choosing seed treatments over foliar fungicides as the better option to control this disease with higher efficiency.

Demethylation inhibitors (DMI), quinone outside inhibitors (QoI), and succinate dehydrogenase inhibitors (SDHI) are three classes of fungicides commonly used in canola to control the blackleg disease. Succinate dehydrogenase is a key component in the electron transport chain and plays a vital role in the tricarboxylic acid cycle (Horsefield et al., 2006). SDHIs inhibit succinate dehydrogenase by blocking the ubiquinone-binding site of the enzyme. This inhibition leads to a limitation of ATP production and growth of the fungus (Avenot and Michailides 2010; Sierotzki and Scalliet 2013). SDHI fungicides are effective against a broad spectrum of fungal diseases. According to Sierotzki and Scalliet (2013), the class of SDHIs was the fastest-growing type in market adaptation due to its high level of activity.

This study focused on assessing the newly developed SDHI seed treatment, pydiflumetofen, on blackleg disease. The pydiflumetofen is an N-methoxy-(phenyl-ethyl)-pyrazole-carboxamide with a broad spectrum (Breunig and Chilvers 2021). This product was developed by Syngenta specifically targeting Soybean sudden death syndrome caused by *F. virguliforme*, Bakanae in rice caused by *G. fujikuroi*, and Blackleg disease in canola.

As pydiflumetofen is developed as a seed care product, it can target early infections more efficiently than a foliar fungicide (Peng et al., 2012). The seed could be a suitable vector for carrying fungal pathogens within and intact on the surface. Furthermore, when the seed is sown, the emerging seedlings are attacked by many soil-borne pathogens (Mancini and Romanazzi 2014; Hubbard and Peng 2018). In Canada, as of 2020, seed-applied fungicides have not been incorporated into their in-season blackleg management toolbox. The main reason was the unavailability of seed-applied fungicides registered for this disease. Therefore, it is exciting that pydiflumetofen has now been registered in Canada and commercially available from 2021 planting season onwards as a part of the integrated approach with crop rotations and cultivar resistance for blackleg disease. Previous studies have shown the success of pydiflumetofen in controlling other pathosystems. As Bai et al. (2021) showed, pydiflumetofen is highly effective against the growth and sporulation of *F. fujikuroi* attacks rice. Furthermore, Breunig and Chilvers (2021) explained

pydiflumetofen could be used in fusarium head blight management in wheat and corn. This study was undertaken to assess the efficacy of SDHI fungicide, pydiflumetofen as a seed treatment controlling seed-borne and air-borne infections of the blackleg pathogen under controlled and field conditions compared to other available fungicides and its potential to be added as a new tool to the blackleg management toolbox.

4.3 Materials and Methods

4.3.1 Plant Material

Susceptible canola cultivar Westar with no *R* genes and moderately resistant (MR) cultivar SY4135 were used as host germplasm. The SY4135 cultivar has been characterized to carry *Rlm3* by inoculating a series of *L. maculans* isolates with known avirulence genotypes, as explained by Zhang et al. (2016). The same host genotypes were used in both controlled and field experiments. The company, Syngenta itself pretreated all the seeds used in controlled and field studies with the corresponding fungicide tested. One kilogram of canola seeds was prewetted with 20 ml of water. Measured seed treatments were added slowly to the treater until the seed treatment was applied to the seeds. A spinning disc inside the mixing bowl atomizes the seed treatment to ensure good primary contact with the canola seed. A secondary mixing for 20–30 s was done as the seed treatment dried on the seeds. This ensures excellent seed coverage of the seed treatment.

4.3.2 Pathogen Isolates Used under Controlled Conditions

Three *L. maculans* isolates D1, D5, and D7, previously characterized by scientists at the University of Melbourne (Zhang et al., 2016), were used for the *in vitro* dose-response study. D3 (Zhang et al., 2016), which was the most virulent isolate among the lab isolate collection was used to see the effectiveness of pydiflumetofen under controlled conditions (Table 4.1). As our lab did not have a GFP-tagged *L. maculans* isolate at that time, isolate LmGFP kindly donated by Dr. Kim Hammond-Kosack (Rothamsted Research, Harpenden, Herts, UK) was used in this study (Eckert et al., 2005).

The isolates were grown on V8 agar medium (200 ml V8 juice, 800 ml distilled water, 15 g agar, 0.75 g $CaCO_3$, 10 ml 0.35% [wt/vol] streptomycin sulphate) and kept on a light bank under fluorescent light for a 24 h period at 20 ± 2°C. After 21 days, the mycelial cultures with pycnidia

were harvested by flooding with sterile distilled water and scraping the cultures gently with a spatula. Two layers of Miracloth were used to filter the pycnidiospore suspension. The filtered inoculum concentration was adjusted to 2×10^7 pycnidiospores/ml.

4.3.3 In vitro Dose-Response Experiment for Pydiflumetofen

The effectiveness of pydiflumetofen to inhibit the growth of *L. maculans* was tested using an in vitro dose–response experiment. *L. maculans* isolates D1-*avrLm1*, D5-*avrLm3*, and D7-*avrLepR2*, each representing highly abundant virulence genes in Canadian prairies, were used in the study. The three isolates were cultured on V8 juice agar medium for 18 days starting with a single pycnidiospore on each plate. A 0.5 cm agar disc with mycelia was excised from the margins of new cultures and transferred to the centre of new 90-mm plates prepared with V8 agar medium amended with 0, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, and 100 mg active ingredient, acetone-dissolved pydiflumetofen, per litre (technical grade from Syngenta Canada). Only acetone was used in the control. Plates were incubated under fluorescent light as previously for 14 days. The experiment was conducted in a completely randomized design with six and four replicates per treatment for the first and second experimental runs, respectively. The radial growth of each isolate was determined by measuring the diameter of a grown colony at its widest point, and a second measurement perpendicular to the first.

Table 4. 1 *Leptosphaeria maculans* Isolates D1, D3, D5, and D7 Used in the Dose-Response Experiment and Controlled Environment Study. The Avirulence genes, host species first isolated from, year cultured, the state/country received from, and references for each isolate have shown.

Isolate	Avirulence genotype												Species	Year cultured	Country	References
	AvrLm1	*AvrLm2*	*AvrLm3*	*AvrLm4*	*AvrLm5*	*AvrLm6*	*AvrLm7*	*AvrLm8*	*AvrLm9*	*AvrLmS*	*AvrLepR1*	*AvrLepR2*				
D1 (IBCN13)	*-	*+	-	-	+	+	-	-	+	+	+	+	*B. napus*	1991	Western Australia	Balesdent et al., 2005
D3 (IBCN16)	-	-	-	-	+	-	-	-	-	-	+	-	*B. napus*	1988	Western Australia	Purwantara et al., 2000
D5 (IBCN18)	+	+	-	+	-	-	+	-	-	+	+	+	*B. napus*	1988	Victoria	Purwantara et al., 2000
D7 (IBCN76)	+	-	+	-	+	+	-	+	-	*nd	+	-	*B. napus*	1987	Western Australia	Purwantara et al., 2000

* +/− indicates the presence/absence of a specific avirulence gene
nd indicates the genotype was not determined

4.3.4 Evaluation of pydiflumetofen under controlled conditions

The effect of pydiflumetofen was evaluated under the controlled environment of a greenhouse attached to the Department of Plant Science, University of Manitoba, Winnipeg, Canada. Greenhouse studies were conducted to compare fungicide performance between the newly developed seed treatment of pydiflumetofen (40 g a.i. pydiflumetofen/100 kg seeds) and the currently available Syngenta fungicide Vibrance Flexi (5 g a.i. Sedaxane, 24 g a.i. difenoconazole, 7.5 g a.i. metalaxyl-M, and 2.5 g a.i. fludioxonil/100 kg seeds). Westar and SY4135 seeds were treated with (a) one of the two fungicides, (b) a combination of both fungicides, or (c) no fungicide (control).

Seeds were sown in Sunshine mix #4 (SunGro Horticulture), under a 16 h photoperiod (18℃ dark and 21℃ light) in a growth chamber. Seven-day-old soil-grown cotyledons were pinched by a needle as one pinch per lobe and inoculated with 10 µl of the relevant spore suspension

4.3.4.1 Evaluation of lesion development and in planta mycelial growth in cotyledons using GFP-tagged isolate under controlled conditions

A GFP-tagged isolate of *L. maculans*, LmGFP, was used for the confocal study. LmGFP was characterized to carry *AvrLm1*, *AvrLm6*, *AvrLm7*, *AvrLepR1*, and *AvrLmS* by a pathogenesis assay conducted using known Rlm genes (Dr Zhongwei Zou, University of Manitoba, Winnipeg, Manitoba, Canada, personal communication).

The experiment was conducted in a completely randomized design. Three replicates (seedlings) were used for each treatment. Seedlings were grown and inoculated as described above. Two seedlings (eight lobes) of each treatment were used in lesion development evaluations and in confocal observations at each time point. To maintain alive cotyledons in growing seedlings, emerging true leaves were removed. Disease lesion sizes were calculated as a percentage of the total leaf area by ImageJ software at 7, 10, and 14 days postinoculation (dpi) (Schneider et al., 2012). The confocal observations were done at the same timepoints under 20× magnification and cotyledon specimens were taken as follows. At 7 dpi, cotyledon pieces 2 × 2 mm^2 in size were taken 2 mm away from the inoculation wounds. At 10 and 14 dpi, the cut pieces of cotyledons (2

× 2 mm^2) were taken 2 mm away from the margins of lesions to ensure collection of the growing fungus. In cotyledons without lesion development, the specimens were collected in the same way as at 7 dpi. The cut pieces were water-mounted on slides. GFP-tagged *L. maculans* mycelia development was visualized using a laser confocal LSM 700 microscope (LSM 700; Zeiss), while GFP was imaged using the excitation of 488 nm with a 515/565 nm bandpass filter. This study was repeated twice.

4.3.4.2 Effect of pydiflumetofen on blackleg disease severity under controlled conditions

In this study, the host genotypes, fungicide treatments, the seeding method, pathogen inoculation, and the growth conditions were the same as above. L. maculans isolate D3 (AvrLm5 and AvrLepR1), which carries the least number of avirulence genes, was the most virulent isolate in the Fernando laboratory isolate collection of around 2000 isolates (Zhang et al., 2016). The true leaves were not removed from inoculated seedlings and the cotyledons were allowed to senesce and gradually fall off from the seedlings. The seedlings were transplanted into individual 2.6 L pots at the 2–3 true-leaf stage. Transplanted pots were moved into the greenhouse with a 16 h photoperiod (18°C dark and 21°C light). Twenty plants were used per treatment arranged in a complete randomized design and the study was repeated twice.

Blackening of the stem base, the main disease symptom, was examined to evaluate the disease severity. The lesion development caused by D3 was similar to that with the GFP-tagged L. maculans isolate. Therefore, the results of lesion development in cotyledons of this experiment are not shown. The disease severity was scored at maturity stage (https://www.canolacouncil.org/canola-encyclopedia/growth-stages/), 7 weeks after transplanting, based on the percentage blackening of stem cross-sections taken at the stem base, and rated on a 0–5 scale (Western Canada Canola/Rapeseed Recommending Committee): 0: no blackening, 1: blackening of ≤25% of the cross-section, 2: blackening of ≥25–50% of the cross-section, 3: blackening of ≥50–75% of the cross-section, 4: blackening of ≥75–100% of the cross-section while possessing seeds, 5: 100% blackened cross-sections with no seeds as shown in Figure 4.1 (Zhang et al., 2016).

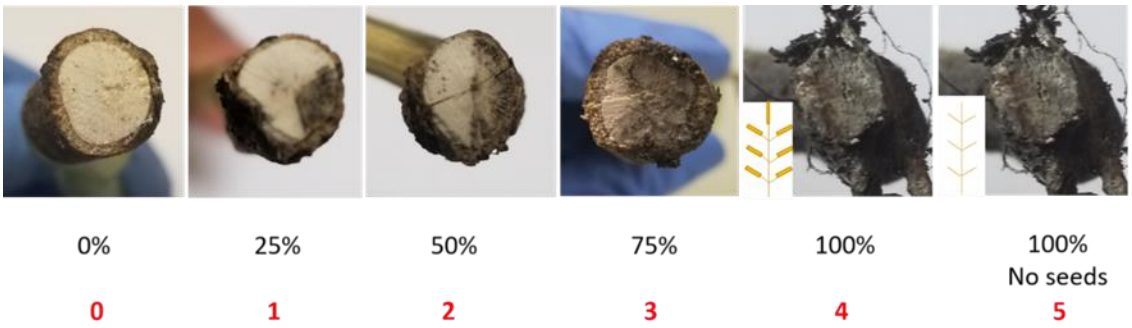


Figure 4. 1 The Scale of Blackening of Stem Base Cross-Sections used to Determine Disease Severity. 0: no blackening, 1: blackening of ≤25% of the cross-section, 2: blackening of ≥25-50% of the cross-section, 3: blackening of ≥50-75% of the cross-section, 4: blackening of ≥75-100% of the cross-section while possessing seeds, 5: 100% blackened cross-sections with no seeds.

4.3.5 Field Evaluation of Seed-Applied Pydiflumetofen

The field experiment was conducted in Ian N. Morrison Research Farm, University of Manitoba, Carman, Manitoba, Canada, in summer 2018 and 2019. Five seed treatments, including the new product, pydiflumetofen were evaluated on the same canola genotypes: Westar and SY4135. To overcome the flea beetle problem which was common in summer season, all seeds were treated with an insecticide in addition to the fungicide. The seeds treated only with the insecticide Cruiser® 5FS (400 g ai Thiamethoxam/100 kg of seed), were used as the control. In addition to pydiflumetofen, three other fungicides currently used for blackleg disease control were used in the field study. The fungicide treatments used in this study were Helix® Vibrance (400 g a.i. thiamethoxam, 24 g a.i. difenoconazole, 7.5 g a.i. Metalaxyl-M , 5 g a.i. Sedaxane, and 2.5 g a.i. fludioxonil/100 kg of seed), Helix® Vibrance + Pydiflumetofen (40 g a.i./100 kg of seed), Prosper® EverGol (400g a.i. clothianidin, 15 g a.i. penflufen, 10 g a.i. trifloxystrobin and 10 g a.i. metalaxyl/100 kg of seeds), and BAS 720 FST (10 g a.i. pyraclostrobin, 10 g a.i. fluxapyroxad, and 8 g a.i. Metalaxyl /100 kg of seeds). All the fungicide seed treatments were carried with an integrated insecticide within. Since pydiflumetofen did not carry an insecticide, it was applied along with Helix® Vibrance, Syngenta's current recommendation for canola which carried Cruiser. The seeds treated with chemicals were supplied by the company to conduct the experiment.

A total of 10 treatments were arranged in a randomized complete block design with four replicates. The plot size was set to two by eight square meters, and two meters apart. Seeding was done at a 9.8 kg ha^{-1} seeding rate and with 0.2 m row spacing on August 13th and 15th of 2018 and 2019, respectively.

4.3.5.1 Fungal Inoculum

A mixture of 19 *L. maculans* isolates representing the blackleg population in prairies was used as the inoculum (Table 4.2). Isolates were cultured on V8 agar medium as explained previously. After two weeks of incubation, pycnidiospores were harvested. Equal amount of spore suspension of each isolate was mixed to prepare the final suspension. The concentrated spore suspension was carried to the field under cold conditions. At the field, Tween 20 added (1 mL/1 L suspension), diluted spore suspension (2 x 10^7 spores/mL) was sprayed using a backpack sprayer.

The spray inoculation was done three times, starting from two weeks after seeding at one-week intervals within the two to four-leaf stage of plants. In addition, diseased stubble collected from the previous season were spread randomly in plots to ensure the infection.

Table 4. 2 Nineteen *Leptosphaeria maculans* Isolates Used in the Field Study and their Avirulence and Virulence Gene Composition. nd[a] – not determined.

Isolate #	Name of *L. maculans* Isolate	Avirulence Genes										Province	Year
		AvrLm1	***AvrLm2***	***AvrLm3***	***AvrLm4***	***AvrLm6***	***AvrLm7***	***AvrLm9***	***AvrLepR1***	***AvrLepR2***	***AvrRlmS***		
1	1-1	a	A	a	A	A	A	a	A	a	a	MB	2011
2	3-1	A	a	a	A	A	A	a	a	a	nd[a]	MB	2011
3	5-1	a	A	a	A	A	A	a	a	a	a	MB	2011
4	8-1	A	a	a	A	A	A	a	A	a	nd	MB	2011
5	21-2	a	A	a	A	A	A	a	a	a	a	MB	2011
6	25-1	a	A	a	A	A	A	a	a	a	a	MB	2011
7	30-3	a	A	a	A	A	A	a	a	a	a	MB	2011
8	41-2	a	A	a	A	A	A	a	a	a	A	MB	2011
9	43-1	a	A	a	A	A	A	a	a	a	a	MB	2011
10	DM65	a	A	a	a	A	A	a	a	a	a	MB	2010
11	DM77	a	A	a	a	A	A	a	a	a	a	MB	2010
12	DM78	A	a	a	A	A	A	a	a	a	nd	MB	2010
13	DM79	a	A	a	a	A	A	a	a	a	A	MB	2010
14	DM81	a	a	a	A	A	A	a	a	a	A	MB	2010
15	DM85	a	A	a	a	A	A	a	a	a	A	MB	2010
16	DM96	a	A	A	a	A	a	a	a	a	A	MB	2010
17	DM118	a	A	A	a	A	a	a	a	a	A	MB	2010
18	J11	a	A	a	A	A	A	a	a	a	a	AB	2010
19	X3	A	a	a	A	A	A	a	a	a	nd	MB	2010

4.3.5.2 Effect of Pydiflumetofen on Canola Blackleg Disease Control under Field Conditions

Since the study conducted under controlled conditions demonstrated the early control of the pathogen, what was considered in the field experiment was the control of disease severity of stem cankers and yield protection led by pydiflumetofen application. Fifty plants from each plot were uprooted, and stems were cut at the stem base. Disease severity was evaluated at maturity stage (https://www.canolacouncil.org/canola-encyclopedia/growth-stages/), 11 weeks after seeding, based on the 0-5 disease severity scale as explained (Zhang et al., 2016).

Seed yields (kg per ha) were taken from plants in 1x1 m^2 area from each plot leaving marginal rows at maturity stage. The number of plants per square meter area in each plot was taken, leaving marginal rows. Plant height in centimeters and number of pods per plant were calculated using 20 and 15 random plants from each plot, respectively.

4.3.6 Data Analysis

4.3.6.1 In vitro Dose-Response Experiment

Mycelial growth of *L. maculans* was modeled to increasing concentrations of pydiflumetofen with a three-parameter sigmoidal function (Equation 1) described by Haanstra et al. (1985) using mixed-model non-linear regression (PROC NLMIXED) adapted from Knezevic et al. (2002). Observations were first divided by the mean mycelial growth of the 0 mg pydiflumetofen L^{-1} treatment (the control) before being fitted to the following model:

[1] $$Y_G = \frac{C}{1+e^{B(\log(X)-\log(A))}}$$

where Y_G is the relative mycelial growth, X the pydiflumetofen concentration, C represents the mycelial growth at 0 g pydiflumetofen concentration, A represents the concentration at which mycelial growth was half of the initial pydiflumetofen concentration (EC50 value). B is a slope parameter that indicates the rate of mycelial growth inhibition. A series of single-degree-of-freedom ESTIMATE statements were constructed to determine differences among the estimates of the EC_{50} value of each *L. maculans* isolate from the sigmoidal model. Parameter estimates were combined when no differences for that parameter among all isolates were detected. The random error term used for the model was the experiment replicates. Initial parameters were optimized by

selecting a set of values minimizing the negative log-likelihood, following a sample code used by Coffey (2016). In brief, a range of probable values was specified for each parameter and increased stepwise within that range. Convergence of the procedure was achieved by utilizing three strategies as needed: (1) abounds statement to keep variance estimates greater than or equal to zero, (2) the relative gradient convergence criterion was set to zero (Kiernan et al., 2012), and (3) the optimization algorithm was set to either quasi-newton, Newton-Raphson, or double-dogleg (SAS Institute 2017). The Akaike information criterion was used to evaluate the fit for each non-linear regression model.

4.3.6.2 Controlled and Field Experiments

Statistical analysis was perfored using SAS 9.4 software (SAS Institute, Carey, NC). In the experiment conducted under a controlled environment, the GLIMMIX procedure was used to test treatment effects for disease severity using the Multinomial (ordered) error distribution with the Cumulative Logit link function. Comparing χ^2 values was used to determine the best model fit. Host genotype, fungicide treatment, and interactions were considered fixed effects, while experimental replication was considered random. ANOVA was conducted within each trial. The Least Squares Means ($p<.05$) were compared using the Tukey-Kramer test. When needed, heterogeneity of variance was corrected using the group statement.

Prior to analysis of field data, residuals were tested for normality for seed yield, the number of pods per plant, plant height, plant stand density using the Shapiro Wilks test in the univariant procedure (PROC UNIVARIATE). Analysis of variance (ANOVA) was used to test treatment effect and their interactions using the mixed procedure (PROC MIXED) of SAS 9.4 for the response variables. The treatment means were not separated as ANOVA was not significant for the above variables. Host genotype, fungicide treatment, site-year, and interactions were considered fixed effects, while experimental replication nested within site-year was considered random. When needed, heterogeneity of variance was corrected using the group statement.

The GLIMMIX procedure was used to test treatment effects and their interactions for disease severity using the beta error distribution and the Cumulative Complementary Log-Log link function with the beta distribution. The model fit was determined by comparing χ^2 values. Host genotype, fungicide treatment, site-year, and interactions were considered fixed effects, while experimental replication nested within site-year and host genotype by fungicide treatment by replication (site-year) were considered random. ANOVA was conducted within each site year. The Least Squares Means ($p < 0.05$) were compared by the Tukey-Kramer test.

4.4 Results

4.4.1 Dose-Response Experiment

Mycelial growth of *L. maculans* was inhibited by increasing concentrations of pydiflumetofen and was required less than 0.4 mg a.i. L^{-1} to reduce the in planta mycelial growth by half (EC50 or 'A' parameter, Table 4.3). This response, however, was unique for each isolate. The D1 (*avrLm1*) isolate was the most resilient among all isolates and required a 9.3-fold increase in pydiflumetofen concentration compared to the most sensitive D5 (*avrLm3*) isolate. For the D7 (*avrLepR2*) isolate, about half the pydiflumetofen was required compared to the D1 and twice as much as the D5 isolate. The rate of mycelial growth inhibition was observed as pydiflumetofen concentration increased (i.e., 'B' parameter) was up to 0.4-times greater in the D1 and D5 isolates than the D7 isolate. No differences were observed among isolates in the initial mycelial growth inhibition rate between the D1 and D5 isolates. Mycelial growth in the 0 mg pydiflumetofen L^{-1} treatment in averaged diameters of 42.7, 39.8, and 67.0 mm for the D1, D5, and D7 isolates, respectively.

Table 4. 3 Parameter Estimates for Three Virulent *Leptosphaeria maculans* Isolates Modelled to an Increasing Concentration of Pydiflumetofen Using a Three-Parameter Sigmoidal Function. Previously collected *L. maculans* isolates D1, D5, and D7 which respectively contained the virulent genes *avrLm1*, *avrLm3,* and *avrLepR2*. Equation 1 describes the sigmoidal function where parameter A represents the concentration of pydiflumetofen at which mycelial growth was half of the 0 mg pydiflumetofen concentration (C_{50} value), parameter B represents the rate of mycelial growth inhibition, and parameter C represents the relative mycelial growth at 0 mg pydiflumetofen concentration. Parameter C was not different among isolates. Standard errors for the parameter estimates are indicated in parentheses.

