Canola Agronomic Research Program (CARP)
FINAL REPORT

The Final Report should fully describe the work completed for the year, noting the personnel involved and any deviations from the original plan and next and/or corrective steps as may be required if deviations are noted. A final financial statement summary of expenses must also be submitted. In the event of major changes within the budget, supporting notes are required. The final report is a summary of activity for the final year and an overview of the entire project.

Project Title: ***Investigating interactions of ascospores and pycnidiospores with blackleg resistance in canola and efficacy of seed applied fungicides in these specific interactions in western Canada***

Research Team Information

Lead Researcher:		
Name	***Institution***	***Project Role***
Dr. Dilantha Fernando	University of Manitoba	Principal Investigator
Research Team Members (add rows as required)		
Name	***Institution***	***Project Role***
Dr. Gary Peng	AAFC Saskatoon	Co-Investigator
Dr. Shuanglong Huang	University of Manitoba	Co-Investigator

Project Start Date: April 1, 2021 **Project Completion Date:** March 31, 2024

Reporting Period: April 1, 2023 **to** March 31, 2024

CARP Project Number: **2021.44**

Instructions: This Final Project Report shall be completed and submitted on March 31st of the fiscal year that the agreement is in effect (upon completion of the project). The Lead Researcher of the project in question shall complete and submit the report on behalf of his/her complete research team.

This Report is a means by which to provide a detailed account upon completion of the project. Final project financial reporting should be provided at this time.

The following template is provided to assist you in completing this task. Please forward the completed document electronically to the CCC contact listed below.

In addition to the Final Research Report, a *Final Research Abstract/Extension Report* is due upon completion of the project, maximum 2-3 pages, to be used for publication on the Funders' websites and in the *Canola Digest*. Content will be used in extension material, for consumers and/or industry. Include an Executive Summary, brief project description, key findings and conclusions (with a summary graph/table or supporting image for the project), translation of key findings into best management practices and/or relevance to the canola sector and future research, and funding acknowledgment as determined in the grant award letter. The Final Extension Report is intended to support messaging to all audiences. Information needs to be clear, concise and in "grower-friendly" language.

Please include the funding acknowledgements outlined in your research agreement in all deliverables (publications, presentations, etc.) from this project.

1. Date of completion & status of activity (please check one)

Date of completion: _March 31, 2024_____

☐ **Ahead of Schedule** ☑ **On Schedule** ☐ **Behind Schedule** ☐ **Completed**

Comments: The production of ascospore and pycnidiospore inoculums in the lab was successfully established, and these type-specific inoculums have been used to test Canadian commercial canola lines in both controlled environment and field conditions, with or without fungicide treatment against blackleg. Disease resistance levels have been evaluated from seedling and adult plant stages, and key data has been collected with analysis. Further data analysis is ongoing. Fungal characterization from the stubbles collected from field trials is still ongoing. Manuscript preparation in ongoing.

2. Summary - Maximum of one page. This must include project objectives, results, and conclusions.

The objectives of this proposed study are to: 1. Develop a protocol to efficiently produce ascospore and pycnidiospore inoculum with defined Avr profile for inoculation of canola seedlings in research and resistance screening by industry; 2. Assess potential interactions of inoculum types (ascospores and pycnidiospores) with blackleg resistance (major and minor); 3. Evaluate the efficacy of seed applied fungicides against the infection by ascospores and pycnidiospores influenced by genetic resistance.

Three approaches were developed and one of these three approaches was optimized for the production of ascospores and pycnidiospores in the lab. These ascospores and pycnidiospores were then used to elucidate the interactions of ascospores and pycnidiospores with commercial canola varieties in controlled environments. A panel of 17 commercial canola lines acquired from seed companies were inoculated with ascospores, pycnidiospores and a combination of the two and assessed for seedling and quantitative resistance, following standard cotyledon inoculation method for seedling resistance (Zhang et al. 2016) and petiole inoculation method for quantitative resistance (Huang et al. 2014). These ascospores and pycnidiospores were also used to evaluate the efficacy of seed applied fungicides against the infection by ascospores and pycnidiospores influenced by genetic resistance under field conditions. The experiments as detailed in below sections were carried out in Carman MB and Melfort SK for the two years 2022 and 2023.

The controlled-environment studies indicated that, as compared to the typical susceptible reactions in Westar, at the seedling stage, resistant, intermediate and susceptible reactions were observed in these 17 commercial canola lines when inoculated with ascospores, pycnidiospores and a combination of the two. While a mixture of ascospores and pycnidiospores has shown the greatest aggressiveness, followed by ascospores and pycnidiospores, respectively. At the adult plant stage, quantitative resistance was present in all the 17 commercial canola lines when inoculated with ascospores, pycnidiospores and a combination of the two, with varied level of quantitative resistance observed across the panel. Similarly to seedling resistance, when interacting with these quantitative resistance, a mixture of ascospores and pycnidiospores has shown the greatest aggressiveness, followed by ascospores and pycnidiospores, respectively.

Data from the two-year (2022 and 2023) field trials set in Carman MB and Melfort SK showed that, a similar trend of the aggressiveness of these spores was also found in both Westar and InVigor L255PC at seedling and adult plant stages, when these inoculums were directly applied to freshly pricked wounds. A reduction in cotyledon rating score at seedling stage and disease severity at adult plant resistance stage was detected when

Westar and InVigor L255PC were applied with Fluopyram fungicide seed treatment. As compared to Westar, the quantitative resistance in InVigor L255PC was effective in lowering disease severity index at adult plant resistance stage.