	Parameter Estimates					
Isolate	**C**		**B**		**A**	
D1	107.3	(2.09)	0.759	(0.049)	0.362[a]	(0.039)[b]
D5	107.3	(2.09)	0.841	(0.061)	0.035	(0.004)
D7	107.3	(2.09)	0.599	(0.037)	0.116	(0.014)

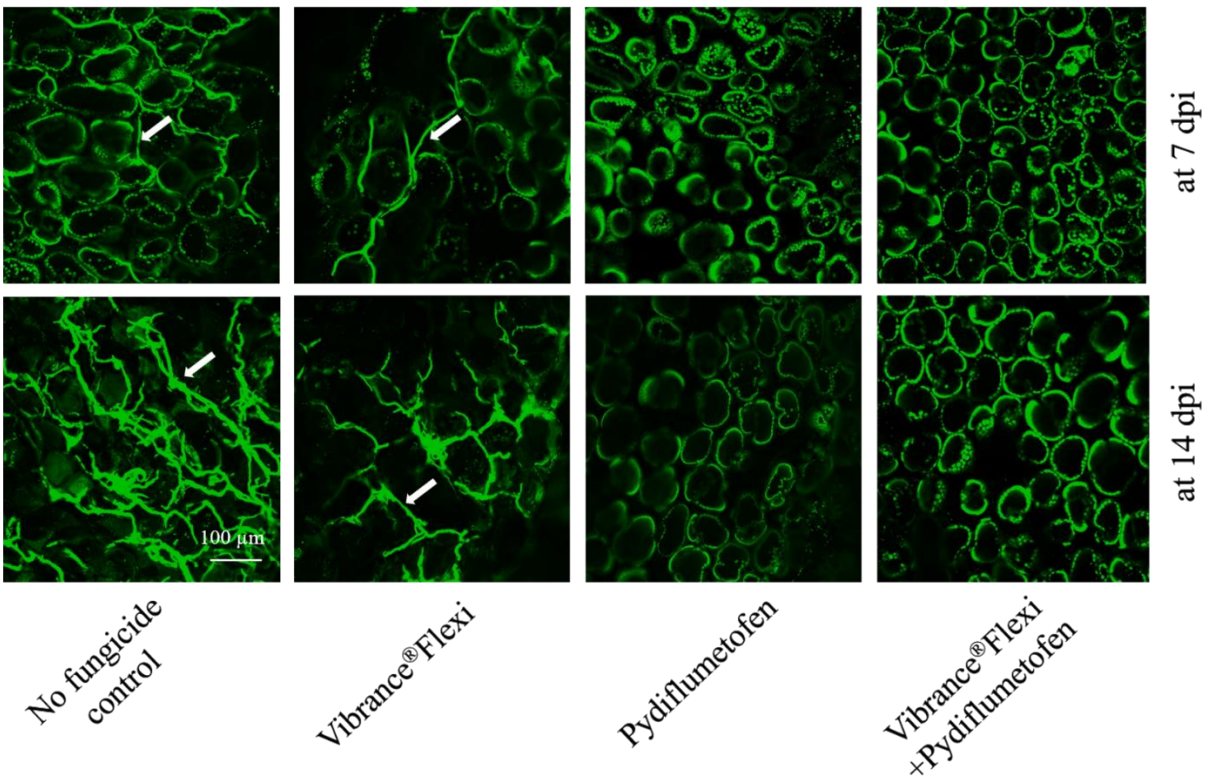
[a] Estimate values b standard error of 'A' parameter for D1 isolate ($P \leq 0.0001$).

4.4.2 Effectiveness of the Seed-Applied Pydiflumetofen under Controlled Environment

4.4.2.1 The Effect of Pydiflumetofen on in situ Mycelial Growth

Out of four treatments used in the study, pydiflumetofen treated alone and accompanied with Vibrance® Flexi showed no mycelial growth in cotyledon apoplasts even after 14 dpi (Figure 1). The control and Vibrance® Flexi alone treated cotyledons clearly showed the GFP signal of *L. maculans* mycelia in cotyledons, as shown in Figure 4.2 starting from seven dpi. At seven dpi, the mycelial growth was higher in cotyledons of the control than cotyledons only treated with Vibrance® Flexi. The above observations were further confirmed by lesions in cotyledons caused by LmGFP (Figure 4.3). The lesion development was not seen in seedlings developed from Pydiflumetofen treated seeds.

(a)


at 7 dpi
at 14 dpi
100 µm
No fungicide control
Vibrance®Flexi
Pydiflumetofen
Vibrance®Flexi +Pydiflumetofen

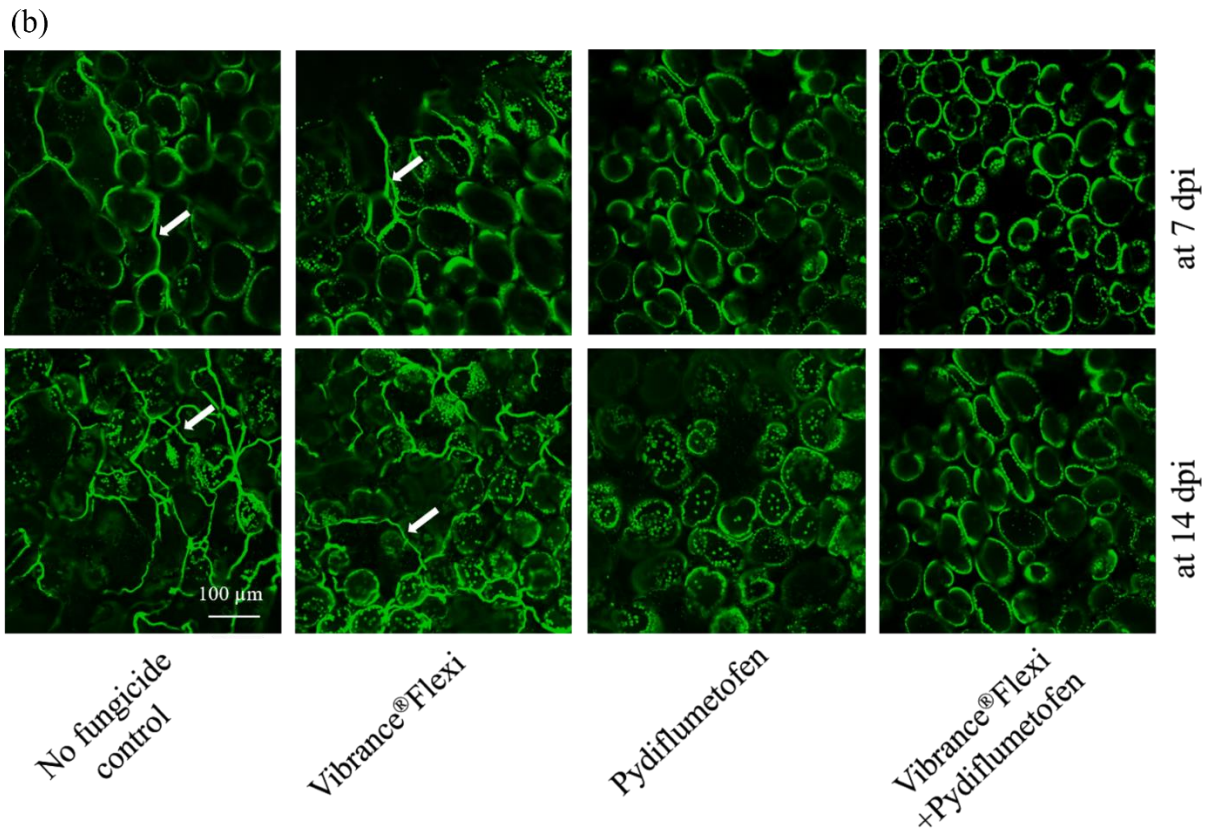


Figure 4. 2 Green Fluorescent Protein-Tagged *Leptosphaeria maculans* Development in Canola Cotyledons Under Controlled Conditions. Green Fluorescent Protein expressed *L. maculans* mycelia was observed in apoplasts of cotyledons treated with no fungicide and Vibrance® Flexi alone treated cotyledons. Observations were done at 7- and 14-dpi. Cotyledons from seeds treated with Pydiflumetofen were wound inoculated with GFP-tagged *L. maculans* isolate. The cotyledons did not show mycelial growth at even 14 days post inoculation. Only chlorophyll autofluorescence was seen in pydiflumetofen treated cotyledons. GFP expressed mycelial growth observed in (a): Westar, and (b): moderately resistant SY4135. Arrowheads have pointed to mycelia.

(a)


No fungicide control
Vibrance®Flexi
Pydiflumetofen
Vibrance®Flexi + Pydiflumetofen
SY4135
Westar

(b)

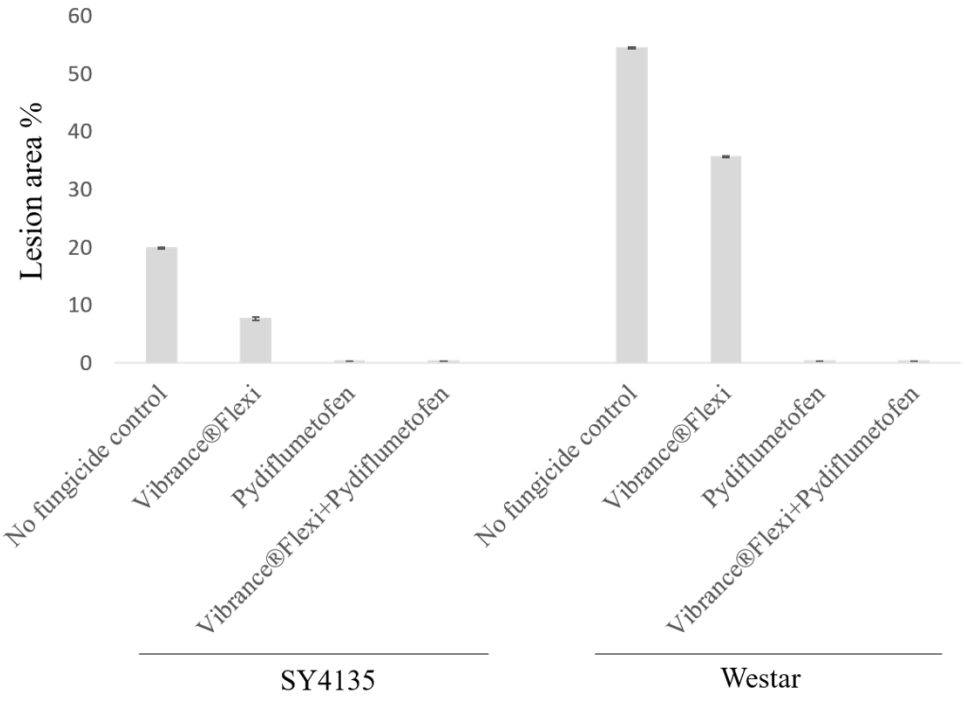

Lesion area %
60
50
40
30
20
10
0
No fungicide control
Vibrance®Flexi
Pydiflumetofen
Vibrance®Flexi+Pydiflumetofen
No fungicide control
Vibrance®Flexi
Pydiflumetofen
Vibrance®Flexi+Pydiflumetofen
SY4135
Westar

Figure 4. 3 *Leptosphaeria maculans* Lesion Development in Canola Cotyledons under Controlled Conditions. Westar and moderately resistant SY4135 cotyledons were wound inoculated with GFP-tagged *L. maculans* isolate. (a): Blackleg disease lesion development on susceptible Westar and moderately resistant SY4135 cotyledons were seen at 14 days post-inoculation in no fungicide control and Vibrance® Flexi alone treated cotyledons. Westar and SY4135 cotyledons from seeds treated with Pydiflumetofen did not show lesion development 14 days post-inoculation. Lesion sizes were more extensive in no fungicide control over the lesions in Vibrance® Flexi alone treated cotyledons. The lesion sizes were larger in Westar cotyledons than in SY4135 cotyledons. (b): Lesion size has shown as a percentage of the total leaf area of both host types calculated by ImageJ.

4.4.2.2 Effect of Pydiflumetofen on Disease Severity

Disease severity was significantly higher in control and in Vibrance® Flexi only treated plants of Westar and SY4135. The disease severity evaluated based on the stem base canker development was significantly higher in plants in control and Vibrance® Flexi only treated plants of Westar and SY4135. Disease severity was significantly lower in both pydiflumetofen used treatments in both host genotypes ($p < 0.05$, $df = 319$) (Figure 4.4).

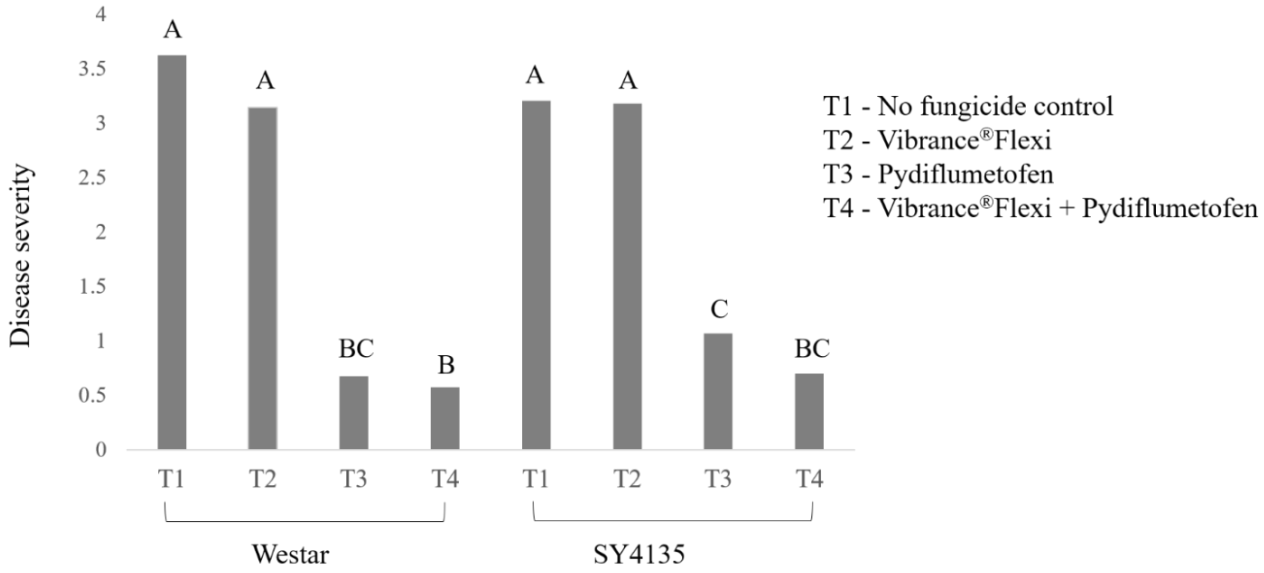


Figure 4. 4 Blackleg Disease Severity on Plant Stems of Westar and SY4135 Plants Developed from Seeds Treated with Three Different Fungicide Seed Treatments and no Fungicide Control under Controlled Conditions. Blackleg disease severity on stems in Westar and SY4135 genotypes were compared among three seed treatment fungicides and no fungicide control and maintained at 22°C/18°C Day and night temperatures and 16 h photoperiod. Disease severity was scored at maturity stage by 0-5 disease severity scale. The four treatments used were no fungicide control, Vibrance® Flexi, pydiflumetofen, and Vibrance® Flexi+ pydiflumetofen. Pydiflumetofen treated cotyledons showed a significantly lower disease severity than other two treatments. Bars with different letters are significantly different based on the Tukey-Kramer test ($p < 0.05$). The graph demonstrates the average results of two experiments.

4.4.3 Evaluation of the Effect of pydiflumetofen under Field Conditions

The drying and lodging of plants were evident in the other four treatments compared to pydiflumetofen treated plots, in which plants looked robust, healthier, greener compared to others in both 2018 and 2019, but the results were better contrasting in 2019 (Figure 4.5; taken on the 1st of August 2019).

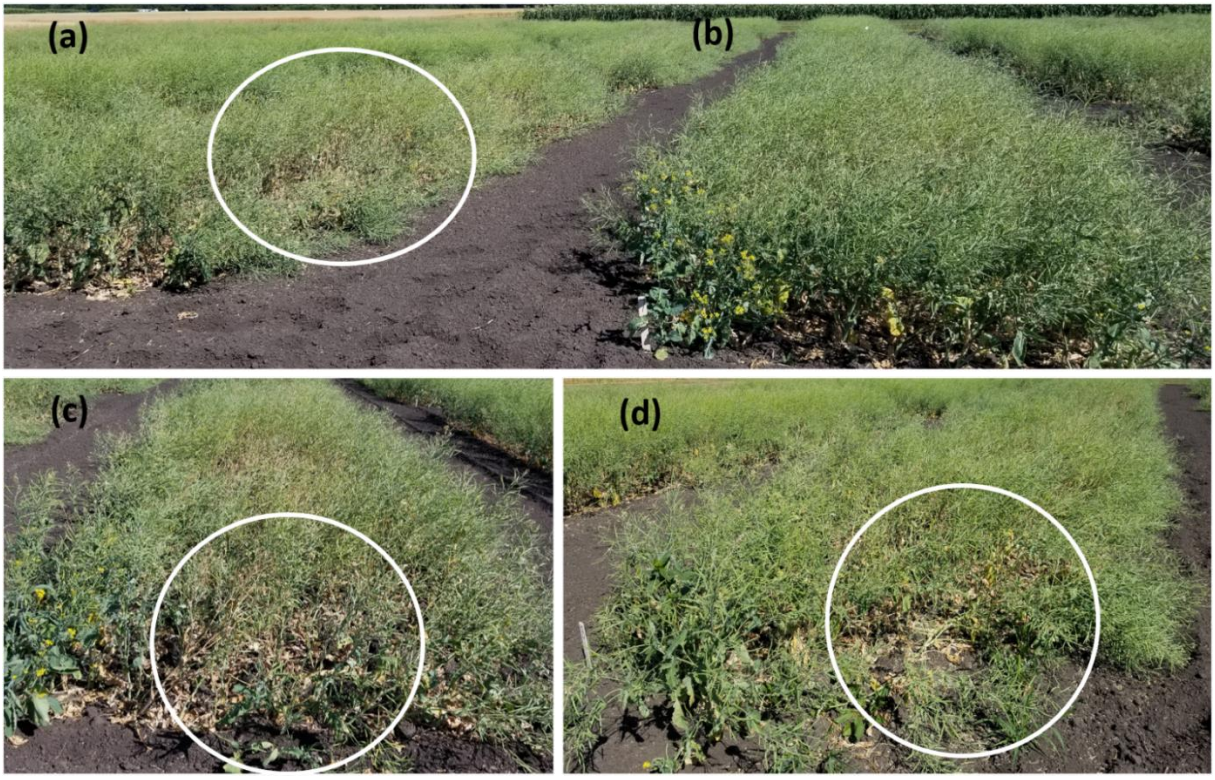


Figure 4. 5 Blackleg Disease Damage of Westar Plants Developed from Seeds Treated with Different Fungicide Seed Treatments under Field Conditions. Westar seeds treated with Helix® Vibrance+Pydiflumetofen, Helix® Vibrance, Prosper® EverGol, BAS 720 FST and no fungicide control used in field experiment. Seedlings were spray inoculated three times with a spore suspension of a mixture of highly abundant 19 *L. maculans* isolates and randomly spreading diseased stubble samples from the previous year. Westar plants grown from seeds treated with pydiflumetofen exhibited a healthier appearance than other treatments considered after two months of seeding in the field (the photos were taken on 1st August 2019). (a): Prosper® EverGol, (b): Helix® Vibrance+Pydiflumetofen, (c): Helix® Vibrance, (d): no fungicide control treated plots have shown.

4.4.3.1 Effect of Pydiflumetofen on Disease Severity

Disease severity assessed using the 0 – 5 disease scale showed the lowest disease severity estimate values in plants developed from pydiflumetofen treated seeds in Westar and SY4135 cultivars. The estimated disease severity in pydiflumetofen treated plants was 1.38, while it was 2.27 in the non-fungicide control ($p < 0.05$). In Westar, disease severity generally was much greater than in SY4135 in both the untreated control and pydiflumetofen treated plants (Figure 4.6).

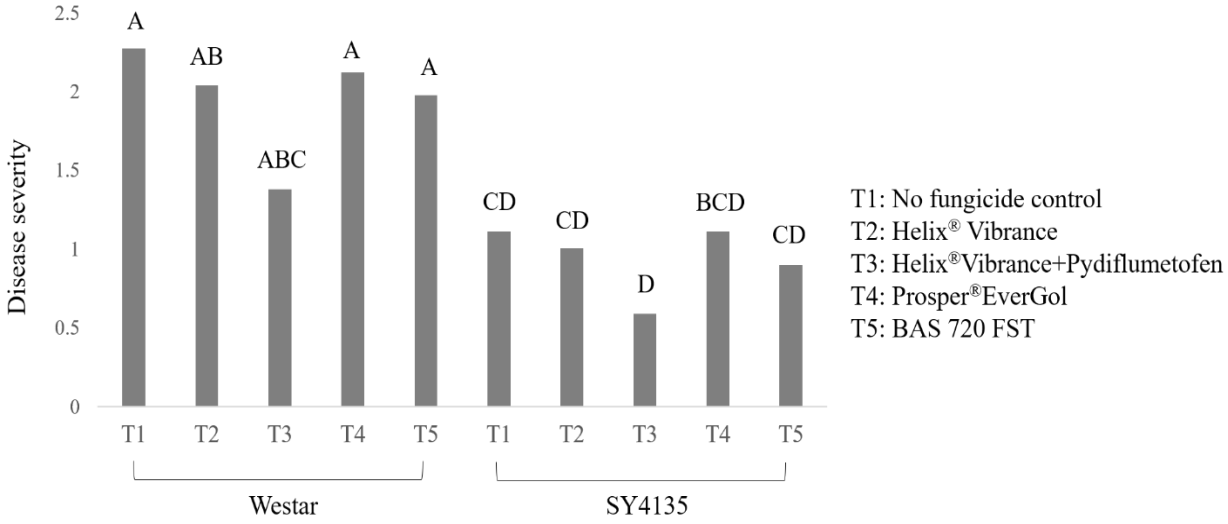


Figure 4. 6 Blackleg Disease Severity on Plant Stems of Westar and SY4135 Plants Developed from Seeds Treated with Four Different Fungicide Seed Treatments and no Fungicide Control under Field Conditions. Westar and SY4135 seeds treated with Helix® Vibrance+Pydiflumetofen, Helix® Vibrance, Prosper® EverGol, BAS 720 FST, and no fungicide control were used in field experiment. The study was performed in a complete randomized block design. Seedlings were sprayed inoculated three times with a spore suspension of a mixture of highly abundant 19 *L. maculans* isolates and random spreading of diseased stubble samples from the previous year. Disease severity assessed using the 0-5 disease scale showed the lowest disease severity estimates in Pydiflumetofen treated plants in both canola cultivars. T1: no fungicide control, T2: Helix® Vibrance, T3: Helix® Vibrance+Pydiflumetofen, T4: Prosper® EverGol, and T5: BAS 720 FST were used as the treatments for the comparative evaluation. The graph represents the average results of the growth year 2018 and 2019. Bars with different letters are significantly different based on the Tukey-Kramer test.

4.4.3.2 Seed Yield Performances

Treatment of SY4315 with pydiflumetofen resulted in the maximum seed yield in this study (1800 kg ha^{-1}). The untreated control and the Prosper treatment resulted in the minimum seed yields, about 25% lower than when treated with pydiflumetofen. Seed yields in SY4315 treated with Helix vibrance and BAS720 FST were intermediate or about 15% greater than the untreated or Prosper treated plants. Seed yield in Westar was much lower in all treatments and ranged from 800 to 950 kg ha^{-1} (Figure 4.7). The number of plants per m^2 area, plant height in cm, and the number of pods per plant were not significant among treatments (data not shown).

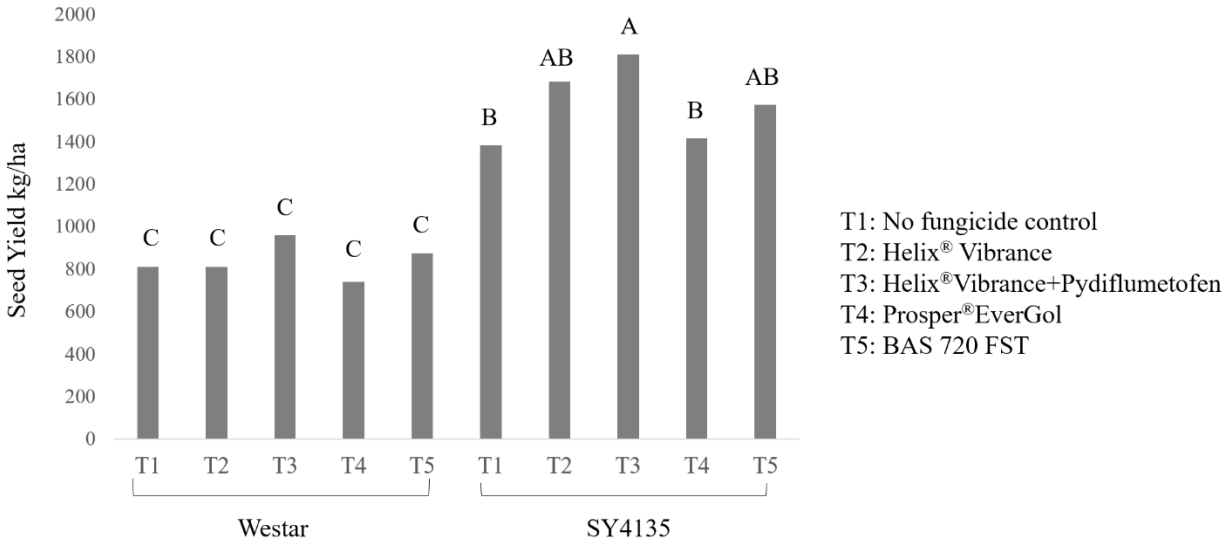


Figure 4. 7 Seed Yield Produced by Westar and SY4135 Plants Developed from Seeds Treated with Four Different Fungicide Seed Treatments along with no Fungicide Control under Field Conditions. Helix® Vibrance+Pydiflumetofen, Helix® Vibrance, Prosper® EverGol, BAS 720 FST, and no fungicide control-treated Westar and SY4135 seeds were used in field experiment. The study was conducted at complete randomized block design. Inoculation of seedlings was done three times of spray inoculation with a spore suspension of a mixture of highly abundant 19 *L. maculans* isolates and by the random spreading of diseased stubble samples from the previous year. Pydiflumetofen treated SY4315 resulted in the greatest seed yield, about 25% higher than the no fungicide control. T1: no fungicide control, T2: Helix® Vibrance, T3: Helix® Vibrance + Pydiflumetofen, T4: Prosper® EverGol, and T5: BAS 720 FST. The graph represents the average results of the growth year 2018 and 2019.

4.5 Discussion

This chapter reports a novel seed applied fungicide for canola that showed a successful control of canola blackleg disease severity, compared to the no fungicide control and selected competitive fungicides currently available in the market for canola blackleg disease. Fluorescence protein studies explicit successful control of the pathogen development by the novel seed treatment in cotyledons grown under the controlled environment.

Pydiflumetofen is a broad-spectrum seed-applied fungicide with SDHI mode of action. Currently available foliar fungicides would not be financially feasible for controlling blackleg disease in canola, which may not be true in some situations depending on the commodity market and pricing. However, more important from an agronomic perspective, since blackleg infection can occur as early as emergence, i.e., cotyledon stage, the timing for foliar fungicide application is a challenge. Moreover, first emergence in canola (commercial fields) rarely occurs simultaneously (Peng et al., 2020). In 2019, soil moisture and rainfall were inconsistent across the prairies; hence the emergence of canola was very uneven (https://www.weather.gov/pub/climate2019SummerReviewFallPreview, accessed the 20th of September 2021). Under such conditions, seed-applied fungicides have a special advantage since the active ingredients are translocated into the seedlings right after germination, protecting the two- to three-leaf stage regardless of when the seedlings finally emerge from the soil. Therefore, seed-applied fungicides will provide consistent results under all growing conditions regardless of when individual seedlings emerge and are vulnerable to an air-borne infection of blackleg.

Pydiflumetofen is recommended to apply along with Helix® Vibrance as same as applied in the field experiments performed. Helix® Vibrance was proven earlier to be effective in controlling seed-borne pathogens and flea beetles, which was a pest threat in canola fields. Pydiflumetofen is a seed-applied fungicide to affect the plant's two- to four-leaf stage. Guo et al., (2008) explained that the two- to four-leaf stage is the most critical in plant life span as the infection that happens during this period can lead to a significant yield reduction. On the other hand, seeds are not well colonized by *L. maculans* (Fernando et al., 2016). Therefore, Pydiflumetofen gives that protection at its best.

Based on the results in the dose-response experiments, both the most sensitive *L. maculans* isolate D5 and the least sensitive isolate D1 to pydiflumetofen were evaluated. That confirms that the standard concentration of pydiflumetofen used to treat the seeds was sufficient to control the

pathogen isolates in a broad range of sensitivity. The radioactive labeling study conducted by Syngenta group Australia showed the effect of pydiflumetofen is stable up to two- to four-leaf stage of the plant (https://www.syngenta.com.au/seedcare). After the six-leaf stage, the effect gets diminished. Furthermore, they observed that this systemic chemical translocation happens throughout the plant via the vascular bundle. This study focused on ensuring the effectiveness of pydiflumetofen on controlling blackleg inoculum under controlled environment and field conditions.

Evaluation of baseline in vitro sensitivity to pydiflumetofen is essential to determine the EC50 value of the fungicide, which is the efficacious concentration to reduce the growth by 50% of the pathogen population (Breunig and Chilvers, 2021). Typically, the EC50 is important in advising on growers' management decisions. Furthermore, establishing sensitivity monitoring programs for the pathogen is necessary to improve our understanding of epidemiological and biological aspects of the target pathogen (Reis et al., 2015). Since we analyzed the EC50 values only for three virulent isolates in Canadian prairies, it is important to test additional isolates in the MB and other canola growing provinces to make suggestions get confirmed. Since the results demonstrated a range in susceptibility for the fungicide, it is advisable to investigate for mutants of SDH genes in the pathogen population to see any resistant strains.

The fungicide effect on the pathogen was always evaluated based on the agronomic, physiological, and biochemical changes occurring in infected plants (Lamichhane et al., 2020; Peng et al., 2021). This study focused on the direct detection of the effect of the fungicide on the pathogen itself. The GFP-tagged *L. maculans* isolate approach was used to detect the fungal mycelial growth *in planta*. The apoplastic growth of fungal mycelia was clearly observed in cotyledons treated only with Vibrance® Flexi and fungicide non-treated seedlings. As shown by Becker et al., (2017) by Laser Capture Microdissection studies, the lesion development on cotyledons initiates at seven dpi in a compatible interaction. Once the fungus is established within the cotyledons, the lesion symptoms appear. Therefore, the efficacy of pydiflumetofen in controlling the fungus in the host was clearly exhibited by suppressing the growth of apoplastic mycelia even after 14 dpi. By electron microscope transmission studies, Bai et al., (2021) revealed pydiflumetofen damages mycelial cell wall and cell membrane and breaks up cells leading to intracellular plasma leakage of *F. fujikuroi* in rice. Most interestingly, Westar did not have mycelial development, which contains no R genes or no known quantitative resistance, simulating

the worst-case scenario (Balesdent et al., 2005). The isolate was D3, one of the most virulent isolates available in our lab isolate collection (Zhang et al., 2016). The above observations confirm pydiflumetofen could defeat the pathogen without even any involvement of host genetic makeup.

In addition, to the direct proof of pydiflumetofen on the pathogen itself, there were several indirect pieces of evidence exhibited by the host plant to confirm the efficacy of pydiflumetofen on the blackleg pathogen. Disease severity reduction was statistically significant in pydiflumetofen treated cotyledons under the controlled environment. Under controlled environment, despite pydiflumetofen being applied individually or in combination with Vibrance® Flexi, pydiflumetofen was significant in controlling the disease severity in stem base canker development.

Under field conditions, the disease severity reduction was exhibited in estimated values in both host genotypes. The disease severity value was one or less in pydiflumetofen treated plants, while others showed more than three disease severity values. As explained in Peng et al., (2020), the effect of the fungicide was more reducing pathogen damage than stopping it. That is explained by the disease severity reduction in two host genotypes that were not statistically different (Peng et al., 2020).

The year 2018 was dry and exhibited a low level of disease, and therefore the opportunity to evaluate the fungicide efficacy was less (https://www.agcanada.com/daily/droughts-effects-linger-on-prairies, reviewed on the 18th of January 2020). However, the 2019 growing season was better by means of disease development. Three times of spray inoculation and spreading of diseased stubble from the previous year are recommended to ensure a high disease pressure as all three inoculations were made sure to get done before ending two- to four-leaf stage until the effect of seed treated pydiflumetofen will remain. On the other hand, the canola plant's two- to four-leaf stage is the most critical stage during which infection caused can lead to a significant yield loss (Guo et al., 2008).

In contrast to the controlled environment, field-grown plants have no control over the persistent infection by the pathogen. However, in the controlled environment, the inoculation was done by hand inoculation on each plant, which was not done in the field study. That would be why the disease severity observed in the controlled environment was higher than that in the field study. Furthermore, there was a statistically significant difference between the disease severity in Westar and SY4135. That could be due to the activation of quantitative resistance in MR SY4135 against

the infection at later stages after the effect of the seed treatment is diminished, and which was lacking in Westar plants (Balesdent et al., 2005). However, the disease severity reduction under field study observed by GLIMMIX statistical analysis was not statistically significant. The average rating for the treatment was converted to a percentage and did the stats on that using the beta distribution, which would be correct for that type of data. The normal distribution is not the best choice (top) for this data as the data constrained at both ends. However, the estimates of disease severity in Westar and SY4135 plants treated with pydiflumetofen were 1.38 and 0.59 ($p \leq 0.05$), respectively. While the no fungicide control and other three fungicide treatments: Helix® Vibrance, Prosper® EverGol, and BAS 720 FST exhibited 2.28, 2.04, 2.00, and 1.94 of disease severity, respectively in Westar plants and 1.10, 1.00, 1.11, and 0.90 of disease severity, respectively in SY4135 plants. The reduction observed in disease severity in pydiflumetofen treated plants compared to other plants confirmed the efficacy of pydiflumetofen as a successful seed treatment to manage the blackleg pathogen and the significance of protecting two- to four-leaf stage to mitigate this devastating disease as explained by Guo et al. (2008).

The agronomic measurements observed did not show a statistically significant difference among treatments as they are not directly related to canola disease severity. Since the ultimate outcome of the crop is the yield, without assessing the effect of the fungicides used on seed yield, it would not be a complete story. Marcroft and Potter (2008) observed that the seed dressing, fluquinconazole, was effective and gave an economic return under extreme disease severity with lower and moderate host genotype resistance. In our case SY4315 treated with pydiflumetofen could result in about 25% more seed yield compared to the control though Westar could not. There could be several reasons behind these observations. The disease severity reduction gained by seed treatment was insufficient to give rise in seed yield (Hwang et al., 2016). On the other hand, this might be due to the yield relies not only on the disease infections but on many physiological factors. However, the results showed the protection at early stage, from cotyledon to four true-leaf stage, leads to less disease severity on stems and higher seed yield estimates at the end.

4.6 Conclusion

As the results confirmed, this novel SDHI seed treatment, pydiflumetofen is not acceptable as a top management tactic which can use solely to control the blackleg pathogen. However, due to the potential of protecting the plant at critical window for infection, pydiflumetofen can be identified as a worthy addition to farmers' integrated management toolbox to mitigate this devastating pathogen *L. maculans* from canola fields. It will help rapidly reduce the emergence of new races, reduce disease in the field, and mitigate trade barriers on seed due to the blackleg-pathogen.

Integrated management of the disease would be more successful when the system is rich in different strategies. The introduction of biological control agent would be another potential addition to the blackleg disease management toolbox.

Less Virulent *Leptosphaeria biglobosa* Immunizes the Canola Plant to Resist Highly Virulent *L. maculans*, the Blackleg Pathogen

K. Rasanie E. Padmathilake[1] and W. G. Dilantha Fernando[1,*]

[1]Department of Plant Science, University of Manitoba, Winnipeg, MB R3T2N2, Canada 1; Dilantha.Fernando@umanitoba.ca
*Correspondence: Dilantha.Fernando@umanitoba.ca

This manuscript was published in Plants: Padmathilake KRE, Fernando WGD. Less Virulent *Leptosphaeria biglobosa* Immunizes the Canola Plant to Resist Highly Virulent *L. maculans*, the Blackleg Pathogen. Plants. 2022; 11(7):996. https://doi.org/10.3390/plants11070996

Author Contributions: W.G.D.F. and K.R.E.P. designed the experiments. K.R.E.P. performed the experiments and analyzed the data. K.R.E.P. wrote the manuscript and W.G.D.F. revised the manuscript. Funding acquisition was done by W.G.D.F. All authors have read and agreed to the published version of the manuscript.

CHAPTER 5. CAN LESS VIRULENT *LEPTOSPHAERIA BIGLOBOSA* IMMUNIZE THE CANOLA PLANT TO RESIST HIGHLY VIRULENT *L. MACULANS*?

5.1 Abstract

Leptosphaeria biglobosa is a less virulent *Leptosphaeria* spp. that causes blackleg disease in canola. Previous studies from our lab have shown that inoculation with the less virulent *L. biglobosa* can boost the resistance of canola plants against the highly virulent *L. maculans*. The objective of this study was to confirm the effectiveness of *L. biglobosa* as a biocontrol agent against *L. maculans* utilizing morphology, fluorescence microscopy, gene quantification, and transcriptomic analysis. The in planta development of two *Leptosphaeria* species inoculated at different time points was assessed using fluorescent protein-tagged isolates which are GFP-tagged *L. maculans* and DsRed-tagged *L. biglobosa*. The growth inhibition of *L. maculans* by pre-and co-inoculated *L. biglobosa* was supported by no lesion development on cotyledons and no or weak fluorescence protein-tagged mycelia under the confocal microscope. The host defense-related genes, *WRKY33*, *PR1*, *APX6*, and *CHI*, were upregulated in *L. biglobosa* inoculated Westar cotyledons compared to *L. maculans* inoculated cotyledons. The quantification of each pathogen through qPCR assay and gene expressions analysis on host defense-related genes by RT-qPCR confirmed the potential of *L. biglobosa* 'brassicae' in the management of the blackleg disease pathogen, *L. maculans* 'brassicae', in canola.

5.2 Introduction

Blackleg is one of the most destructive diseases in canola (*Brassica napus* L.) growing areas and causes more than CAD $ 900 million annual loss per growing season worldwide (Zhang and Fernando, 2018). Two *Leptosphaeria* species are associated with blackleg (Mendes-Pereira et al., 2003), and they are *Leptosphaeria maculans (Desm.) Ces. & de Not. (anamorph: Phoma lingam (Tode ex Fr.) Desm.)* and *L. biglobosa* Shoemaker & Brun (anamorph = *P. biglobosus*) (Williams and Fitt 1999; Rouxel and Balesdent 2005). *L. maculans* isolates have been categorized into two sub clades 'brassicae' and 'lepidii', while *L. biglobosa* have been categorized into seven isolate groups, and they are 'brassicae', 'canadensis', 'thlaspii', 'erysimii', 'australensis', 'occiaustralensis' and 'americensis' respectively (Mendes-Pereira et at., 2003; Vincenot et al., 2008; Zou et al., 2019). *L. biglobosa* 'brassicae' is the most common species and has been found in most canola growing regions (Liu et al., 2014). These species co-exist in most *B. napus* growing areas of the world (West et al., 1999).

L. maculans and *L. biglobosa* were described as virulent and weakly virulent strains, respectively (West et al., 2001). Although *L. maculans* is more aggressive than *L. biglobosa*, their life cycles are similar (Paul and Rawlinson 1992). The two species are genetically different and exhibit distinct disease symptoms on the canola plant (West et al., 2001). *L. maculans* produces grey lesions with dark brown margins and black color pycnidia. The pathogen moves towards the stem through leaf petiole to the stem base and causes the main characteristic symptom of the disease, blackened stem base canker, which was the reason for the name of the disease 'blackleg'. *L. biglobosa* caused leaf lesions to be smaller, darker, and confined to the upper stem (Fitt et al., 2006). *L. maculans* is a hemibiotrophic fungus, while *L. biglobosa* is a necrotrophic fungus with a comparatively shorter biotrophic stage in its life cycle. Furthermore, *L. biglobosa* is known to start its expression of hydrolases, a characteristic for necrotrophic stage earlier than *L. maculans* (Lowe et al., 2014).

According to Grandaubert et al. (2014), species-specific genes of the two species did not exhibit attributed function. In contrast, genes with functions related to causing disease (e.g. CAZys and proteases) were conserved between the two species. *L. maculans* species contain larger AT-rich isochores compared to *L. biglobosa.* Grandaubert et al. (2014) further explained the presence of species-specific effector genes in AT-rich isochores of *L. maculans* 'brassicae'species which was very few in *L. biglobosa* 'brassicae'. Therefore, they suggested that the concerted expression

of these species-specific SSPs in *L. maculans* suppress the host PTI, which was less or lack in *L. biglobosa* species leading *L. maculans* to be virulent and *L. biglobosa* to be weakly virulent.

Several studies demonstrated the resistance induction of the canola plant by *L. biglobosa* and suppression of *L. maculans* infection when *L. biglobosa* was pre- or co-inoculated with *L. maculans*. This suppression may be due to 1) the competition arising between the two species during infection and colonization within the host plant and 2) the induction of resistance in the host plant (Mahuku et al., 1996; Chen and Fernando 2006). The enhanced plant defense in the host activated by biotic or abiotic factors is induced resistance (Baker, 2000). Induction of disease resistance by pre- or co-inoculation with an avirulent or weakly virulent strain has been shown against many pathogenic strains and is known to be a broad-spectrum defense that protects the host from subsequent infections. For instance, hampering of control strategies against the virulent *F. graminearum* in the disease complex causes FHB by the presence of the weakly pathogenic *F. poae* (Tan et al., 2021) and tomato biocontrol activity of the *F. oxysporum* Fo47 endophyte against the pathogen *F. oxysporum* f. sp. *lycopersici* causing tomato wilt disease (de Lamo et al., 2021). The activated defense responses include 1) oxidative burst, which can cause cell death by which restricting the pathogen at the inoculation site (Heath 1998), and 2) changes in cell wall composition in inhibiting pathogen penetration and secondary metabolite production, which can act as antimicrobial compounds (Hammerschmidt 1999).

Studying defense-related gene expressions is expected in the investigation of different plant-pathogen interactions. Phytohormones, such as salicylic acid (SA), ethylene (ET), and jasmonic acid (JA), coordinate defense responses by activation of defense-related gene expressions. SA-mediated defense response plays a central role in local and systemic acquiredsystemic acquired resistance (SAR) against biotrophic pathogens. The ET/JA-mediated response contributes to the defense against necrotrophic pathogens. SA target gene *PR1* and the JA marker gene *PDF1.2* (Li et al., 2019; Yang and Fernando 2021). The respiratory burst oxidase homologues (RBOHs) are a major source of reactive oxygen species (ROS) during plant-microbe interactions (Otulak-Kozieł et al., 2020). On the other hand, Ascorbate peroxidases (APXs) are another critical group of enzymes adjusting ROS levels in the cells (Sharma et al., 2012). Furthermore, transcription factors play important roles in defense responses. Transcription factor WRKY33 is essential for defense toward the necrotrophic pathogens (Birkenbihl et al., 2012), and

plant-specific transcription factor WRKY70 is a common component in SA- and JA-mediated signal pathways (Li et al., 2004).

The focus of this study was to investigate the potential of *L. biglobosa* in inducing canola plant resistance against the virulent species *L. maculans* through a combination of (1) assessment outer lesion developments, (2) confocal microscopic observations of fluorescent protein-tagged isolate growth *in planta*, and (3) quantification of each isolate development by qPCR and (4) defense-related host gene expressions by RT-qPCR.

5.3 Material and Methods

5.3.1 Pathogen Isolates

GFP tagged *L. maculans* 'brassicae' isolate (refers to LmGFP) and red fluorescent protein, DsRed, tagged *L. biglobosa* 'brassicae' (refers to LbDsRed) received from Dr. Kim Hammond-Kosack group (Eckert et al., 2005) were used in this study. The isolates were grown in V8 agar plates and maintained under a light bank for 24 h period at 20°C ± 2°C. After 21 days, the mycelial cultures with pycnidia were harvested by flooding culture plates with sterilized distilled water and scraping the fungal cultures gently by a heat sterilized spatula. Then, two layers of Mira-cloth-filtered pycnidiospore suspension were adjusted to a final concentration of 2 x 10^7 pycnidiospores /mL using a hemocytometer.

5.3.2 Plant Growth and Inoculation

Susceptible *B. napus* cultivar, Westar, with no known *R* genes or quantitative resistance (Hubbard and Peng 2018) was grown in Sunshine mix #4 (SunGro Horticulture, http://www.sungro.com/).), under 16 h photoperiod (18 ℃ dark and 21 ℃ light) in a growth chamber at the Department of Plant Science, University of Manitoba. The cotyledons of seven-day-old seedlings were needle-punctured as one per lobe and inoculated with 10 µL of pycnidia suspension of each isolate or sterile distilled water as mock, respectively. Five different inocula types were used in addition to the control (mock) as Treatment 1: LbDsRed inoculation; Treatment 2: LmGFP inoculation; Treatment 3: LbDsRed and LmGFP co-inoculation; Treatment 4: LbDsRed followed by LmGFP inoculation (in 24 h interval); Treatment 5: LmGFP followed by

LbDsRed inoculation (in 24 h interval). In treatment 3, *L. maculans* and *L. biglobosa* spore suspensions were mixed in equal volumes to prepare the co-inoculum (Figure 5.1). For treatments 3 and 4, the first inoculation was followed by another pinching of the same site to do the second inoculation as 24 hours of callus formation of the pinched site acts as a barrier for the second inoculation (Ikeuchi et al., 2017).

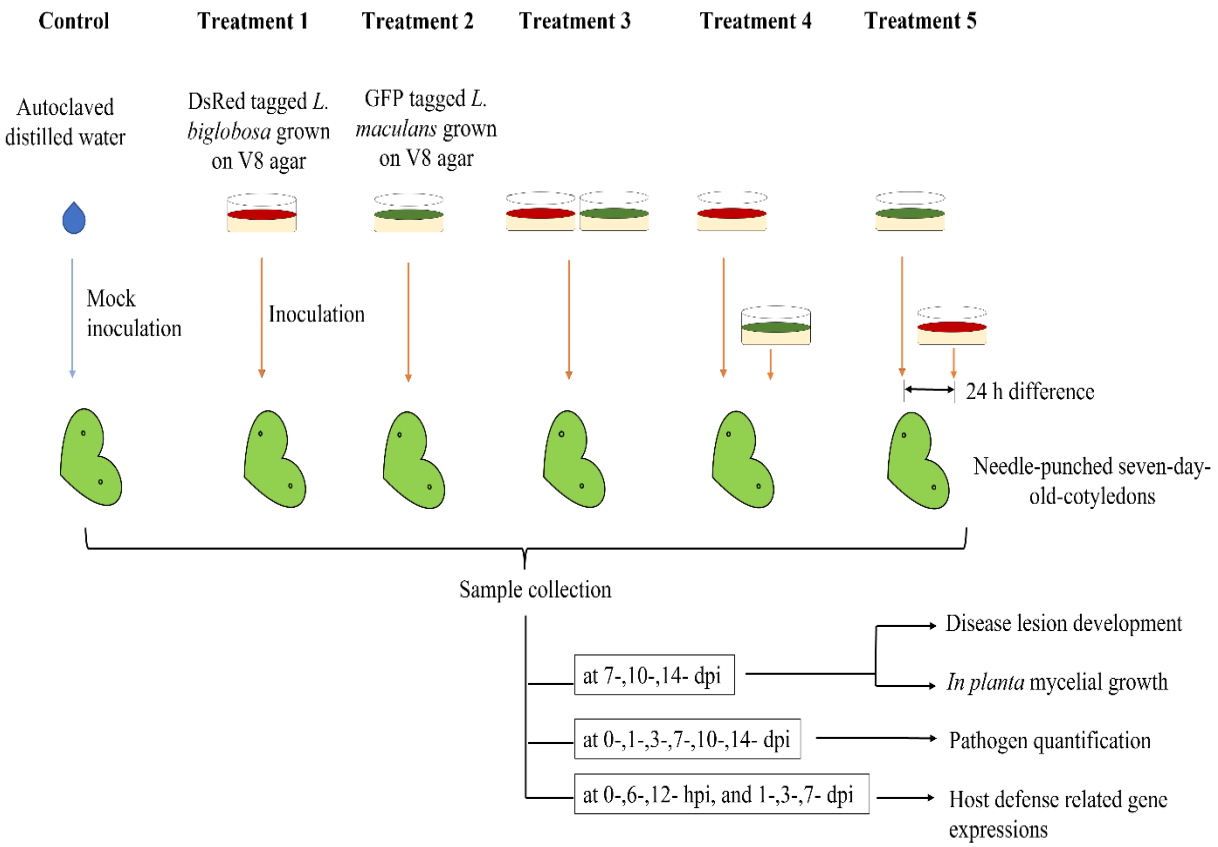


Figure 5. 1 Schematic Representation of the Research Design. Seven-day-old Westar cotyledons were wound inoculated with Control: distilled water treatment; Treatment 1: LbDsRed inoculation; Treatment 2: LmGFP inoculation; Treatment 3: LbDsRed and LmGFP co-inoculation; Treatment 4: LbDsRed followed by LmGFP inoculation (in 24 h interval); Treatment 5: LmGFP followed by LbDsRed inoculation (in 24 h interval). Inoculated cotyledons were collected at different timepoints to analyze the lesion development, *in planta* mycelial development, pathogen quantification and host defense related gene expressions.

5.3.4 Disease Lesion Development on Cotyledons

Cotyledon lesion developments in each treatment were observed at 7-, 10-, and 14- days post inoculation (dpi) and the lesion development were calculated as percentage of the total cotyledon area by ImageJ software (Schneider et al., 2012).

5.3.5 Confocal Observation of *in Planta* Mycelial Development

The collected cotyledons from each treatment at 7-, 10- and 14- dpi were observed by laser confocal LSM 700 microscope (LSM 700; Zeiss, Jena, Germany). At seven dpi cotyledon pieces of 2 mm x 2 mm size were cut 2 mm away from the pinched wounds made on cotyledons. At 10- and 14- dpi, the cut pieces of cotyledons with lesions were taken two millimeters away from the margin of disease lesions to ensure of having the growing fungus. The cut pieces were water mounted on slides. The cut pieces were water mounted on slides, and GFP tagged *L. maculans* mycelia and DsRed tagged *L. biglobosa* development were observed by laser confocal LSM 700 microscope. LmGFP was imaged using the excitation of BP 470/40 nm and emission of BP 525/50, and LbDsRed was visualized with the excitation of BP 550/25 nm and emission of BP 605/70. The study was repeated two times.

5.3.6 PCR identification of *Leptosphaeria maculans* 'brassicae' and *L. biglobosa* 'brassicae' Isolates in Inoculated Cotyledons

Cotyledons of LmGFP and LbDsRed separately and co-inoculated were collected at seven dpi as two cotyledons per replicate for three replicates. Genomic DNA extraction from inoculated Westar cotyledons was performed using the CTAB method that Liban et al., (2016) explained with modifications. Freeze-dried cotyledons were grounded into a fine powder and mixed with lysis buffer (Tris, EDTA, CTAB, and NaCl), incubated at 65°C for 20 min, extracted with phenol: chloroform: isoamyl alcohol (25:24:1), and precipitated with 5M NaCl and 95% ethanol, and washed by 70% ethanol twice. The DNA pellet was dissolved in 100 µL sterile distilled water following the final centrifugation.

Species-specific primers LmacF/LmacR ((LmacF, 5'-CTT GCC CAC CAA TTG GAT CCC CTA -3'; LmacR, 5'-GCA AAA TGT GCT GCG CTC CAGG-3' for *L. maculans*) and

LbigF/LmacR (LbigF, 5'-ATC AGG GGA TTG GTG TCA GCA GTT GA-3'; LmacR, 5'-GCA AAA TGT GCT GCG CTC CAGG-3'for *L. biglobosa*) were used for PCR identification of the isolates as described by Liu et al., (2006). PCRs were performed in 20 µL volumes, made up of 10 µL of 2x Taq Mix (FroggaBio), with 0.8 µL of each primer (10 mM) and 100 ng total of sample gDNA and sterile distilled water to volume up. PCR was performed on a T100 Thermal cycler (Bio-Rad), and the amplification program set was an initial denaturation period of 95°C for 2 min, followed by 30 cycles of 95°C for 15 s, 70°C for 30 s and 72°C for 1 min, followed by an additional primer-extension period of 72°C for 10 min. PCR products were resolved by electrophoresis through a 1% (w/v) agarose gel.

5.3.7 Quantification of Pathogen Isolates Using qPCR

Fungal suspensions of both isolates of LmGFP and LbDsRed with mycelia, pycnidia, and pycnidiospores were collected from a single spore grown V8 agar plate of each isolate in sterile distilled water. Genomic DNA of fungal suspensions was extracted using the modified CTAB method, as Liban et al., (2016) described with minor modifications. The extracted procedure followed was the same as explained above except initial steps. The fungal suspensions were centrifuged to remove out the aqueous portion of fungal biomass. The samples were mixed with a lysis buffer and lysed with 5-8 ceramic beads at 10,000 rpm for one min. The rest of the procedure was the same as described above.

Cotyledons of each treatment were collected at 0-, 1-, 3-, 7-, 11- dpi as two cotyledons per replicate for three replicates. Genomic DNA extraction was extracted from inoculated Westar cotyledons as described above. The amounts of DNA of *L. maculans* and *L. biglobosa* present in each pure fungal pathogen samples and pathogen inoculated cotyledon samples were quantified using SYBR Green qPCR with species-specific primer pairs LmacF/LmacR and LbigF/LmacR were used for *L. maculans* and *L. biglobosa*, respectively as described by Liu et al., (2006). The qPCR reaction mixtures were prepared to a total volume of 20 µL made up of 10 µL of Bio-Rad CFX Connect™ Real-Time System with SYBR® Green Supermix (Bio-Rad, USA), 0.8 µL (10 mM) of forward primer, and 0.8 µL (10 mM) of reverse primer, 100 ng total of sample gDNA and sterile distilled water to volume up as per manufacturer's instructions.

PCRs were performed with the amplification programs set as follows: 50°C for 2 min, 95°C for 3 min, 40 cycles of 95°C for 5 s, 60°C for 10 s and 72°C for 30 s. Melting curve analysis

was performed by increasing 0.5°C at 3 s/step from 55 to 95°C. In each qPCR run, a standard dilution series consisting of 10000, 1000, 100, 10, and 1 pg of DNA of *L. maculans* "brassicae" or *L. biglobosa* "brassicae" pure culture was used to produce a standard curve. The amount of *L. maculans* DNA or *L. biglobosa* DNA for each inoculated cotyledon sample was estimated using the standard curve. Results were expressed as the amount (pg) of *L. maculans* or *L. biglobosa* DNA in 100 ng total DNA from diseased plant tissue. The study was repeated two times (Huang et al., 2014).

5.3.8 Defense Related Gene Expression

Inoculated cotyledons collected at 0-, 6-, 12- hour post inoculation (hpi), and 1-, 3-, 7- dpi were promptly frozen in liquid nitrogen. Frozen cotyledons were homogenized in liquid nitrogen with a sterilized porcelain mortar and pestle. Plant total RNA was extracted with PureLink® Plant RNA Reagent (Invitrogen, Carlsbad, CA) and treated with a TURBO DNA-free™ Kit (Ambion, https://www.thermofisher.com) according to the manufacturer's instructions. Reverse transcription of the first-strand cDNA was achieved using the 1st cDNA Synthesis Kit (Thermo Scientific, Waltham, MA) with one μg total RNA. For the 10 μL of total qPCR volume, 2.1 μL of 100-fold diluted cDNA, five μL of Bio-Rad CFX Connect™ Real Time System with SYBR® Green Supermix (Bio-Rad, USA), and 0.4 μL of each primer (10 mM; defense related genes and used primers have shown in Table 5.1) were used for PCR reaction. Actin was used as the reference gene in the analysis. Real time quantitative PCR was performed on a CFX96 Real-Time Instrument (Bio-Rad) with the amplification program of 95°C for 2 min, 40 cycles of 95°C for 5 s, 60°C for 10 s, and 72°C for 30 s. Melting curve analysis was performed by increasing 0.5°C at 3 s/step from 55 to 95°C. The list of primers used have shown in Table 5.1 (based on Zhang 2016; Yang 2021). The relative gene expression level was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The study was repeated two times.

Table 5.1 List of Selected Defense Related Genes in Canola Used in Gene Expression Analyses and the Primer Pairs Used for each Gene

Gene	Full name	Defense Signaling Pathway	Forward Primer (5'3')	Reverse Primer (5'3')
WRKY70	WRKY transcription factor 70	Salicyclic acid signaling pathway	ACATACATAGGAAACCACACG	ACTTGGACTATCTTCAGAATGC
PR1	Pathogenesis-related protein 1	Salicyclic acid pathway	GGCTAACTATAACCACGATTC	GTTCCACCATTGTTACACC
WRKY33	WRKY transcription factor 33	Jasmonic acid signaling pathway	TGTCGGACAGCTTGGGAAAG	AGAGGACGGTTACAACTGGAGAAA
PDF1.2	Plant defensin 1.2	Ethylene and Jasmonic acid pathway	AAATGCTTCCTGCGACAACG	AGTCCACGTCTCCGATCTCT
RbohD	Respiratory burst oxidase homolog protein D	Reactive Oxygen Species Production	TATCCTCAAGGACATCATCAG	TATCCTCAAGGACATCATCAG
APX6	Ascorbate peroxidase	Reactive Oxygen Species Scavenging	AGTTCGTAGCTGCTAAATATT	GGAGTTGTTATTACCAAGAAA
CHI	Chitinase	Pathogen chitin degradation	TGCTACATAGAAGAAATAAACGG	TTCCATGATAGTTGAATCGG
Actin	Actin	Reference gene used in the assay	CTGGAATTGCTGACCGTATGAG	GTTGGAAAGTGCTGAGGGATG

5.3.9 Statistical Analysis

Unless specified, the analyses of samples used three biological replicates. statistical analyses were performed using SAS 9.4 software. The data were log-transformed to maintain the homogeneity and normality of residuals. Analysis of variance (ANOVA) was conducted using the MIXED model to determine the significance of the treatments. The Fisher's the least significant difference (LSD) at the 0.05 probability level was applied to percentage lesion development, defense gene expression and DNA quantity of each isolate.

5.4 Results

5.4.1 Disease Development in Cotyledons

The disease lesion caused by LbDsRed was limited to the surrounding inoculation sites of each lobe of the cotyledon (Figure 5.2). Significantly largest and second largest disease lesions were observed in cotyledons in which *L. maculans* was used as the sole inoculum and pre-inoculum as shown in Figure 5.1. In those two treatments, black color pycnidia were visible on disease lesions. Lesion development on LbDsRed and LmGFP co-inoculated cotyledons and LbDsRed pre-inoculated cotyledons were not statistically different from the lesions seen in LmDsRed solely inoculated cotyledons ($p < 0.05$) (Figure 5.2).

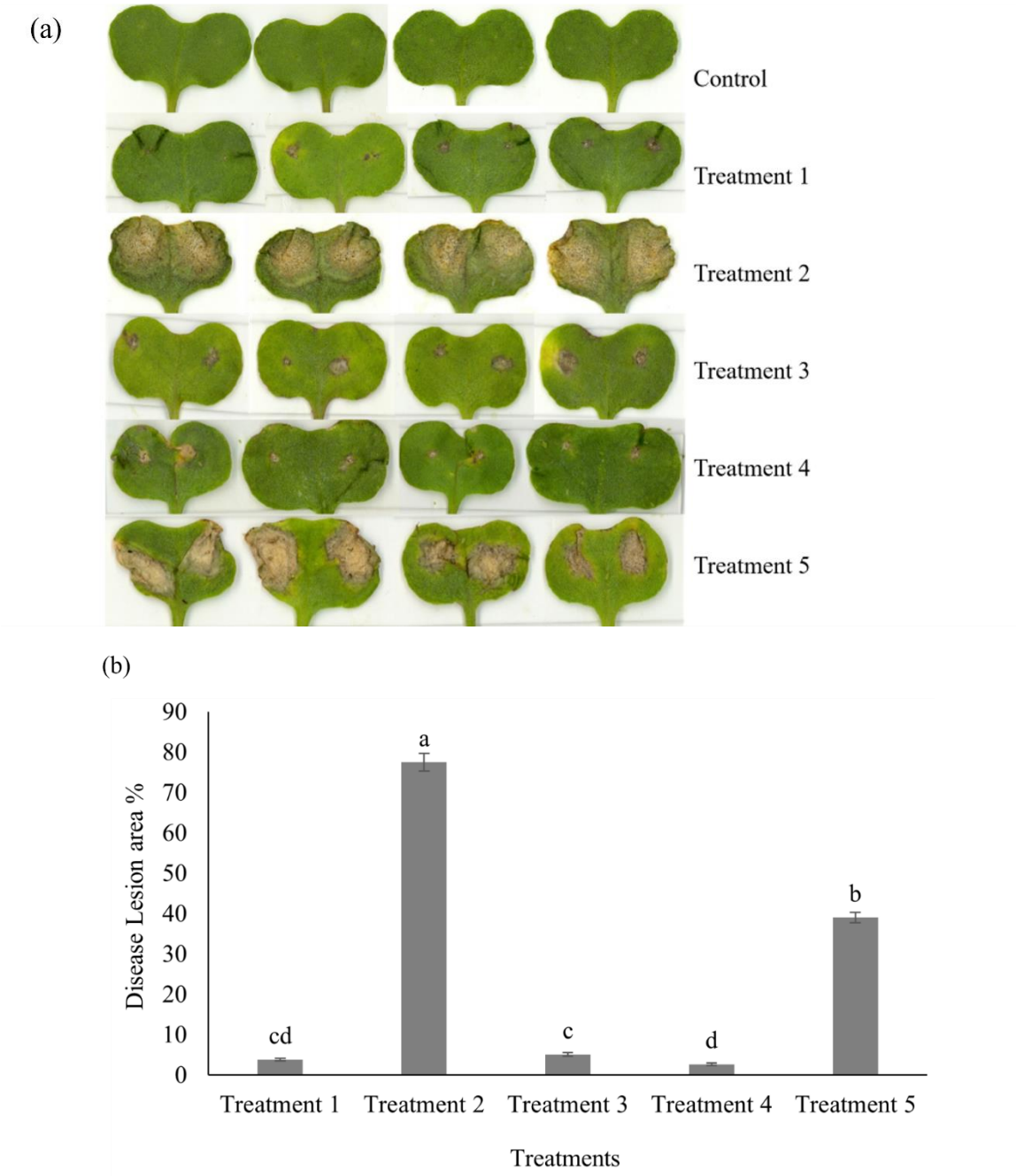


Figure 5. 2 Blackleg Disease Lesion Development in Westar Cotyledons at 14 Days Post Inoculation under Controlled Conditions with *Leptosphaeria maculans* 'brassicae' *and L. biglobosa* 'brassicae' Inoculations. Seven-day-old cotyledons were wound inoculated and observed the lesion development in time course. Cotyledons inoculated with only GFP tagged *L. maculans* (LmGFP) and cotyledons pre inoculated with LmGFP showed clear lesion development at 14 dpi. Control: distilled water treatment; Treatment 1: LbDsRed inoculation; Treatment 2: LmGFP inoculation; Treatment 3: LbDsRed and LmGFP co-inoculation; Treatment 4: LbDsRed

followed by LmGFP inoculation (in 24 h interval); Treatment 5: LmGFP followed by LbDsRed inoculation (in 24 h interval). a: scanned images of disease lesion development in cotyledons; b: Lesion size has shown as a percentage of the total leaf area of both host types calculated by ImageJ ($p \leq 0.05$).

5.4.2 Presence of *Leptosphaeria* spp. in Inoculated Westar Cotyledons

PCR products of genomic DNA extracted from infected cotyledons inoculated with only LmGFP and only LbDsRed showed gel bands of 444 bp and 331 bp sizes for LbDsRed and LmGFP, respectively. Co-inoculated cotyledons with both isolates displayed both bands (Figure 5.3).

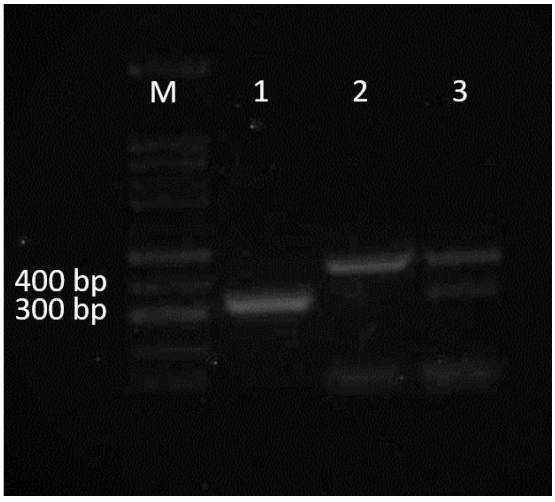


Figure 5. 3 Gel Image of Species-Specific PCR Products of *Leptosphaeria maculans* 'brassicae' and *L. biglobosa* 'brassicae' Isolates in Inoculated Westar Cotyledons. PCR identification of *L. biglobosa* and *L. maculans* with species specific primers, LmacF/LmacR and LbigF/LmacR in a multiplex PCR. *L. biglobosa* 'brassicae' and *L. maculans* 'brassicae' gave PCR bands of 444 bp and 331 bp, respectively. PCR products were resolved by electrophoresis through a 1% (w/v) agarose gel. Lane1: *L. maculans* 'brassicae' inoculated cotyledon gDNA; Lane 2: *L. biglobosa* 'brassicae' inoculated cotyledon gDNA; 3: both isolates co-inoculated cotyledon gDNA; M: 100 bp DNA ladder.

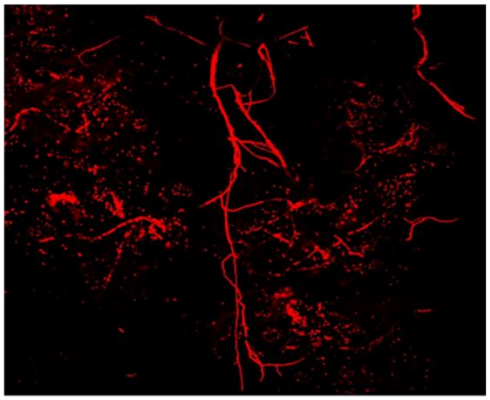
5.4.3 *L. biglobosa* Inhibits the *L. maculans* Growth in *B. napus* Cotyledons

Confocal observations at 14 dpi of cotyledons inoculated with LbDsRed only showed the LbDsRed mycelia, but comparatively in less density (Figure 5.4.a). The DNA quantity of LbDsRed was reduced around 33 times from 1- to 7- dpi (Figure 5.4.b). In cotyledons that inoculated solely by LmGFP, confocal microscopic observations showed densely grown mass of LmGFP (Figure 5.4.c). *L. maculans* DNA quantity increased starting from three dpi onwards. Moreover, fungal DNA quantity was raised at an exponential rate from seven dpi onwards (Figure 5.4.d). The amount of LmGFP DNA was increased 10 times from 7- to 14- dpi.

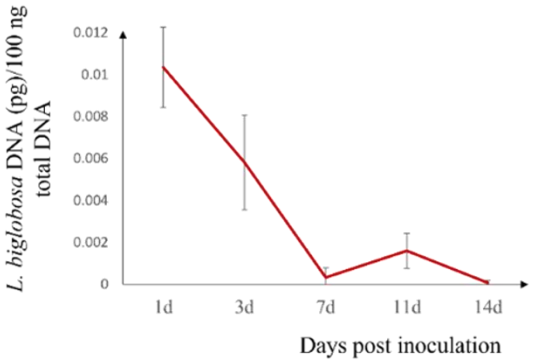
The co-inoculated cotyledons displayed both LbDsRed and LmGFP signals as shown in Figure 5.4.e. The quantification study clearly demonstrated the reduction of LbDsRed in a similar pattern in cotyledons treated only with LbDsRed. However, the reduction of DNA of LmGFP was also observed in those cotyledons (Figure 5.4.f). Cotyledons pre inoculated with LbDsRed one day before prior to the LmGFP inoculation showed only LbDsRed mycelia in confocal observations. The quantification study demonstrated a clear growth inhibition of LmGFP from 3- to 14- dpi (Figure 5.4.h).

On the other hand, LmGFP inoculation followed with LbDsRed inoculation with 1-day interval showed densely developed LmGFP in confocal images and raising of LmGFP DNA quantity starting from seven dpi similarly to the LmGFP solely treated cotyledons. LbDsRed was not observed in the confocal observations and the DNA quantity of LbDsRed was reduced with time as shown in Figure 5.4.j.

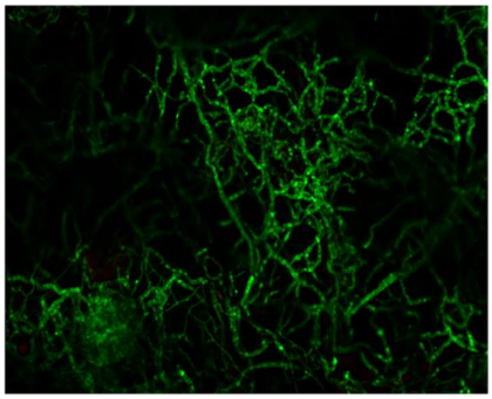
a.



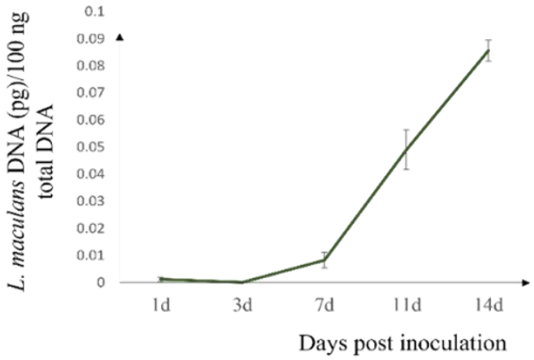
b.


L. biglobosa DNA (pg)/100 ng total DNA
0.012
0.01
0.008
0.006
0.004
0.002
0
1d
3d
7d
11d
14d
Days post inoculation

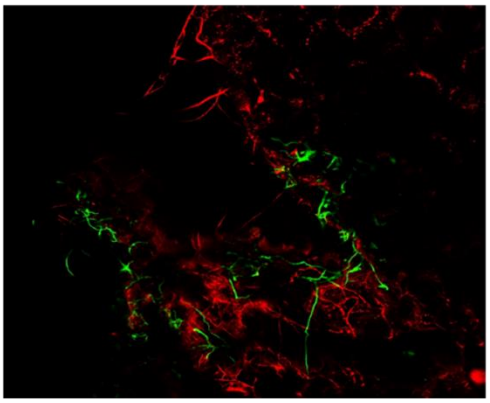
c.



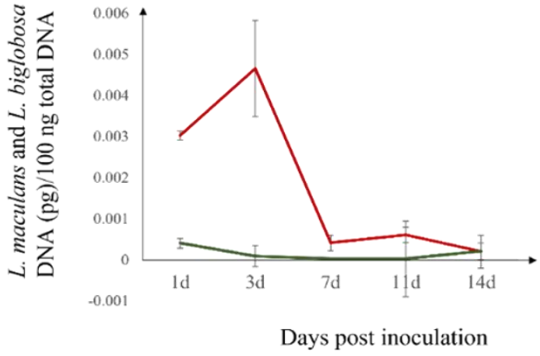
d.


L. maculans DNA (pg)/100 ng total DNA
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
1d
3d
7d
11d
14d
Days post inoculation

e.



f.


L. maculans and L. biglobosa DNA (pg)/100 ng total DNA
0.006
0.005
0.004
0.003
0.002
0.001
0
-0.001
1d
3d
7d
11d
14d
Days post inoculation

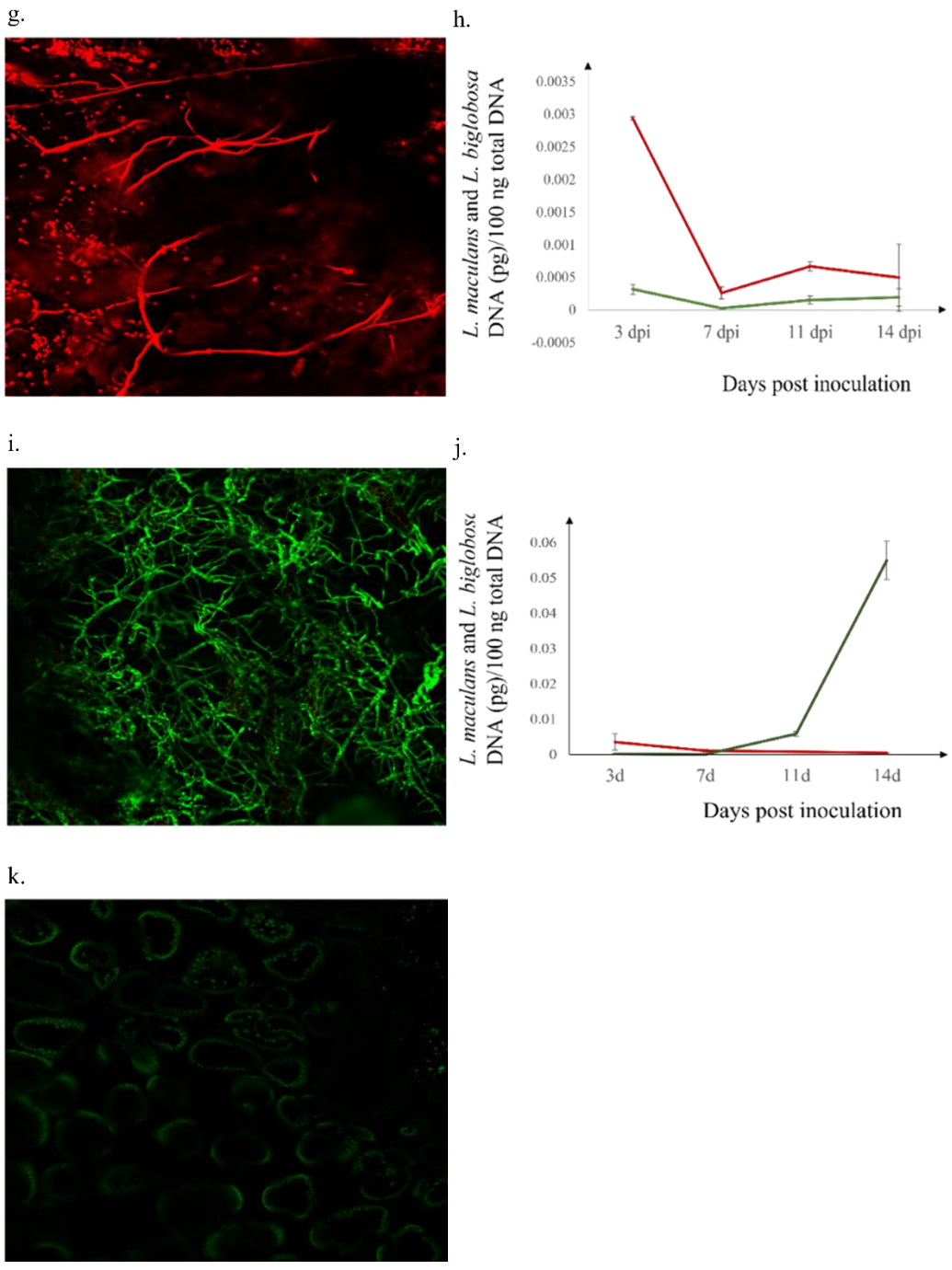


Figure 5. 4 DsRed-tagged *L. biglobosa* (LbDsRed) and GFP-tagged *L. maculans* (LmGFP) in planta development of Westar cotyledons under controlled environment at 14 days post-inoculation. a: Confocal microscopic observations of fluorescence protein-tagged *Leptosphaeria* isolates (10X magnification). **b**: Quantification of each isolate in planta at 3-, 7-, 11-, and 14- dpi. LbDsRed and LmGFP mycelia shown in fluorescent red and green colors, respectively. In graphs,

red and green colors represent LbDsRed and LmGFP DNA, respectively. **a, b**: LbDsRed inoculation; **c, d**: LmGFP inoculation; **e, f**: LbDsRed and LmGFP co-inoculation; **g, h**: LbDsRed followed by LmGFP inoculation with one day interval; **i, j**: LmGFP followed by LbDsRed inoculation with one-day interval; **k**: Chlorophyll autofluorescence of cellular chloroplasts of cotyledons in control.

5.4.4 Expression of Defense Genes

Except *WRKY70* and *PDF1.2*, all other surveyed genes, *PR1, WRKY33, RbohD, APX6* were upregulated in *L. biglobosa* inoculated cotyledons compared to the other two methods of inoculations. *CHI, Chitinase*, which is important in degradation of pathogen cell walls, was expressed significantly higher in *L. biglobosa* inoculated cotyledons (Figure 5.5.4). As shown by Figure 5.6, ROS burst marker gene, *RbohD* started to upregulate starting from three dpi onwards at an exponential rate.

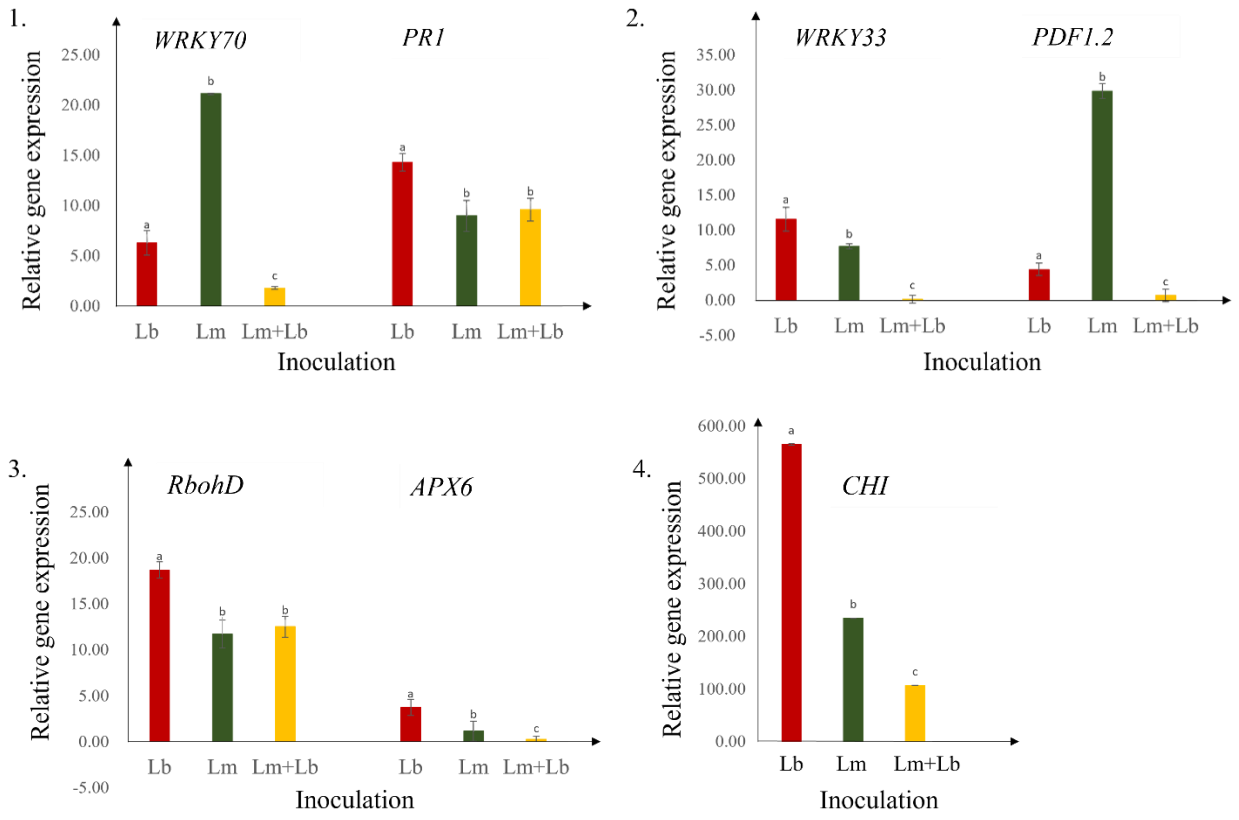


Figure 5. 5 Relative Transcript Levels of Several Important Genes Involved in Host Defense Signaling Pathways Assessed by RT-qPCR at 7 Days Post Inoculation. 1: Relative gene expression of *WRKY70* and *PR1*; 2: Relative gene expression of *WRKY33* and PDF1.2; 3: Relative gene expression of *RbohD* and *APX6*; and 4: Relative gene expression of *CHI*; in Westar cotyledons inoculated with only DsRed tagged *L. biglobosa* (Lb), with only GFP tagged *L. maculans* (Lm) and co-inoculated with both isolates (Lm+Lb).

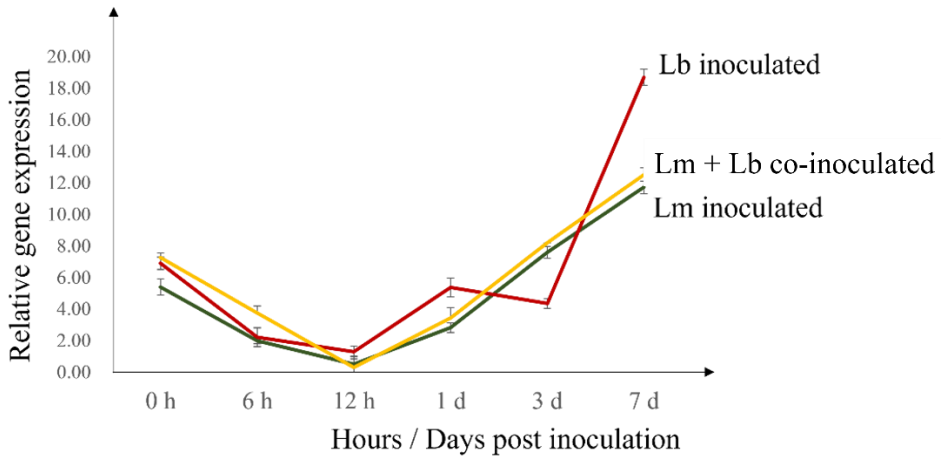


Figure 5. 6 Expression Profile of *RbohD* in Westar Cotyledons Infected with LmGFP, LbDsRed as Sole and Combined Inocula. *RbohD* expression was upregulated at an exponential rate from 3- to 7- dpi. The observations were taken at 0-, 6-,12- hpi and 1-, 3-, 7- dpi.

5.5 Discussion

This study demonstrated the resistance inducing potential of a less virulent isolate, *L. biglobosa* 'brassicae' in the susceptible canola variety Westar against virulent *L. maculans* 'brassicae'. The successful control of *L. maculans* was demonstrated by pre-and co-inoculation of *L. biglobosa* by studies of cotyledon lesion development, in planta mycelial development, pathogen quantification, and gene expressions related to host defense.

The avirulence nature of *L. biglobosa* 'brassicae' on the susceptible Westar was evidenced by (1) the restricted disease lesion at the inoculation site, (2) reduction of LbDsRed DNA quantity by 33 folds from 3- to 7- dpi, and (3) low density of *L. biglobosa* mycelia under the confocal microscope. The restricted disease lesion caused by *L. biglobosa* could be related to the oxidative burst that arises in the host plant during the pathogen infection (Lowe et al. 2014). Lowe et al. (2014) demonstrated that the oxidative burst occurrence is higher in the host infected with *L. biglobosa* than in the host infected by *L. maculans*. *RbohD* is the oxidative burst marker gene of canola and RbohD-dependent ROS is involved in the plant defense (Suzuki et al., 2011). *RbohD* was upregulated starting from 3 dpi onwards in this study. As the principal ROS producer, *RbohD* leads the hypersensitive response (HR) and induce SAR against pathogen attacks (Keller et al. 1996; Suzuki et al., 2011). The on-site oxidative burst and localized cell death are two of the typical behaviors during HR caused by gene-for-gene interaction (Bestwick et al. 1997). Upregulation of *RbohD* in the host during *L. biglobosa* infection could be one of the main reasons that host became resistant, and the pathogen was inhibited and limited to the infection site. The SAR induced by *RbohD* could favor the controlling of subsequent *L. maculans* infections (Keller et al. 1996).

On the other hand, *APX6* was significantly upregulated in Westar after being inoculated with *L. biglobosa*, compared to the plants inoculated with *L. maculans*. *APX6* plays a leading role in eliminating intracellular ROS and protecting plants from oxidative burst and damage consequences. As discussed above, *RbohD*, the source of ROS, was also upregulated in *L. biglobosa* inoculated cotyledons and then led to cell death. Cell death could benefit the necrotrophic pathogen *L. biglobosa* (Spoel et al., 2007). Spoel et al., (2007) described that sacrificing infected cells by triggering cell death is a defense strategy against biotrophic pathogens. In contrast, the maintenance of plant cell viability can be used as a defense mechanism against necrotrophic pathogens (Spoel et al., 2007). Based on these observations, the higher expression of

APX6 could be used to maintain the balance of cell death caused by upregulated *RbohD* in *L. biglobosa* infected cotyledons.

The hypothesis was further supported by the expression patterns of the selected defense-related genes. In the canola blackleg pathosystem, the host becomes resistant by the early recognition of *L. maculans* by gene-for-gene interaction and the interaction is known as incompatible. SA signaling pathway related *WRKY70* initiates the process and is upregulated to higher levels as early as 3 dpi when the interaction is incompatible. However, the Westar plant's compatible interaction with *L. maculans* in this study exhibited the upregulation of *WRKY70* from 7 dpi onwards due to the late recognition of the pathogen (Beckar et al. 2017, Yang et al. 2021). Interestingly, Westar did not show an apparent upregulation of *WRKY70* in the interaction with *L. biglobosa* at 7 dpi compared to its level at 3 dpi (data have shown only for 7 dpi). Similar results were obtained by Lowe et al., (2014). On the other hand, *WRKY33*, a gene related to the JA signaling pathway, was highly upregulated in Westar infected by *L. biglobosa* than by *L. maculans*. Previous studies revealed that responses against biotrophic pathogens are generally regulated by SA (Vlot et al., 2009), while reactions towards necrotrophs are mediated by JA and ethylene (Farmer et al., 2003). Therefore, the upregulation of only *WRKY33* in response to *L. biglobosa* infections further confirms *L. biglobosa* as a necrotrophy, but *L. maculans* is a hemibiotroph, which initiates its lifespan as a biotroph. *PDF1.2* is considered as an ethylene-response-factor marker gene and JA marker gene (Li et al., 2019). The increment of *PDF1.2* in LmGFP inoculated seedlings at 7 dpi onwards can be explained by the switching of the pathogen's trophic nature into necrotrophy (Li et al., 2019).

PR1 expression was upregulated highly in *L. biglobosa* inoculated Westar cotyledons compared to *L. maculans* inoculated ones. The *PR1* gene is often used as a marker for SAR. The upregulation of *PR1* and the resulting SAR will induce a defense response against subsequent infection in host plants (Henry et al. 2013). The defense-related genes of *RbohD*, *APX6*, *PR1*, *WRKY33*, *PDF1.2*, and *CHI* began to upregulate from 3 dpi onwards (results not shown except for *RbohD*), which is consistent with the observed reduction in the *L. maculans* quantity from 3 dpi onwards in planta.

Chitinase showed a significant upregulation at 7 dpi in plants inoculated by *L. biglobosa* compared to the other plants. Chitinase belongs to the glycosyl hydrolase family, catalyzing the hydrolysis of glycosidic bonds in chitin, a key component of the fungal cell wall (Kumar et al.

2018). Lowe et al., (2014) observed a significantly higher chitinase expression in *L. biglobosa* 'canadensis' inoculated plants compared to the ones inoculated by *L. maculans*. Therefore, the upregulation of chitinase must play a significant role in plant resistance against *L. biglobosa* in Westar.

As demonstrated by Zou et al., (2019), different *L. biglobosa* isolates showed different levels of virulence on Westar. Interestingly, the population of *L. biglobosa* 'brassicae' reduced from 1 dpi onwards in susceptible Westar cotyledons in this study. In contrast, *L. maculans* showed its general virulence when LmGFP was inoculated solely on Westar cotyledons. As the Westar makes a compatible interaction with the LmGFP, no early recognition of the pathogen by the host plant and no triggering of effector-triggered immunity (Becker et al., 2017). Therefore, the pathogen colonized within the host tissues at its biotrophic stage without doing any apparent damage upon host cells and showed an incremental increase in the *L. maculans* quantity observed from 7 dpi onwards as demonstrated by previous studies (Li et al. 2008; Becker et al. 2017; Yang and Fernando 2021). The lesion development was prominent as the virulent pathogen kills more and more tissues needed for the survival of the later necrotrophic stage (Li et al. 2008).

Interestingly, the virulence of *L. maculans* was hidden entirely when it was co-inoculated or post-inoculated one day after *L. biglobosa*. The restraining of the *L. maculans* in the cotyledons was evidenced by restricted lesion development images and PCR quantification studies. The inhibition of *L. maculans* did not occur when the cotyledons were inoculated with *L. maculans* prior to *L. biglobosa* inoculation. The above observation suggests that *L. biglobosa* inoculation induces the host defense system and prime for any subsequent infections by *L. maculans*.

Both *L. maculans* and *L. biglobosa* coexist in most regions in which Brassica crops are grown. For instance, Dilmaghani et al., (2009) revealed the existence of *L. maculans* and *L. biglobosa* species within the same field in the American continent, extending from Chile to Canada. *L. maculans* is considered more damaging to the crop than *L. biglobosa* (Fitt et al., 2008). Though damage, symptoms, and other biological features of *L. maculans* and *L. biglobosa* have been studied thoroughly; different ecological niches and other epidemiological features such as the timing of infection remained unrevealed (Jacques et al., 2021). As West et al., (2002) and Mazáková et al., (2017) demonstrated two *Leptosphaeria* spp. exhibited different niches even in the same host plant. Liu et al., (2006) demonstrated the possibility of controlling the incidence of stem canker caused by *L. maculans* with the antagonistic interactions or the induction of systemic

resistance caused by *L. biglobosa* in the host plant. Furthermore, Jacques et al., (2021) suggested that though there is no sign of exclusion between two *Leptosphaeria* spp., there could be defense response induction, resource competition, and topographic exclusion. Martyn (1991) and Mahuku et al., (1996) reported that closely related pathogens could better induce resistance to the target pathogen. Although these two species have co-existed in the ecosystem for a long period of time, no recombination has been reported so far. Therefore, there is no or very less concern about developing more virulent L. biglobosa as there's no recombination between two species

The reduction of in planta mycelial development and disease lesion development, pathogen quantification, as well as transcriptomic observations of plant defense gene expressions confirms *L. biglobosa* could be used as a biological control in the diversification of the integrated management system of *L. maculans* in order to reduce the selection pressure exerted on host resistant genes. Further studies are required to determine the time of application of *L. biglobosa* in the field to prime the plant against this devastating pathogen.

5.6 Conclusion

Fluorescent protein tagged *Leptosphaeria* isolates demonstrated a successful in planta control of virulent *L. maculans* in cotyledons pre- or co-inoculated with *L. biglobosa.* Moreover, lesion development on cotyledons, host defense-related gene expressions, and pathogen quantification studies in time course studies confirmed the above results. The outcome of the study concludes that *L. biglobosa* 'brassicae' can inhibit the destructive pathogen *L. maculans* in susceptible canola varieties. The reliability of this biological control agent under field conditions and the applicability of *L. biglobosa* into the integrated management approaches have to be studied further.

CHAPTER 6. GENERAL DISCUSSION

This thesis provided insight into host resistance genes underlying disease defense mechanisms and pathogen virulence in one of the most economically significant *B. napus-L. maculans* pathosystem. Furthermore, an integrated management strategy including good agronomic practices and stewardship of blackleg resistance is vital in managing this devastating blackleg disease in western Canada. This thesis delivers three such management tools necessary for the blackleg management toolbox.

Mainly grown Canadian canola resistant genotype, i.e., *Rlm3*, (Zhang et al., 2016), has been overcome by the pathogen after about three decades of growth in the field, and disease incidence of blackleg has kept increasing in recent years (Canadian disease survey, http://phytopath.ca/publication/cpds). Understanding defence-related host genes can facilitate the development of more resistant varieties using efficient breeding strategies. The introduction of new resistant genotypes to commercial canola variety pools has been raised as a pivotal necessity to counter this devastating pathogen *L. maculans.* Based on the avirulence proportion in the pathogen population and stability of the host genotype, *Rlm7* is suggested as the next potential genotype to be introduced into Canadian canola production. An integrated management system instead of a single method is the best way to control the blackleg disease. Therefore, evaluating a seed treatment fungicide would be an excellent tool for the integrated management toolbox. Pydiflumetofen is a broad-spectrum seed-applied fungicide with an SDHI mode of action. In addition, the analysis of the biocontrol ability of weaky virulent *L. biglobosa* on *L. maculans* would be another option that can be another tool.

In-depth understanding of the *B. napus-L. maculans* pathosystem is a primary need to control this devastating disease efficiently and effectively. The *Rlm7-AvrLm7* pathosystem was selected since *AvrLm7* is one of the most abundant avirulence genes in the pathogen population in Canadian prairies, and *B. napus Rlm7* genotype has been reported from other countries as one of the stable host genotypes which has been introduced so far. Therefore, *Rlm7* would be the next potential host-resistant gene introduced into the Canadian canola seed market. Understanding host genes behind the host defense and pathogen genes behind its virulence would support manipulating those key genes to maintain the *Rlm7* genotype for a long time without being overcome by the pathogen.

A genome-wide transcriptional study conducted in a time-course at 0-, 1-, 3-, 7-, and 11-dpi in *Rlm7* genotype compared to Westar exhibited the activation of defense-related genes in a host background along with and without the *R* gene. The compatible and incompatible interactions created with the *AvrLm7* isolate and its mutant demonstrated how the gene-for-gene interaction initiates in incompatible systems. The early activation of defense-related transcripts leads the host to be resistant against the pathogen.

Among thousands of host genes in disease defense responses, key genes involved in key defense mechanisms against the pathogen in incompatible vs compatible interactions were highlighted. Host genes of the 01-23-2-1 involved in incompatible interaction with *AvrLm7* compared to its compatible interaction (without *AvrLm7*) in resistant gene background were studied. The same was evaluated in Westar, which lacks resistant gene background.

Hormone signaling pathways including, JA, SA, and ET, significantly involve host defense against *L. maculans* infections (An and Mou 2011; Fu and Dong 2013). In hemibiotrophic pathogens, the above signaling mechanisms vary between incompatible and compatible interactions and their trophic nature (Fu and Dong 2013; Garg et al., 2013).

Salicyclic acid plays a vital role in plant defense, taking part in PTI, ETI, signal transduction, and systemic acquired resistance (SAR) (Pieterse et al., 1998; Durrant and Dong 2004; Salzmanetal 2005; Tsuda et al., 2009). Reduced SA accumulation incompatible interactions: Rlm-umavr7, Westar-UMAvr7, and Westar-umavr7 can relate to the lower basal defense, which leads to susceptibility (de Torres-Zabala et al., 2007; Mohr and Cahill, 2007). SA has an antagonistic and synergistic impact on other hormonal pathways such as JA and ET (Koornneef and Pieterse 2008). High expressions of JA and ET signaling and biosynthesis genes at 7- and 11-dpi, especially in incompatible interactions, can be explained as they are the vital phytohormones in plant defense against necrotrophic pathogens, and the hemibiotrophic pathogen survived as a necrotroph at those two-time points (Berens et al., 2017). Such upregulation of genes related to JA and ET pathway was not seen in Rlm7-UMAvr7 incompatible interaction in which the pathogen is under control and predicted to remain in its biotrophic stage itself. Upregulation of SA pathway-related genes but not JA and ET-related genes further suggested that the pathogen remains biotrophic at even 7- and 11- dpi in the incompatible interaction. The above observation could be supported by Chowdhury et al. (2017). They explained that the biotrophy-to-necrotrophy switch was delayed in incompatible interaction.

Salicyclic acid is necessary for SAR, and SAR is characterized by an increase in endogenous SA and activation of pathogenesis-related (PR) genes (such as PR1:GSBRNA2T00150001001) and enhanced plant resistance to a broad spectrum of virulent pathogens (Ryals et al., 1996; Fan and Dong 2002). The outstanding high expression of GSBRNA2T00150001001 at 3-dpi in the incompatible interaction, 01-23-2-1-UMAvr7, which was not seen in compatible interactions, could be proof of the early recognition and activation of plant defense due to gene-for-gene interaction. Incompatible interactions lacking gene-for-gene interaction, the host would not recognize the pathogen until it reached the QS and initiated the infection. As Padder et al. (2018) explained, QS is a density-dependent microbes' signaling mechanism. The signaling compounds called autoinducers reach the threshold level, which leads to synchronizing the expression of virulence factors as a function of the fungal density to overcome the host defense. This late recognition led the host plant to fail from the battle as it would be too late to initiate the defense mechanisms.

Pathogen effectors are virulence factors developed to overcome host basal resistance. Effectors involve reprogramming and modulating plant immunity by overcoming PTI (Stergiopoulos and de Wit 2009). The upregulation of effectors was higher in later time points, i.e., 7- and 11- dpi. Several effectors only seen in 11 dpi, which were not seen in the biotrophic stage. Tan et al., (2010) explained necrotrophs used effectors to disable susceptible hosts before colonization.

Pathogen CAZymes are enzymes such as cellulase, hemicellulase, and pectinase that are prominent in degrading host cell walls (O'Connell et al., 2012). Cell wall and pectin degrading enzymes get upregulated to facilitate the pathogen entering the plant system (O'Connell et al., 2012). Then they help the pathogen establish itself within the host system. Differentially expressed CAZymes were higher in three compatible interactions, where the pathogen colonized and infected the host successfully compared to the incompatible interaction in which the pathogen was under control.

Similar to CAZymes, pathogen peptidases were also highly expressed in compatible interactions compared to the incompatible interaction specially at 7- and 11- dpi. Fungal pathogens use proteases to degrade plant antimicrobial proteins, such as pathogenesis-related proteins, including plant chitinases, and challenge plant immunity (Jashni et al., 2015). The lower expression of proteases in incompatible interaction could be explained as gene-for-gene interaction

activated in incompatible interaction leads the host plant to activate its proteases and protease inhibitors which can modify or damage pathogen proteases (Jashni et al., 2015). The upregulation of DE proteases was observed in compatible interactions. A thorough understanding of the pathosystem will support further breeding programs of host-resistant genes and maintain the *Rlm7* for a more extended period without breaking down.

Since the pathogen has already overcome the Canadian canola resistance, it is necessary to introduce new host genotypes to the canola seed market. Until the ongoing breeding programs get succeeded there should be alternatives to face the battle against this destructive pathogen. Fungicides would be the best option that could be thought of in such a situation. On the other hand, having effective fungicides would be supportive to deal with any outbreaks on the way.

Pydiflumetofen is a broad-spectrum seed-applied fungicide with an SDHI mode of action. Seed treatments are more economically feasible to use in canola as the disease can occur in the crop at any stage in the life span, so the timing for foliar fungicide application is a challenge. Furthermore, the first emergence of canola rarely co-occurs all at the same time due to inconsistent weather patterns (Peng et al., 2020). Under such conditions, seed-applied fungicides have a real advantage over foliar ones. Since the active ingredients are translocated into the seedlings right after germination, protecting the two to three-leaf stage regardless of when the seedlings finally emerge from the soil, seed-applied fungicides will provide consistent results under all growing conditions irrespective of when individual seedlings emergence.

The efficacy of pydiflumetofen in controlling the fungus *in planta* was exhibited by suppressing the growth of apoplastic mycelia even after 14 dpi under confocal microscopy studies in moderately resistant SY4135 and even in Westar. Westar contains no *R* genes or no known quantitative resistance, simulating the worst-case scenario of the situation (Balesdent et al., 2005). Bai et al. (2021) revealed that pydiflumetofen damages the mycelial cell wall and membrane and breaks up cells, leading to intracellular plasma leakage of *F. fujikuroi* in rice via electron microscope transmission studies. Under a controlled environment, despite pydiflumetofen being applied individually or in combination with Vibrance® Flexi, pydiflumetofen was significantly effective in controlling the blackleg disease severity of stem cankers.

However, the disease severity reduction observed by GLIMMIX statistical analysis in the field study was not statistically significant. The disease severity estimates in Westar and SY4135 treated with pydiflumetofen were 1.38 and 0.59 ($p \leq 0.05$). In contrast, the no fungicide control

and other three fungicide treatments: Helix® Vibrance, Prosper® EverGol, and BAS 720 FST exhibited 1.10, 1.00, 1.11, and 0.9 of disease severity, respectively, in SY4135 plants and 2.2725, 2.04, 2.00, and 1.94 disease severity, in Westar plants. Moreover, SY4315 treated with pydiflumetofen could result in about 25% more seed yield than the control. However, disease severity reduction in pydiflumetofen treated plants compared to other plants confirmed the efficacy of pydiflumetofen as a successful seed treatment to manage the blackleg pathogen. As the results confirmed, this novel SDHI seed treatment, pydiflumetofen, would be an excellent addition to farmers' integrated management toolbox to mitigate this devastating pathogen, *L. maculans,* from canola fields and mitigate trade barriers.

Mahuku et al. (1996) explained that pathogens closely related to challenging isolates are better resistance inducers than nonpathogens or pathogens of unrelated hosts. Single leaf inoculation following induction of systemic resistance has also been demonstrated in other host-pathogen interactions (Kuc 1982). *L. biglobosa*, weakly virulent and not economically destructive, appeared to induce resistance against *L. maculans* in canola (Chen and Fernando, 2005; Liu et al., 2006). This study demonstrated the potential of weakly virulent *L. biglobosa* 'brassicae' in inducing resistance of susceptible canola variety Westar against virulent *L. maculans'* brassicae'. In incompatible interaction of canola cultivars with resistant genes and *L. maculans* isolates with corresponding avirulence gens. However, Westar develops a compatible interaction with the *L. maculans* isolates used as Westar lacks any of the resistant genes. The pre-and co-inoculation of *L. biglobosa* on Westar cotyledons exhibited successful control of *L. maculans* by controlling cotyledon lesion development and planta mycelial development upregulation of defence-related genes and pathogen quantification studies.

Pre- and co-inoculated *L. biglobosa* induction of Westar resistance and *in planta* control of *L. maculans* mycelial growth were demonstrated by GFP tagged *L. maculans*, and DsRed tagged *L. biglobosa* isolates used. The lesions caused by *L. biglobosa* were only limited to the inoculation sites, which could be related to the oxidative burst that arises in the host plant with the inoculation of the *L. biglobosa* isolate (Lowe et al., 2014). According to previous studies, the oxidative burst in the host plant is higher with *L. biglobosa* infection than with *L. maculans* infection. The number of *L. biglobosa* mycelia species-specific *ITS* region was reduced by 33 folds from 3 dpi to 7 dpi. The confocal observations in the time course study also confirmed the *L. biglobosa* 'brassicae' is an avirulent isolate on Westar.

RbohD, the oxidative burst marker gene of canola, involves plant defense against pathogens associated with the hypersensitive response (Zusuki et al., 2011; Otulak-Kozieł et al., 2020) was raised. Furthermore, *RbohD* was reported to induce systemic acquired resistance (SAR) and hypersensitive response (HR) reactions (Keller et al., 1996). *L. biglobosa* infection led up-regulation of *RbohD* in the host plant must be one of the main reasons the host becomes resistant and the pathogen becomes less virulent or avirulent and limited to the infection site. On the other hand, the *RbohD* induces SAR, which controls the subsequent *L. maculans* infections (Keller et al., 1996). *APX6,* which plays a central role in eliminating intracellular ROS and protects plants from the oxidative burst and consequences damage, is also upregulated with *L biglobosa* infection to balance the cotyledon cell death, which could be beneficial for necrotrophic *L. biglobosa* Later on (Spoel et al., 2007).

WRKY70 is closely related to the SA pathway, and *WRKY33* is related to JA and ET pathways. Therefore, the higher upregulation of *WRKY70* and *WRKY33* in response to *L. maculans* and *L. biglobosa* infections, respectively, further confirms *L. maculans* is a hemibiotroph, which commences its lifecycle as a biotroph and *L. biglobosa* survives as a necrotroph.

PR1 is significant in SAR induction and known as a marker for SAR. Upregulation of *PR1* led SAR to enhance defense response in the host against subsequent infections (Henry et al., 2013; Zhai et al., 2021). Upregulation of *PR1* was higher in *L. biglobosa* inoculated Westar cotyledons compared to *L. maculans* inoculated ones.

L. biglobosa inoculated plants showed upregulated expressions of other defence-related genes: *RbohD, APX6, PR1, WRKY33, PDF1.2, CHI* from 3-dpi, and it might be a reason for *L. maculans* control in the host. Specially *CHI,* upregulation was significantly higher in *L. biglobosa* inoculated plants. Chitinase degrades chitin, a key fungal cell wall component, with no harm to the host plant (Kumar et al., 2018). Howlett et al. (2014) exhibited significant upregulation of chitinase in *L. biglobosa* 'canadensis' inoculated hosts compared to *L. maculans'* brassicae' inoculated ones. Therefore, this study confirms the positive relationship between upregulation of chitin and induced plant resistance in Westar plants against *L. biglobosa'* brassicae' in this study. Chen and Fernando (2005) and Lowe et al. (2014) showed the induction of other defence-related genes: callose and lignin deposition, β-1.3-glucanase and peroxidases in this pathosystem.

Different *L. biglobosa* isolates show a different level of virulence on Westar (Zou et al., 2019). In this study, *L. biglobosa'* brassicae' reduced its population in susceptible Westar

cotyledons from 1-dpi onwards. In contrast, *L. maculans* performed a compatible interaction and made disease in susceptible Westar cotyledons. The virulency of *L. maculans* was ceased entirely when it was co-inoculated or post-inoculated with *L. biglobosa* inoculation. The above obserbvation suggests that *L. biglobosa* inoculation induces host resistance and is prime for any subsequent infections of *L. maculans*. The results suggested that *L. biglobosa* could be used as a biological control tool in the integrated management toolbox for canola blackleg disease. However, further studies must be carried out to analyze the feasibility of using this method under field conditions.

CHAPTER 7. OVERALL CONCLUSION AND CONTRIBUTION TO THE KNOWLEDGE

Based on the abundance of the pathogen avirulence genes frequencies and the stability of the host *R* gene *Rlm7* can be the next potential to be introduced. In-depth understanding of the interaction of *Rlm7*under resistant and susceptible interactions would help use this canola germplasm for a long without breaking down. Newly developed seed treatment, pydiflumetofen can be used as an alternative pathogen management tool to avoid any outbreaks of the pathogen until novel *R* genes come to the market. On the other hand, less virulent *L. biglobosa* can be used as an effective biological control of the pathogen *L. maculan.* This study investigated three approaches that can be successfully used in mitigating of devastating disease of blackleg in canola.

According to the best of our knowledge, this is the first transcriptomic study done using the same host and pathogen isolate background of *B. napus-L. maculans* pathosystem. Better understanding the *Rlm7-AvrLm7* pathosystem will assist in seeing the potential of introducing *Rlm7* to the market and maintaining *Rlm7* for a longer time without breakdown. In addition, the resistant genes and pathways of host and virulence genes of the pathogen can encourage more future studies to assist in countering this destructive pathogen *L. maculans.*

As the pathogen and the disease incidence have overcome the primarily current grown *Rlm3* has increased, it is very effective to have a seed-applied fungicide that could be used to avoid any breakouts of the disease. Pydiflumetofen is a seed-applied fungicide that would be economically feasible and more effective in controlling the disease.

Using weakly virulent *L. biglobosa* would be another interesting potential tool that could be used in the integrated management of *L maculans.*

CHAPTER 8. FUTURE DIRECTIONS

The key defence-related genes in the host involved in incompatible interactions over compatible interactions and the pathogen virulence genes involved in successful disease development must be further analyzed and characterized by functional complementation studies. The knowing on perfect molecular markers could assist in effective breeding programs for resistant canola genotypes.

Though pydiflumetofen showed low disease severity and high yield gain estimates, the results were not statistically significant under field conditions. Therefore, more field tests are needed to confirm the success of the fungicide in field.

Further studies are needed to investigate whether *L. biglobosa* is getting controlled in Westar due to the basic PTI involved or if *L. biglobosa* 'brassicae' produces an avirulent effector or any other molecule which can be recognized by undiscovered resistant protein or any other protein or molecule of Westar which can lead to gene-for-gene interaction going on behind Westar-*L. biglobosa* interaction.

The results suggested that *L. biglobosa* could be used as a biological control in the integrated management of canola blackleg disease. However, further studies have to be carried out to analyze the feasibility of using this under field conditions and also need to be optimized as it can be used successfully under field conditions.

CHAPTER 9. REFERENCES

Agrawal, G.K., Rakwal, R. and Jwa, N.S. 2000. Rice (*Oryza sativa* L.) *OsPR1b* gene is phytohormonally regulated in close interaction with light signals. *Biochem Biophys Res Commun*. 278(2): 290-8.

Akbudak M.A., Yildiz S. and Filiz E. 2020. Pathogenesis related protein-1 (PR-1) genes in tomato (Solanum lycopersicum L.): Bioinformatics analyses and expression profiles in response to drought stress. *Genomics*. 112(6): 4089-4099.

An, C. and Mou, Z. 2011. Salicylic acid and its function in plant immunity. J. Integr. *Plant Biol.* 53, 412–428.

Avenot, H.F. and Michailides, T.J. 2010. Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. *J. Crop Prot*. 29: 643-651.

Bai, Y., Gu, C.Y., Pan, R., Abid, M., Zang, H.Y., Yang, X., Tan, G. and Chen, Y. 2021. Activity of A Novel Succinate Dehydrogenase Inhibitor Fungicide Pydiflumetofen against *Fusarium fujikuroi* causing Rice Bakanae Disease. *Plant Dis.* 105(10): 3208-3217.

Balesdent, M.H., Barbetti, M.J., Li, H., Sivasithamparam, K., Gout, L., and Rouxel, T. 2005. Analysis of *Leptosphaeria maculans* race structure in a worldwide collection of isolates. *Phytopathol*. 95: 1061-1071.

Balesdent, M.H., Fudal, I., Ollivier, B., Bally, P., Grandaubert, J., Eber, F., Chevre, A.M., Leflon, M. and Rouxel, T. 2013. The dispensable chromosome of *Leptosphaeria maculans* shelters an effector gene conferring avirulence towards *Brassica rapa*. *New Phytol.* 198(3):887–898.

Baker, C. 2000. Systemic acquired resistance. Pages 198-217 in: Molecular Plant Pathology. Annual Plant Reviews, Vol. 4. M. Dickinson and J. Beynon, eds. Sheffield Academic Press, Sheffield, U.K.

Becker, M.G., Haddadi, P., Wan, J., Adam, L., Walker, P., Larkan, N.J., Daayf, F., Borhan, M.H. and Belmonte, M.F. 2019. Transcriptome analysis of Rlm2-mediated host immunity in the *napus–Leptosphaeria maculans* pathosystem. *Mol. Plant-Microbe Interact*. 32(8): 1001-1012.

Becker, M.G., Zhang, X., Walker, P.L., Wan, J.C., Millar, J.L., Khan, D., Granger, M.J., Cavers, J.D., Chan, A.C., Fernando, D.W. and Belmonte, M.F. 2017. Transcriptome analysis of the *Brassica napus–Leptosphaeria maculans* pathosystem identifies receptor, signaling and structural genes underlying plant resistance. *Plant J*. 90(3): 573-586.

Berens, M.L., Berry, H.M., Mine, A., Argueso, C.T. and Tsuda, K. 2017. Evolution of hormone signaling networks in plant defense. *Annu. Rev. Phytopathol*. 55: 401–425.

Bestwick, C.S., Brown, I.R., Bennett, M.H.R., and Mansfield, J.W. 1997. Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas syringae* pv *phaseolicola*. *Plant Cell* 9, 209–221

Birkenbihl, R.P., Diezel, C., and Somssich, I.E. 2012. Arabidopsis WRKY33 is a key transcriptional regulator of hormonal and metabolic responses toward Botrytis cinerea infection. *Plant Physiol*. 159, 266–285.

Bolger, A.M., Lohse, M., and Usadel, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30: 2114–2120.

Borhan, M. H., Van de Wouw, A. P., and Larkan, N. J. 2022. Molecular Interactions Between *Leptosphaeria maculans* and *Brassica* Species. *Annu Rev Phytopathol*, 10.1146/annurev-phyto-021621-120602. Advance online publication.

Bousset, L, Sprague, SJ, Thrall, PH, and Barrett, LG. 2018. Spatio-temporal connectivity and host resistance influence evolutionary and epidemiological dynamics of the canola pathogen Leptosphaeria maculans. *Evol Appl.* 11: 1354– 1370.

Brassica napus Genome Resources. Available online: http://www.genoscope.cns.fr/brassicanapus/ (accessed 15th-30th October 2015).

Breunig, M. and Chilvers, M.I. 2021. Baseline sensitivity of *Fusarium graminearum* from wheat, corn, dry bean and soybean to pydiflumetofen in Michigan, USA. *Crop Protection*, 140, 105419, ISSN 0261-2194.

Canola Council of Canada 2020. Industry overview. Available at: http:// www.canolacouncil.org/markets-stats/industry-overview/ (accessed 1 September 2020).

Castel, B., Ngou, P.M., Cevik, V., Redkar, A., Kim, D.S., Yang, Y., Ding, P., and Jones, J.D.G 2019. Diverse NLR immune receptors activate defence via the RPW8-NLR NRG1. New Phytol. 222, 966–980.

Coffey, T. 2016. Fitting Threshold Models using the SAS® Procedures NLIN and NLMIXED. Pages 1–11 in Proceedings of SAS Conference for Western Users of SAS Software 2016, San Francisco, CA: SAS Institute.

Chen, Y., and Fernando, W.G.D. 2006. Induced resistance to blackleg (*Leptosphaeria maculans*) disease of canola (Brassica napus) caused by a weakly virulent isolate of *Leptosphaeria biglobosa*. *Plant Dis.* 90, 1059–1064.

Chen, X., Tong, C., Zhang, X., Song, A., Hu, M., Dong, W., Chen, F., Wang, Y., Tu, J., Liu, S., Tang, H. and Zhang, L. 2021 A high-quality Brassica napus genome reveals expansion of transposable elements, sub genome evolution and disease resistance. *Plant Biotechnol. J.* https://doi.org/10.1111/pbi.13493.

Chowdhury, S., Basu, A., and Kundu, S. 2017. Biotrophy-necrotrophy switch in pathogen evoke differential response in resistant and susceptible sesame involving multiple signaling pathways at different phases. *Sci. Rep*. 7:17251.

Cools, H. J., Hawkins, N. J., and Fraaije, B. A. 2013. Constraints on the evolution of azole resistance in plant pathogenic fungi. *Plant Pathol*. 62, 36-42.

Cools, H. J., and Ishii, H. 2002. Pre-treatment of cucumber plants with acibenzolar-S-methyl systemically primes a phenylalanine ammonia lyase gene (PAL1) for enhanced expression upon attack with a pathogenic fungus. *Physiol Mol Plant Pathol.* 61(5), 273-280.

Dangl, J.L., Horvath, D.M. and Staskawicz, B.J. 2013. Pivoting the plant immune system from dissection to deployment. *Science* 341: 746–751.

de Lamo, F.J., Spijkers, S.B., and Takken, F.L.W. 2021. Protection to Tomato Wilt Disease Conferred by the Nonpathogen Fusarium oxysporum Fo47 is More Effective Than that Conferred by Avirulent Strains. *Phytopathol.* 111, 253–257.

Delourme, R., Chèvre, A.M., Brun, H., Rouxel, T., Balesdent, M.H., Dias, J.S., Salisbury, P., Renard, M., and Rimmer, S.R. 2006. Major gene and polygenic resistance to *Leptosphaeria maculans* in oilseed rape (*Brassica napus*). *Eur. J. Plant. Pathol*. 114, 41–52.

Dilmaghani, A., Balesdent, M.H., Didier, J.P., Wu, C., Davey, J., Barbetti, M. J., Li, H., Moreno-Rico, O., Phillips, D., Despeghel, J.P., Vincenot, L., Gout, L. and Rouxel, T. 2009. The *Leptosphaeria maculans Leptosphaeria biglobosa* species complex in the American continent. *Plant Pathol*. 58: 1044–1058.

Dodds, P. N., and Rathjen, J. P. 2010. Plant immunity: towards an integrated view of plant–pathogen interactions. *Nat Rev Genet*. 11(8), 539-548.

Does, D.V.d., Leon-Reyes, A., Koornneef, A., Verk, M.C.V., Rodenburg, N., Pauwels, L., Goossens, A., Körbes, A.P., Memelink, J.; Ritsema, T., et al. 2013. Salicylic acid suppresses jasmonic acid signaling downstream of SCFCOI1-JAZ by targeting GCC promoter motifs via transcription factor ORA59. Plant Cell 25, 744–761.

Dolatabadian, A., Cornelsen, J., Huang, S., Zou Z., and Fernando W., G., D. 2022. Sustainability on the farm: breeding for resistance and management of major canola diseases in Canada contributing towards an IPM approach, *Can. J. Plant Pathol.* 44:2, 157-190.

Durrant, W. E., and Dong, X. 2004. Systemic acquired resistance. Annu. Rev. Phytopathol. 42, 185-209.

Eckert, M., Maguire, K., Urban, M., Foster, S., Fitt, B., Lucas, J., and Hammond-Kosack, K. 2005. *Agrobacterium tumefaciens*-mediated transformation of *Leptosphaeria* spp. and *Oculimacula* spp. with the reef coral gene DsRed and the jellyfish gene *gfp*, *FEMS Microbiol. Lett*. 253, 67–74.

Eckert, M. R., Rossall, S., Selley, A., and Fitt, B. D. 2010. Effects of fungicides on in vitro spore germination and mycelial growth of the phytopathogens *Leptosphaeria maculans* and *L. biglobosa* (phoma stem canker of oilseed rape). *Pest Managt Sci.* 66(4), 396-405.

Farmer, E.E., Alme´ras, E. and Krishnamurthy, V. 2003. Jasmonates and related oxylipins in plant responses to pathogenesis and herbivory. *Curr Opin Plant Biol* 6: 372–378.

Fernando, W.D.G. and Zhang, X. 2017. Insights into fighting against blackleg disease of *Brassica napus* in Canada. *Crop Pasture Sci.* 69,40-47.

Fernando, W.G., Zhang, X., and Amarasinghe, C.C. 2016. Detection of Leptosphaeria maculans and Leptosphaeria biglobosa Causing Blackleg Disease in Canola from Canadian Canola Seed Lots and Dockage. *Plants*, 5(1)-12.

Fernando, W.D., Zhang, X., Selin, C., Zou, Z., Liban, S.H., McLaren, D.L., Kubinec, A., Parks, P.S., Rashid, M.H., Padmathilake, K.R.E. and Rong, L. 2018. A six-year investigation of the dynamics of Avirulence allele profiles, blackleg incidence, and mating type alleles of *Leptosphaeria maculans* population associated with canola crops in Manitoba. Canada. *Plant Dis.* 12, 790–798.

Fisher, N., and Meunier, B. 2008. Molecular basis of resistance to cytochrome bc1 inhibitors. *FEMS yeast res*. 8(2), 183-192.

Fitt, B. D. L., Brun, H., Barbetti, M. J. and Rimmer, S. R. 2006. World-wide importance of phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa)* on oilseed rape (*Brassica napus*). *Eur. J. Plant Pathol*. 114: 3–15.

Fitt, B. D., Hu, B. C., Li, Z. Q., Liu, S. Y., Lange, R. M., Kharbanda, P. D., Butterworth, M.H. and White, R. P. 2008. Strategies to prevent spread of *Leptosphaeria maculans* (phoma stem canker) onto oilseed rape crops in China; costs and benefits. *Plant pathol*. *57*(4): 652-664.

Flor, H.H. 1971. Current status of the gene-for-gene concept. *Ann Rev Phytopathol*. 9: 275– 296.

Fu, F., Liu, X., Wang, R., Zhai, C., Peng, G., Yu, F., and Fernando, W.G.D. 2019. Fine mapping of *Brassica napus* blackleg resistance gene *Rlm1* through bulked segregant RNA sequencing. S*ci Rep.* 9(1):14600.

Fu, Z.Q., and Dong, X. 2013. Systemic acquired resistance: turning local infection into global defense. *Annu. Rev. Plant Biol*. 64: 839–863.

Fudal, I., Ross, S., Gout, L., Blaise, F., Kuhn, M.L., Eckert, M.R., Cattolico, L., Bernard-Samain, S., Balesdent, M.H., and Rouxel, T. 2007. Heterochromatin-like regions as ecological niches for avirulence genes in the Leptosphaeria maculans genome: Map-based cloning of AvrLm6. *Mol. Plant-Microbe Interact*. 20, 459–470.

Garalde, D. R., Snell, E. A., Jachimowicz, D., Sipos, B., Lloyd, J. H., Bruce, M. and Turner, D. J. (2018). Highly parallel direct RNA sequencing on an array of nanopores. *Nat methods*. 15(3), 201-206.

Ghanbarnia, K., Dilantha Fernando, W. G., and Crow, G. 2011. Comparison of disease severity and incidence at different growth stages of naturally infected canola plants under field conditions

by pycnidiospores of Phoma lingam as a main source of inoculum. *Can J Plant Pathol*, 33(3), 355-363.

Ghanbarnia, K., Fudal, I., Larkan, N.J., Links, M.G., Balesdent, M.H., Profotova, B., Fernando, W.D., Rouxel, T., and Borhan, M.H. 2015. Rapid identification of the *Leptosphaeria maculans* avirulence gene *AvrLm2*, using an intraspecific comparative genomics approach. Mol. Plant. Pathol. 6, 1–11.

Ghanbarnia, K., Ma, L., Larkan, N.J., Haddadi, P., Fernando, W.G.D., and Borhan, M.H. 2018. *Leptosphaeria maculans AvrLm9*: A new player in the game of hide and seek with *AvrLm4-7*. Mol. Plant Pathol. 19, 1754–1764.

Garg, H., Li, H., Sivasithamparam, K. and Barbetti, M. J. 2013. Differentially expressed proteins and associated histological and disease progression changes in cotyledon tissue of a resistant and susceptible genotype of *Brassica napus* infected with Sclerotinia sclerotiorum. *PLoS ONE* 8, e65205.

Grandaubert, J., Lowe, R. G., Soyer, J. L., Schoch, C. L., Van de Wouw, A. P., Fudal, I., Robbertse, B., Lapalu, N., Links, M. G., Ollivier, B., Linglin, J., Barbe, V., Mangenot, S., Cruaud, C., Borhan, H., Howlett, B. J., Balesdent, M. H., and Rouxel, T. 2014. Transposable element-assisted evolution and adaptation to host plant within the Leptosphaeria maculans-Leptosphaeria biglobosa species complex of fungal pathogens. *BMC genomics*, 15, 891.

Gudelj, I., Fitt, B. D., & Van den Bosch, F. 2004. Evolution of sibling fungal plant pathogens in relation to host specialization. *Phytopathology*, 94(7), 789-795.

Guo, X.W., Fernando, W.G.D. and Entz, M. 2008. Dynamics of infection by *Leptosphaeria maculans* on canola (*Brassica napus*) as influenced by crop rotation and tillage. *Arch. Phytopathol. Pflanzenschutz.* 41: 57-66.

Gout, L., Fudal, I., Kuhn, M.L., Blaise, F., Eckert, M., Cattolico, L., Balesdent, M.H., and Rouxel, T. 2006. Lost in the middle of nowhere: The AvrLm1 avirulence gene of the dothideomycete Leptosphaeria maculans. *Mol. Microbiol*. 60, 67–80.

Gugel, R.K. and Petrie, G.A. 2009. History, occurrence, impact, and control of blackleg of rapeseed. *Can J of Plant Pathol*. 36-45.

Hacquard S, Spaepen S, Garrido-Oter R, and Schulze-Lefert P. 2017. Interplay between innate immunity and the plant microbiota. *Annu Rev Phytopathol*. 55: 565–589.

Haddadi, P., Ma, L., Wang, H. and Borhan, M. H. 2016. Genome wide transcriptomic analyses provide insights into lifestyle transition and efector repertoire of *Leptosphaeria maculans* during the colonization of *Brassica napus* seedlings. *Mol Plant Pathol*. 17: 1196–210.

Hadrami, A., Fernando, W. G., and Daayf, F. 2010. Variations in relative humidity modulate Leptosphaeria spp. pathogenicity and interfere with canola mechanisms of defence. *Eur j plant pathol.* 126(2), 187-202.

Hammerschmidt, R. 1999. Induced disease resistance: How do induced plants stop pathogens? Physiol. Mol. Plant Pathol. 55:77-84. Heath, M. C. (1998). Apoptosis programmed cell death and the hypersensitive cell death. *Eur. J. Plant Pathol*. 104:117-124.

Haanstra, L., Doelman, P. and Voshaar, J.H.O. 1985. The use of sigmoidal dose response curves in soil ecotoxicological research. *Plant Soil*, 84, 293-297.

Hayden, K. J., Garbelotto, M., Knaus, B. J., Cronn, R. C., Rai, H., and Wright, J. W. 2014. Dual RNA-seq of the plant pathogen Phytophthora ramorum and its tanoak host. *Tree Genet Genomes* 10(3), 489-502.

Hayward. (2012) Introduction-oilseed Brassicas In: Genetics, genomics and breeding of oilseed Brassicas pp 1-13

Heath, M. C. 1998. Apoptosis programmed cell death and the hypersensitive cell death. *Eur. J. Plant Pathol*. 104:117-124.

Henry, E., Yadeta, K. A. and Coaker, G. 2013. Recognition of bacterial plant pathogens: Local, systemic, and transgenerational immunity. *New Phytol*. 199: 908–915.

Horsefield, R., Yankovskaya, V., Sexton, G., Whittingham, W., Shiomi, K., Omura, S., Byrne, B., Cecchini, G. and Iwata, S. 2006. Structural and computational analysis of the quinone-binding site of complex II (succinate-ubiquinone oxidoreductase): a mechanism of electron transfer and proton conduction during ubiquinone reduction. *J. Biol. Chem.* 281: 7309-7316.

Huang Y.J., Karandeni-Dewage C.S. and Fitt, B.D.L. 2014. Importance of *Leptosphaeria biglobosa* as a cause of phoma stem canker on winter oilseed rape in the UK. *Asp. Appl. Biol.* 127: 117-122.

Huang, S., Zhang, X. and Fernando, W.G.D. 2020. Directing trophic divergence in Plant-Pathogen Interactions: Antagonistic Phytohormones with No Doubt? *Front Plant Sci*. 11:600063.

Hubbard, M. and Peng, G. 2018. The quantitative resistance against *Leptosphaeria maculans* (blackleg) in Canadian canola cultivars remains effective under increased temperatures. *Plant Pathol.* 67:1329–1338.

Hwang, S.H., Strelkov, S.E., Peng, G., Ahmed, H., Zhou, Q. and Turnbull, G. 2016. Blackleg (*Leptosphaeria maculans*) severity and yield loss in canola in Alberta, Canada. *Plants* 5: 31.

Ikeuchi, M., Iwase, A., Rymen, B., Lambolez, A., Kojima, M., Takebayashi, Y., Heyman, J., Watanabe, S., Seo, M., De Veylder, L., Sakakibara, H. and Sugimoto, K. 2017. Wounding triggers callus formation via dynamic hormonal and transcriptional changes. *Plant physiol.* 175(3):1158–1174.

Jacques, N., Balesdent, M., Rouxel, T. and Laval, V. 2021. New specific quantitative real-time PCR assays shed light on the epidemiology of two species of the *Leptosphaeria maculans*–*Leptosphaeria biglobosa* species complex. *Plant Pathol*. 70(3): 643–54.

Jashni, M. K., Mehrabi, R., Collemare, J., Mesarich, C. H. and de Wit, P. J. 2015. The battle in the apoplast: further insights into the roles of proteases and their inhibitors in plant-pathogen interactions. Front. Plant Sci. 6: 584.

Jones, J. D., and Dangl, J. L. 2006. The plant immune system. *Nature*, 444 (7117), 323-329.

Juska, A., Busch, L., and Tanaka, K. 1997. The blackleg epidemic in Canadian rapeseed as a "normal agricultural accident". *Ecol Appl.* 7(4), 1350-1356.

Jung, H. S., Won, J. S., and Kim, S. W. 2009. An improvement of the performance of multiple-aperture SAR interferometry (MAI). IEEE Trans Geosci Remote Sens IEEE T GEOSCI REMOTE 47(8), 2859-2869.

Keller, H., Blein, J.P., Bonnet, P. and Ricci, P. 1996. Physiological and Molecular Characteristics of Elicitin-Induced Systemic Acquired Resistance in Tobacco. *Plant Physiol*. 110(2): 365-376.

Kiernan, K., Tao, J. and Gibbs, P. 2012. Tips and Strategies for Mixed Modeling with SAS/STAT® Procedures. Pages 1–18 in SAS Global Forum 2012—Statistics and Data Analysis. Cary, NC: SAS Institute.

Kloppholz, S., Kuhn, H. and Requena, N. 2011. A secreted fungal effector of Glomus intraradices promotes symbiotic biotrophy. *Curr Biol*. 21(14): 1204-9.

Knezevic, S.Z., Evans, S.P, Blankenship, E.E., Van Acker, R.C. and Lindquist, J.L. 2002. Critical period for weed control: the concept and data analysis. *Weed Sci.* 50: 773-786.

Koornneef, A., and Pieterse, C. M. J. 2008. Cross talk in defense signaling. *Plant Physiol*. 146: 839-844.

Kostik, V., Memeti, S., and Bauer, B. 2013. Fatty acid composition of edible oils and fats. J. Hyg. Eng. Des. 4, 112-116.

Kraepiel, Y., and Barny, M. A. 2016. Gram-negative phytopathogenic bacteria, all hemibiotrophs after all? *Mol. Plant Pathol*. 17: 313–316.

Kumar, M., Brar, A., Yadav, M., Chawade, A., Vivekanand, V. and Pareek, N. 2018. chitinases—Potential Candidates for Enhanced Plant Resistance towards Fungal Pathogens. *Agriculture* 8(7):88.

Kutcher, H. R., Brandt, S. A., Smith, E. G., Ulrich, D., Malhi, S. S., & Johnston, A. M. (2013). Blackleg disease of canola mitigated by resistant cultivars and four-year crop rotations in western Canada. *Can J Plant Pathol*. 35(2), 209-221.

Kutcher, H.R., Yu, F. and Brun, H. 2010. Improving blackleg disease management of *Brassica napus* from knowledge of genetic interactions with *Leptosphaeria maculans*, *Can J Plant Pathol*. 32:1, 29-34.

Lamichhane, J.R., You, M.P., Laudinot, V., Barbetti, M.J. and Aubertot, J.N. 2020. Revisiting sustainability of fungicide seed treatments for field crops. *Plant Dis*. 104: 610-623.

Larkan, N.J.; Lydiate, D.J.; Parkin, I.A.; Nelson, M.N.; Epp, D.J.; Cowling, W.A.; Rimmer, S.R.; Borhan, M.H. 2013. The Brassica napus blackleg resistance gene LepR3 encodes a receptor-like protein triggered by the Leptosphaeria maculans effector AVRLM1. New Phytol. 197, 595–605.

Larkan, N.J.; Ma, L.; Borhan, M.H. 2015. The Brassica napus receptor-like protein RLM2 is encoded by a second allele of the LepR3/Rlm2 blackleg resistance locus. Plant Biotechnol. J. 13, 983–992.

Lee, S. J. and Rose, J. K. 2010. Mediation of the transition from biotrophy to necrotrophy in hemibiotrophic plant pathogens by secreted effector proteins. *Plant Signal. Behav.* *5*(6): 769–772.

Leptosphaeria maculans - Genome assembly - Ensembl Fungi. Available online: https://fungi.ensembl.org/Leptosphaeria_maculans/Info/Annotation/ (accessed 1st-15th September 2015).

Li, C., Barker, S.J., Gilchrist, D.G., Lincoln, J.E. and Cowling, W.A. 2008. *Leptosphaeria maculans* elicits apoptosis coincident with leaf lesion formation and hyphal advance in *Brassica napus*. *Plant Signal. Behav.* 21: 1143–1153.

Li, N., Han, X., Feng, D., Yuan, D. and Huang, L. J. 2019. Signaling crosstalk between salicylic acid and ethylene/jasmonate in plant defense: Do We Understand What They Are Whispering? *Int J Mol Sci.* 20(3): 671.

Liban, S. H., Cross, D. J., Kutcher, H. R., Peng, G., and Fernando, W. G. D. 2016. Race structure and frequency of avirulence genes in the western Canadian *Leptosphaeria maculans* pathogen population, the causal agent of blackleg in Brassica species. *Plant Pathol*. 65: 1161-1169.

Liu, C. 2014. Evaluation of fungicides for management of blackleg disease on canola and QoI-fungicide resistance in *Leptosphaeria maculans* in western Canada. Master's Thesis, University of Manitoba, Canada.

Liu, Z., Latunde-Dada, A.O., Hall, A.M. and Fitt, B.D.L. 2014. Phoma stem canker disease on oilseed rape (*Brassica napus*) in China is caused by *Leptosphaeria biglobosa* ‘brassicae’. *Eur. J. Plant Pathol*. 140: 841–857.

Liu, S.Y., Liu, Z., Fitt, B.D.L., Evans, N., Foster, S.J., Huang, Y.J., Latunde-Dada, A.O. and Lucas, J.A. 2006. Resistance to *Leptosphaeria maculans* (phoma stem canker) in *Brassica napus* (oilseed rape) induced by *L. biglobosa* and chemical defence activators in field and controlled environments. *Plant Pathol.* 55:401–412.

Liu, S., Liu, R., Latunde-Dada, A. O., Cools, H. J., Foster, S. J., Huang, Y., and Fitt, B. D. (2007). Comparison of Leptosphaeria biglobosa-induced and chemically induced systemic resistance to L. maculans in Brassica napus. *Chinese Sci Bull*. 52(8), 1053-1062.

Livak, K.J. and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) *Methods*. 25(4): 402-8.

Long, Y., Wang, Z., Sun, Z., Fernando, W.G.D., McVetty, B.E.P., Li, G. 2011. Identification of two blackleg resistance genes and fine mapping of one of these two genes in a *Brassica napus* canola variety 'surpass 400'. *Theor. Appl. Genet*. 122, 1223–1231.

Love, M., Anders, S., and Huber, W. 2014. Differential analysis of count data–the DESeq2 package. *GenomeBiol*. 15: 550.

Lowe, R.G.T., Cassin, A., Grandaubert, J., Clark, B.L., Van de Wouw, A.P., Rouxel, T. and Howlett, B.L. 2014. Genomes and Transcriptomes of Partners in Plant-Fungal- Interactions between Canola (*Brassica napus*) and Two *Leptosphaeria* Species. *PLoS ONE* 9(7): e103098.

Mahuku, G.S., Hall, R. and Goodwin, P.H. 1996. Co-infection and induction of systemic acquired resistance by weakly and highly virulent isolates of *Leptosphaeria maculans* in oilseed rape. *Physiol. Mol. Plant Pathol*. 49:61–72.

Mancini, V. and Romanazzi, G. 2014. Seed treatments to control seedborne fungal pathogens of vegetable crops. *Pest Manag. Sci.* 70, 860-868.

Marcroft, S. J., Elliott, V. L., Cozijnsen, A. J., Salisbury, P. A., Howlett, B. J., and Van de Wouw, A. P. 2012. Identifying resistance genes to Leptosphaeria maculans in Australian Brassica napus cultivars based on reactions to isolates with known avirulence genotypes. *Crop Pasture Sci*. 63(4), 338-350.

Marcroft, S.J. and Potter, T.D. 2008. The fungicide fluquinconazole applied as a seed dressing to canola reduces *Leptosphaeria maculans* (blackleg) severity in south-eastern Australia. *Australas. Plant Pathol.* 37, 396-401.

Marcroft, S.J., Sprague, S.J., Pymer, S.J., Salisbury, P.A. and Howlett, B.J. 2004. Crop isolation, not extended rotation length, reduces blackleg (*Leptosphaeria maculans*) severity of canola (*Brassica napus*) in south-eastern Australia. *Aust. J. Exp. Agric*. 44, 601-606.

Markell, S., Gulya, T., McKay, K., Hutter, M., Hollingsworth, C., Ulstad, V. Koch, R. and Knudsvig, A. 2009. Widespread occurrence of the aecial stage of sunflower rust caused by Puccinia helianthi in North Dakota and Minnesota in 2008. *Plant Dis*. 93(6), 668-668.

Martyn, J.D. 1991. Induced resistance to fusarium wilt of watermelon under simulated field conditions. *Plant Dis.* 75:874-877.

Mazáková, J., Urban, J., Zouhar, M. and Ryšánek, P. 2017. Analysis of *Leptosphaeria* species complex causing phoma leaf spot and stem canker of winter oilseed rape (*Brassica napus*) in the Czech Republic. *Crop Pasture Sci*. 68:254–264.

McKeon, T. A., Hayes, D. G., Hildebrand, D. F., and Weselake, R. J. 2016. Introduction to industrial oil crops. In: Industrial Oil Crops (pp. 1-13). AOCS Press.

Mendes-Pereira, E., Balesdent, M.H., Hortense, B. and Rouxel, T. 2003. Molecular phylogeny of the *Leptosphaeria maculans–L. biglobosa* species complex. *Mycol. Res*. 107:1287–1304.

Mitrousia, G. K., Huang, Y. J., Qi, A., Sidique, S., and Fitt, B. 2018. Effectiveness of *Rlm7* resistance against *Leptosphaeria maculans* (phoma stem canker) in UK winter oilseed rape cultivars. *Plant pathol*. 67(6): 1339–1353.

Mohr, P.G.; Cahill, D.M. 2007. Suppression by ABA of salicylic acid and lignin accumulation and the expression of multiple genes, in Arabidopsis infected with Pseudomonas syringae pv. tomato. Funct. Integr. Genomics 7, 181–191

Mur, L. A., Kenton, P., Atzorn, R., Miersch, O. and Wasternack, C. 2016. The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. *Plant Physiol.* 140: 249–262.

O'Connell, R. J., Thon, M. R., Hacquard, S., Amyotte, S. G., Kleemann, J., Torres, M. F., Damm, U., Buiate, E. A., Epstein, L., Alkan, N., Altmüller, J., Alvarado-Balderrama, L., Bauser, C. A., Becker, C., Birren, B. W., Chen, Z., Choi, J., Crouch, J. A., Duvick, J. P., Farman, M. A. and Vaillancourt, L. J. 2012. Lifestyle transitions in plant pathogenic Colletotrichum fungi deciphered by genome and transcriptome analyses. *Nat. Genet*. 44(9): 1060–1065.

Ohm, R.A.; Feau, N.; Henrissat, B.; Schoch, C.L.; Horwitz, B.A.; Barry, K.W.; Condon, B.J.; Copeland, A.C.; Dhillon, B.; Glaser, F.; Altmüller, J. 2012. Diverse lifestyles and strategies of

plant pathogenesis encoded in the genomes of eighteen Dothideomycetes fungi. PLoS Pathog. 8, e1003037.

Oliva, R.; Win, J.; Raffaele, S.; Boutemy, L.; Bozkurt, T.O.; Chaparro-Garcia, A.; Segretin, M.E.; Stam, R.; Schornack, S.; Cano, L.M.; et al. 2010. Recent developments in effector biology of filamentous plant pathogens. Cell. Microbiol. 12, 705–715.

OmicsBox—Bioinformatics Made Easy. BioBam Bioinformatics, March 3, 2019. Available online: https://www.biobam.com/omicsbox. (Accessed on 5th May 2019).

Otulak-Kozieł, K., Kozieł, E., Bujarski, J. J., Frankowska-Łukawska, J., and Torres, M. A. 2020. Respiratory Burst Oxidase Homologs *RBOHD* and RBOHF as Key Modulating Components of Response in Turnip Mosaic Virus-*Arabidopsisthaliana* (L.) Heyhn System. *Int. J. Mol. Sci.* 21(22): 8510.

Otulak-Kozieł, K., Kozieł, E., and Valverde, R. A. 2019. The Respiratory Burst Oxidase Homolog D (*RbohD*) Cell and Tissue Distribution in Potato-Potato Virus Y (PVYNTN) Hypersensitive and Susceptible Reactions. *Int. J. Mol. Sci.* 20(11): 2741.

Padder, S.A., Prasad, R., and Shah, A.H. 2018. Quorum sensing: A less known mode of communication among fungi. *Microbiol. Res*. 210: 51-58 ISSN 0944-5013.

Parlange, F., Daverdin, G., Fudal, I., Kuhn, M.L., Balesdent, M.H., Blaise, F., Grezes-Besset, B. and Rouxel, T. 2009. *Leptosphaeria maculans* avirulence gene *AvrLm4-7* confers a dual recognition specificity by the Rlm4 and Rlm7 resistance genes of oilseed rape, and circumvents *Rlm4*-mediated recognition through a single amino acid change. *Mol. Microbiol. 71*, 851–863.

Paul, V.H. and Rawlinson, C.J. 1992. Diseases and pests of oilseed rape; Gelsenkirchen-Buer: Verlag Theodore Mann, Germany.

Peng, G., Fernando, W.G.D., Kirkham, C.L., Lange, R., McLaren, D., Kutcher, H.R. et al. 2012. Mitigating the risk of blackleg disease of canola using fungicide strategies. In: Proceedings of 2012 Soils and Crops Workshop; 13 March 2012, Saskatoon (Canada). p. 8.

Peng, G., Liu, C., Fernando, D.W.G., Lang, R., McLaren, D.L., Johnson, E.N. et al. (2021) Early fungicide treatment reduces blackleg on canola but yield benefit is realized only on susceptible cultivars under high disease pressure. *Canadian Journal of Plant Pathology*, 43, 384–393.

Peng, G., Liu, X., McLaren, D.L., McGregor, L. & Yu, F. (2020) Seed treatment with the fungicide fluopyram limits cotyledon infection by *Leptosphaeria maculans* and reduces blackleg of canola. *Canadian Journal of Plant Pathology*, 42, 480–492.

Pieterse, C.M.J., Leon-Reyes, A., Van der Ent, S., Van Wees, S.C.M. 2009. Networking by small-molecule hormones in plant immunity. *Nat. Chem. Biol*. 5: 308–316.

Pieterse, C.M., van Wees, S.C., van Pelt, J.A., Knoester,M., Laan, R., Gerrits, H., Weisbeek, P.J. and van Loon, L.C. 1998. A novel signaling pathway controlling induced systemic resistance in Arabidopsis. *Plant Cell* 10: 1571–1580.

Petit-Houdenot, Y. and Fudal, I. 2017. Complex Interactions between Fungal Avirulence Genes and Their Corresponding Plant Resistance Genes and Consequences for Disease Resistance Management. *Front. Plant Sci*. 8, 1072.

Plissonneau, C.; Hartmann, F.E.; Croll, D. 2016. Pangenome analyses of the wheat pathogen Zymoseptoria tritici reveal the structural basis of a highly plastic eukaryotic genome. BMC Biol. 16, 1–16.

Plissonneau, C.; Benevenuto, J.; Mohd-Assaad, N.; Fouché, S.; Hartmann, F.E.; Croll, D. 2017. Using population and comparative genomics to understand the genetic basis of effector-driven fungal pathogen evolution. Front. Plant Sci. 8, 119.

Raman, R., Taylor, B., Marcroft, S., Stiller, J., Eckermann, P., Coombes, N., and Raman, H. 2012. Molecular mapping of qualitative and quantitative loci for resistance to *Leptosphaeria maculans* causing blackleg disease in canola (*Brassica napus* L.). *Theor Appl Genet*. 125(2), 405-418.

Reis, E.M., Zanatta, M., Carmona, M., and Menten, J.O.M. 2015. Relationship between IC50 determined in vitro/in vivo and the fungicide rate used in the Field. *Summa Phytopathol.* 41, 49–53.

Rimmer, S. R. 2006. Resistance genes to *Leptosphaeria maculans* in *Brassica napus*. *Can. J. Plant Pathol*. 28: 288–297.

Robinson, M.D., McCarthy, D.J. and Smyth, G.K. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26: 139–140.

Rouxel, T. and Balesdent, M.H. 2005. The stem canker (blackleg) fungus, *Leptosphaeria maculans*, enters the genomic era. *Mol. Plant Pathol*. 6:225–241.

Rouxel, T., Penaud, A., Pinochet, X., Brun, H. and Gout, L. 2003. A 10-year survey of populations of *Leptosphaeria maculans* in France indicates a rapid adaptation towards the Rlm1 resistance gene of oilseed rape. *Eur. J. Plant Pathol*. 109: 871–881.

SAS Institute Inc. 2017. The NLMixed Procedure. Pages 6723–6837 in SAS/STAT 14.3 User's Guide. Cary, NC: SAS Institute.

Schneider, C.A.; Rasband, W.S.; Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis (2012). Nat. Methods 9, 671–675.

Sexton, A.C. and Howlett, B.J. 2001. Green Fluorescent Protein (GFP) as a reporter in the Brassica—Leptosphaeria maculans interaction. Physiol. *Mol. Plant Pathol*. 58, 13–21.

Sharma, R.K., Patel, S., Pargaien, K.C. (2012). Synthesis, characterization and properties of Mn-doped ZnO nanocrystals. *Adv. Nat. Sci. Nanosci. Nanotechnol*. *3*, 035005.

Shoemaker, R.A. and Brun, H. 2001. The teleomorph of the weakly aggressive segregate of *Leptosphaeria maculans*. *Can. J Bot.* 79:412–419.

Sierotzki, H. and Scalliet, G. 2013. A review of current knowledge of resistance aspects for the next-generation succinate dehydrogenase inhibitor fungicides. *Phytopathol*. 103: 880–887.

Sonah, H., Zhang, X., Deshmukh, R. K., Borhan, M. H., Fernando, W. D., and Belanger, R. R. 2016. Comparative transcriptomic analysis of virulence factors in Leptosphaeria maculans during compatible and incompatible interactions with canola. *Front plant sci*. 7, 1784.

Spoel, S. H., Johnson, J. S. and Dong, X. 2007. Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. *Proc. Natl Acad. Sci. USA* 104: 18842–18847.

Sprague, S. J., Kirkegaard, J. A., Hamblin, P., and Graham, J. 2007. Responses to blackleg fungicides in southern New South Wales. In Proc. 15th Biennial Australian Research Assembly Conf–Brassicas (pp. 192-195).

Stark, R., Grzelak, M., and Hadfield, J. 2019. RNA sequencing: the teenage years. *Nat Rev Genet.* 20(11), 631-656.

Stergiopoulos, I. and de Wit, P.J.G.M. 2009. Fungal effector proteins. *Annu. Rev. Phytopathol.* 47, 233–263.

Stotz, H.U., Harvey, P.J., Haddadi, P., Mashanova, A., Kukol, A., Larkan, N.J., Borhan, M.H. and Fitt, B.D.L. 2018. Genomic evidence for genes encoding leucine-rich repeat receptors linked to resistance against the eukaryotic extra- and intracellular *Brassica napus* pathogens *Leptosphaeria maculans* and *Plasmodiophora brassicae*. *PLoS ONE* 13(6): e0198201.

Stotz, H. U., Mitrousia, G. K., de Wit, P. J., and Fitt, B. D. 2014. Effector-triggered defence against apoplastic fungal pathogens. *Trends Plant Sc.* 19(8), 491-500.

Summanwar, A.; Basu, U.; Kav, N.N.; Rahman, H. 2021. Identification of lncRNAs in response to infection by Plasmodiophora brassicae in Brassica napus and development of lncRNA-based SSR markers. Genome 64, 547–566.

Suzuki, N., Miller, G., Morales, J., Shulaev, V., Torres, M.A. and Mittler. R. 2011. Respiratory burst oxidases: the engines of ROS signaling. *Curr Opin Plant Biol*. 14(6):691-9.

Tamaoki, D., Seo, S., Yamada, S., Kano, A., Miyamoto, A., Shishido, H., Miyoshi, S., Taniguchi, S., Akimitsu, K., and Gomi, K. 2013. Jasmonic acid and salicylic acid activate a common defense system in rice. *Plant Signal. Behav*. 8(6): e24260.

Tan, K. C., Oliver, R. P., Solomon, P. S., and Moffat, C. S. 2010. Proteinaceous necrotrophic effectors in fungal virulence. *Funct. Plant Biol.* 37(10): 907-912.

Thomma, B. P., Nürnberger, T., and Joosten, M. H. 2011. Of PAMPs and effectors: the blurred PTI-ETI dichotomy. *Plant cell*, 23(1), 4-15.

Trapnell, C., Pachter, L., and Salzberg, S.L. 2009. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* 25:1105–1111.

Tsuda, K., Mine, A., Bethke, G., Igarashi, D., Botanga, C. J., Tsuda, Y., Glazebrook, J., Sato, M., and Katagiri, F. 2013. Dual regulation of gene expression mediated by extended MAPK activation and salicylic acid contributes to robust innate immunity in Arabidopsis thaliana. PLoS genet. 9(12), e1004015.

Tsuda, K. and Katagiri, F. 2010. Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. *Curr. Opin. Plant Biol.* 13: 459–465.

Tsuda, K., Sato, M., Stoddard, T., Glazebrook, J., and Katagiri, F. 2009. Network properties of robust immunity in plants. *PLoS Genet*. 5:e1000772.

Van de Wouw, A. P., Cozijnsen, A. J., Hane, J. K., Brunner, P. C., McDonald, B. A., Oliver, R. P. and Howlett, B.J. 2010. Evolution of linked avirulence effectors in *Leptosphaeria maculans* is affected by genomic environment and exposure to resistance genes in host plants. *PLoS Pathog*. 6: 1–15.

Van de Wouw, A.P., and Howlett, B.J. 2020. Advances in understanding the Leptosphaeria maculans - Brassica pathosystem and their impact on disease management. *Can. J. Plant Pathol*. 42: 149-163.

Van de Wouw, A.P., Idnurm, A., Davidson, J.A., Sprague, S. J., Khangura, R.K., Ware, A.H. Lindbeck, K.D. and Marcroft, S.J. 2016. Fungal diseases of canola in Australia: identification of trends, threats and potential therapies. *Australasian Plant Pathol*. 45: 415–423.

Van de Wouw, A.P., Lowe, R.G.T., Elliott, C.E., Dubois, D.J. and Howlett, B.J. 2014. Avirulence gene, *AvrLmJ1*, of *L. maculans*. *Mol. Plant Pathol.* 15: 523-530.

Van der Does, D., Leon-Reyes, A., Koornneef, A., Van Verk, M.C., Rodenburg, N., Pauwels, L., Goossens, A., Körbes, A.P., Memelink, J., Ritsema, T., Van Wees, S.C.M. and Pieterse, C.M.J. 2013. Salicylic acid suppresses jasmonic acid signaling downstream of SCFCOI1-JAZ by targeting GCC promoter motifs via transcription factor ORA59. *Plant Cell* 25: 744–761.

Vincenot, L., Balesdent, M.H., Li, H., Barbetti, M.J., Sivasithamparam, K., Gout, L. and Rouxel, T. 2008. Occurrence of a new subclade of *Leptosphaeria biglobosa* in Western Australia. *Phytopathol*. 98:321–329.

Vlot, A.C., Dempsey,D.A., and Klessig, D.F. 2009. Salicylic Acid, a multi-faceted hormone to combat disease. *Annu. Rev. Phytopathol*. 47: 177–206.

Vlot, A. C., Klessig, D. F. and Park, S. W. 2008. Systemic acquired resistance: The elusive signal(s). *Curr. Opin. Plant Biol*. 11: 436-442.

West, J.S., Balesdent, M.H., Rouxel, T., Nancy, J.P., Huang, Y.J., Rouxel, B.J., Steed, J.M., Fitt, B.D.L. and Schmit, J. 2002. Colonisation of winter oilseed rape tissues by A/Tox+ and B/Tox0 *Leptosphaeria maculans* (phoma stem canker) in France and England. *Plant Pathol*. 51:311–321.

West, J.S., Biddulph, J.E., Fitt, B.D.L., and Gladders, P. 1999. Epidemiology of *Leptosphaeria maculans* in relation to forecasting stem canker severity on winter oilseed rape in the UK.. *Appl. Biol*. 135:535–546.

West, J. S., Kharbanda, P. D., Barbetti, M. J. and Fitt, B. D. L. 2001. Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. *Plant Pathol.* 50: 10–27.

Westermann, A. J., Barquist, L., and Vogel, J. 2017. Resolving host–pathogen interactions by dual RNA-seq. *PLoS pathog*. 13(2), e1006033.

Westermann, A. J., Gorski, S. A. and Vogel, J. (2012). Dual RNA-seq of pathogen and host. *Nat Rev Microbiol*. 10(9), 618-630.

Williams, R.H. and Fitt, B.D.L. 1999. Differentiating A and B groups of *Leptosphaeria maculans*, causal agent of stem canker (blackleg) of winter oilseed rape. *Plant Pathol*. 48:161–75.

Wouw, A.P.V.d.; Cozijnsen, A.J.; Hane, J.K.; Brunner, P.C.; McDonald, B.A.; Oliver, R.P.; Howlett, B.J. 2010. Evolution of linked avirulence effectors in *Leptosphaeria maculans* is affected by genomic environment and exposure to resistance genes in host plants. *PLoS Pathog*. *6*, 1–15.

Wouw, A.P.V.d.; Marcroft, S.J.; Ware, A.; Lindbeck, K.; Khangura, R.; Howlett, B.J. 2014. Breakdown of resistance to the fungal disease, blackleg, is averted in commercial canola (Brassica napus) crops in Australia. Field Crops Res. 166, 144–151.

Yang, C. 2021. The regulation of intrinsic signaling in *Brassica napus* defending against *Leptosphaeria maculans*. PhD thesis, University of Manitoba, Winnipeg, MB, Canada, 172-173.

Yang, H. 2018. Identification of candidate genes for blackleg resistance in the new *Brassica juncea* canola. PhD Thesis, The University of Queensland, Brisbane, Australia.

Yang, C. and Fernando, W.G.D. 2021. Hormonal Responses to Susceptible, Intermediate, and Resistant Interactions in the *Brassica napus–Leptosphaeria maculans* Pathosystem. *Int. J. Mol. Sci*. 22: 4714.

Yu, F.; Lydiate, D.J.; Hahn, K.; Kuzmicz, S.; Hammond, C.; Rimmer, S.R. Identification and mapping of a novel blackleg resistance locus LepR4 in the progenies from Brassica napus × B. rapa subsp. sylvestris. In Proceedings of the 12th Innovation in Rapeseed and Canola, Wuhan, China, 26–30 March 2007.

Yu, F.; Lydiate, D.J.; Rimmer, S.R. (2008). Identification and mapping of a third blackleg resistance locus in Brassica napus derived from B. rapa subsp. Sylvestris. Genome 51, 64–72.

Zhai, C., Liu, X., Song, T., Yu, F., and Peng, G. 2021. Genome-wide transcriptome reveals mechanisms underlying Rlm1-mediated blackleg resistance on canola. *Sci. rep* 11(1): 1-17.

Zhang, X. 2016. Exploring disease resistance in the *Brassica napus- Leptosphaeria maculans pathosystem.* PhD thesis, University of Manitoba, Winnipeg, MB, Canada, 197.

Zhang, X., and Fernando, W. G. D. 2018. Insights into fighting against blackleg disease of *Brassica napus* in Canada. *Crop Pasture Sci*. 69: 40–47.

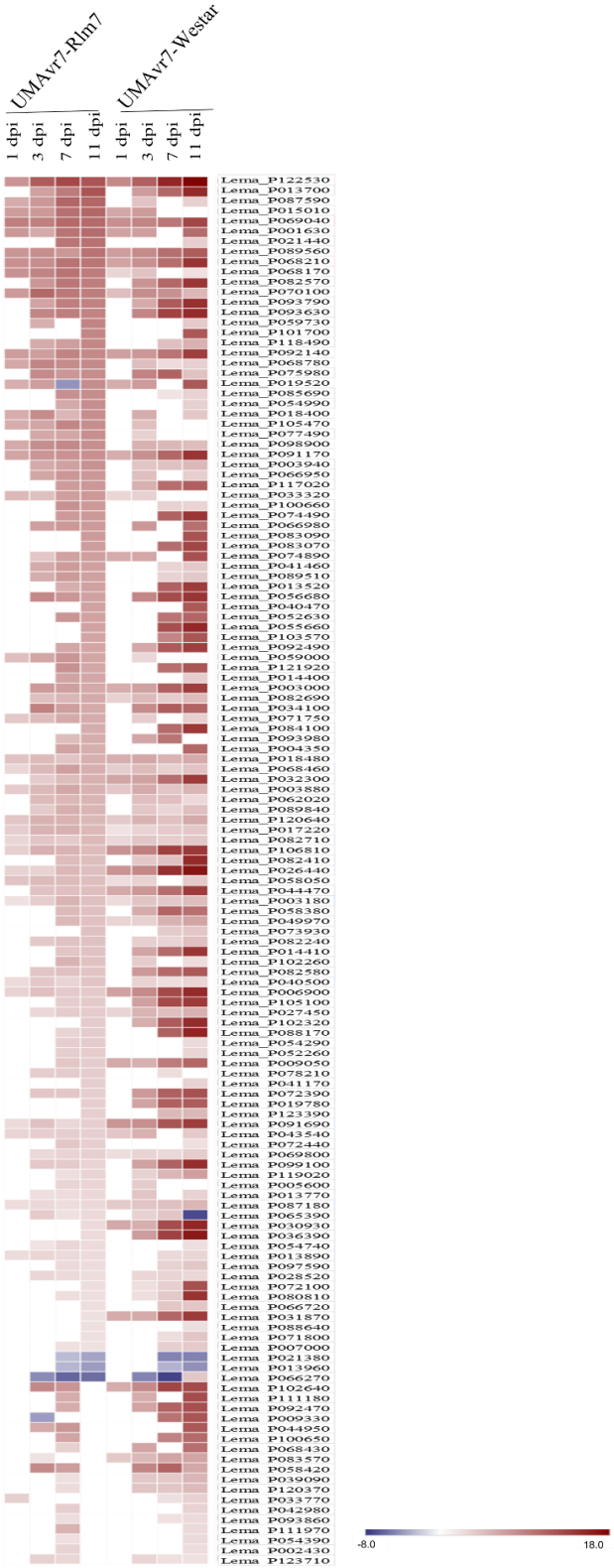
Zhang, X., Peng, G., Kutcher, H.R., Balesdent, M.H., Delourme, R., and Fernando, W.G.D. 2016. Breakdown of *Rlm3* resistance in the *Brassica napus–Leptosphaeria maculans* pathosystem in western Canada. *Eur. J. Plant Pathol.* 145, 659–674.

Zhen, X., Bingxiao, Y., Jianyao, S., Jun, T., Xin, W., Keran, Z., Jiyun, L., Qun, L., Meizhong,L., Yiwen, D. and Zuhua, D. 2019. A nucleotide-binding site-leucine-rich repeat receptor pair confers broad-spectrum disease resistance through physical association in rice Phil. *Trans. R. Soc*. B37420180308.

Zou, Z., Liu, F., Selin, C. and Fernando, W.G.D. 2020. Generation and Characterization of a Virulent *Leptosphaeria maculans* Isolate Carrying a Mutated AvrLm7 Gene Using the CRISPR/Cas9 System. *Front. Microbiol.* 11:1969.

Zou, Z., Zhang, X., Parks, P., du Toit. L.J., Van de Wouw, A.P. and Fernando, W.G.D. 2019. A New Subclade of *Leptosphaeria biglobosa* Identified from *Brassica rapa*. *Int. J. Mol. Sci*. 20(7):1668.

APPENDICES


UMAvr7-Rlm7
UMAvr7-Westar
1 dpi
3 dpi
7 dpi
11 dpi
1 dpi
3 dpi
7 dpi
11 dpi
-8.0
18.0

Appendix 1: Differentially expressed Cazyme genes of *Leptosphaeria maculans* isolates, UMAvr7 and umavr7 *in planta* of Rlm7.Differentially expressed Cazymes genes of UMAvr7 and umavr7 isolates *in planta* of Rlm7 at 1, 3-, 7-, and 11- dpi compared to zero dpi. Logarithm of absolute value of fold change cutoff (logFC) ≥ 2 and ≤ -2 with false discovery rate (FDR) ≤ 0.05 were considered.

Appendix 2: Differentially expressed genes (DEGs) in UMAvr7 compared to umavr7 in Axenic culture. UMAvr7 isolate showed 114 DEGs compared to umavr7 in in vitro with expression log of absolute value of fold change cutoff (logFC) ≥ 2 and false discovery rate (FDR) ≤ 0.05 were considered.

Gene locus	Log2 Fold Change	FDR p-value correction
Lema_P001940	9.51	4.0979E-295
Lema_P001950	8.90	1.5584E-111
Lema_P001960	5.86	1.1446E-169
Lema_P113300	4.86	1.41803E-98
Lema_P007210	4.84	1.9681E-113
Lema_P105290	4.71	2.2307E-142
Lema_P004820	4.67	3.05714E-53
Lema_P020960	4.50	2.94213E-88
Lema_P019630	4.20	2.43506E-73
Lema_P019640	4.14	1.17187E-77
Lema_P105280	3.96	2.95928E-55
Lema_P029920	3.93	7.1182E-198
Lema_P109850	3.93	5.4742E-129
Lema_P048740	3.92	0.049309236
Lema_P110640	3.80	6.9109E-106
Lema_P002480	3.67	2.27949E-60
Lema_P116240	3.64	1.37115E-50
Lema_P051470	3.62	8.47278E-81
Lema_P124100	3.53	2.96317E-83
Lema_P005170	3.53	0.049309236
Lema_P116170	3.53	6.17969E-55
Lema_P067150	3.49	2.91884E-75
Lema_P116460	3.42	1.02742E-51
Lema_P051480	3.34	1.325E-100
Lema_P118290	3.33	4.39695E-83
Lema_P112380	3.27	3.1833E-117

Lema_P004780	3.25	3.11884E-43
Lema_P006920	3.24	5.5316E-113
Lema_P060030	3.21	5.0406E-53
Lema_P073150	3.20	4.1134E-199
Lema_P016760	3.20	1.15427E-84
Lema_P074180	3.17	1.71756E-63
Lema_P115080	3.16	1.351E-109
Lema_P114820	3.10	2.1175E-55
Lema_P087320	3.04	1.02126E-48
Lema_P067600	3.01	3.8603E-111
Lema_P048340	3.00	1.86522E-96
Lema_P087800	3.00	1.05706E-90
Lema_P016030	2.98	3.19255E-47
Lema_P083420	2.95	6.32777E-82
Lema_P087810	2.95	1.86099E-84
Lema_P116190	2.95	8.4746E-55
Lema_P032080	2.92	6.22955E-93
Lema_P021040	2.89	2.1056E-99
Lema_P022070	2.87	3.75511E-94
Lema_P110690	2.84	9.1801E-131
Lema_P057900	2.82	1.0829E-103
Lema_P034940	2.78	2.20628E-79
Lema_P077070	2.77	7.05183E-61
Lema_P093520	2.72	1.85349E-62
Lema_P103220	2.69	8.15585E-43
Lema_P078930	2.67	8.47748E-87
Lema_P010440	2.67	9.55827E-46
Lema_P002320	2.66	2.31015E-64
Lema_P039910	2.63	0
Lema_P063620	2.55	9.2406E-128
Lema_P113070	2.49	0

Lema_P113100	2.48	0
Lema_P063060	2.48	3.2019E-101
Lema_P034930	2.47	2.3298E-71
Lema_P087610	2.45	1.29016E-93
Lema_P009910	2.45	5.4094E-107
Lema_P001850	2.44	0
Lema_P004530	2.44	2.23944E-57
Lema_P113090	2.43	0
Lema_P010470	2.42	0
Lema_P060020	2.38	1.83867E-93
Lema_P099810	2.37	4.9907E-119
Lema_P012380	2.35	3.73618E-44
Lema_P039000	2.30	2.99357E-47
Lema_P059260	2.27	1.93156E-43
Lema_P113130	2.25	0
Lema_P056470	2.25	1.13524E-42
Lema_P073380	2.25	2.34446E-81
Lema_P113120	2.23	0
Lema_P104820	2.22	2.3496E-67
Lema_P066320	2.18	9.78125E-60
Lema_P113110	2.15	0
Lema_P021830	2.12	6.94102E-46
Lema_P037530	2.06	0
Lema_P025430	2.06	0
Lema_P011200	2.05	3.1841E-89
Lema_P093680	2.03	1.15947E-62
Lema_P023990	2.02	0
Lema_P116780	2.01	1.13524E-42

Appendix 3: Identified Brassica resistance (R) genes

No.	*R* gene	Species	References
1	*Rlm1*	*B. napus*	Fu et al. (2019)
2	***Rlm2***	*B. napus*	Larkan et al. (2013)
3	*Rlm3*	*B. napus*	Balesdent et al. (2013)
4	***Rlm4***	*B. napus*	Parlange et al. (2009)
5	*Rlm5*	Re-synthesized, *B. juncea*	Ghanbarnia et al. (2018)
6	*Rlm6*	*B. juncea*	Yang (2018)
7	***Rlm7***	*B. napus*	Parlange et al. (2009)
8	*Rlm8*	*B. rapa*	Larkan et al. (2016)
9	***Rlm9***	*B. napus*	Rimmer (2006)
10	*Rlm10*	*B. nigra*	Petit-Houdenot et al. (2019)
11	*Rlm11*	*B. rapa*	Balesdent et al. (2013)
12	*Rlm12*	*B. napus*	Raman et al. (2020)
13	*Rlm13*	*B. napus*	Raman et al. (2021)
14	*LepR1*	Re-synthesized *B. rapa* subsp. sylvestris	Cantila et al. (2021)
15	*LepR2*	Re-synthesized *B. oleracea*, *B. rapa* subsp. sylvestris	Cantila et al. (2021)
16	***LepR3***	Re-synthesized *B. rapa* subsp. sylvestris	Larkan et al. (2013)
17	*LepR4a,b*	Re-synthesized *B. oleracea*, *B. rapa* subsp. sylvestris	Yu et al. (2012)
18	*RlmS*	Re-synthesized *B. rapa* subsp. sylvestris	Cantila et al., (2021)
19	*BLMR1*	*B. napus* Surpass 400	Long et al. (2011)
20	*BLMR2*	*B. napus* Surpass 400	Long et al. (2011)
21	*LMJR1*	*B. juncea*	Cantila et al. (2021)
22	*LMJR2*	*B. juncea*	Cantila et al. (2021)
23	*rjlm2*	*B. juncea*	Cantila et al. (2021)

Out of 23 resistance genes identified, five resistance genes have been cloned (**bold**).

Note: Adapted from Dolatabadian et al., 2021 and Borhan et al., 2022

Appendix 4: List of Abbreviations

ANOVA	Analysis of variance
Cas9	CRISPR-associated protein 9
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CTAB	Cetyltrimethylammonium bromide
DMI	Demethylation inhibitors
DPI	Days post inoculation
DsRed	Red fluorescent protein from Discosoma sp.
ET	Ethylene
ETI	Effector-triggered immunity
FPKM	Fragments Per Kilobase of gene per Million mapped reads
GFP	Green fluorescent protein
HPI	Hours post inoculation
HR	Hypersensitive response
ICBN	International Blackleg of Crucifers Network
i.e.	Active ingredient
JA	Jasmonic acid
MAPKs	Mitogen-activated protein kinases
MR	Moderately resistant
NBS-LRR	Nucleotide binding site-leucine-rich repeats
NGS	Next-generation sequencing
ORF	Open reading frame
QoI	Quinone outside inhibitors
QTL	Quantitative trait Loci
RNA-seq	RNA sequencing
SA	Salicylic acid
SDHI	Succinate dehydrogenase inhibitor