In conclusion, this is the first investigation into the infection by pycnidio- and asco-spores, as well as a mixture via wounds under the influence of cultivar resistance and fungicide seed treatment. The mixture of ascospores and pycnidiospores exhibited the greatest aggressiveness level, followed by ascospores and pycnidiospores respectively, in both in-door and field conditions. When the inoculums were directly applied through fresh wounds, the efficacy of Fluopyram seed treatment improves, this corroborates with previous findings (Huang et al. 2023), but not when canola plants were infected with natural inoculums (Huang et al. 2024). Canadian canola lines showed varied levels of quantitative resistance against different spore types.

3. Introduction – Brief project background, rationale, and objectives.

In the Brassica napus - Leptosphaeria maculans pathosystem, inoculum types, the sexual ascospores and the asexual pycnidiospores, are critical for blackleg disease development and epidemiology, where environmental conditions could have huge impacts. Briefly, in Europe, ascospore showers are believed to be the major inoculum source. In Australia, the inoculum can be ascospores as the major source, accompanied also by pycnidiospores (Barbetti, 1976; Marcroft et al., 2004). In western Canada, pycnidiospores appear to be an important source of inoculum in infection and disease development (Petrie, 1995; Guo and Fernando, 2005; Ghanbarnia et al., 2011; Dilmaghani et al., 2013; Zhang and Fernando, 2018), while it seems sexual reproduction is not inhibited in western Canada especially Manitoba as revealed in a long-term study (Fernando et al., 2018). Guo and Fernando, 2005; Ghanbarnia et al. 2011; and Zhang and Fernando, 2018 [all from the Fernando lab] clearly showed the importance of pycnidiospores as primary inoculum along with blackleg infection in Western Canada. The Canola Council of Canada now uses this information in educational material to farmers and ag reps. However, it remains largely unknown how these inoculum types (ascospores vs. pycnidiospores) interact with canola blackleg resistance (major and minor), especially under the influence of fungicide directly applied to the seeds in western Canada, such as the newly adopted fungicides FluopyramTM (Peng et al., 2020). This proposed research project precisely identifies the knowledge gap that is as important as current practices like resistance gene identifications and R gene rotations for blackleg disease management in western Canada. By investigating the specific interactions of different spore types with canola varieties, paralleled by seed fungicide treatments, the anticipated outcome would benefit our canola growers, i.e., to select the most economically efficient blackleg management option, and canola industry, i.e., to test the resistance levels of their elite lines with these type-specific fungal spores.

4. Methods – Include approaches, experimental design, methodology, materials, sites, etc. Major changes from original plan should be cited and the reason(s) for the change should be specified.

4.1 Three approaches were developed for the productions of ascospores and pycnidiospores from canola growers' fields in western Canada. The production of pycnidiospores used an in-house protocol as detailed in Fernando et al. (2018), while ascospores were produced in the lab from (I) in vitro inoculated stubbles and (II) in planta inoculated stubbles and (III) the in vitro crossing of opposite mating types.

Approach I: Ascospores production from *in vitro* inoculated stubbles

1. Stubbles collected from canola fields were autoclaved mimicking the decay of stubbles in natural field conditions.
2. These stubbles were submerged in a mixture of pycnidiospores (1×10^7 spores/mL) with opposite mating types (MAT1-1 and MAT1-2) overnight.
3. The inoculated stubbles were placed on moisturized sands in a container.
4. The container was transferred into a growth incubator with black lights (ultraviolet light of wavelength 350 nm) and 15 ℃ continuously for 2 months. The moisture inside the container was maintained by water sprays 3-5 times a week.
5. The stubbles were gently collected and rinsed under the running tap water and soaked in tap water for 1 hour, and placed into the channels of the spore liberator attached with a vacuum pump.
6. The ascospores were captured on a piece of Milenex tape attached on a microscope slide using double-sided tape.
7. The production of ascospores were confirmed by examining the number of ascospores under a Laxco light microscope.
8. The stubbles were then kept in -20 ℃ for further uses.

Approach II: Ascospores production from *in planta* inoculated stubbles

1. The susceptible canola variety "Westar" plants were grown in a growth chamber set at 21°C and 16°C with a 16 h photoperiod with relative humidity about 50-60% for seven days.
2. The seedlings were inoculated by a mixture of pycnidiospores (1×10^7 spores/mL) with opposite mating types (MAT1-1 and MAT1-2).
3. The inoculated seedlings were transferred to 4-inch pots and grown on benches in a greenhouse till early maturation (about 3 months).
4. The infected stubbles were harvested and kept for air drying in room temperature conditions for one week.
5. Follow steps 3-8 in Approach I.

Approach III: A new approach developed for optimized ascospore and pycnidiospore production

Given the efficiency of initial two approaches developed in the previous year was relatively low, a third approach, the in vitro crossing of opposite mating types, has been developed and optimized to scale up ascospore and pycnidiospore inoculum production. Key steps and optimizations leading to this new and more efficient approach to produce ascospores from pycnidiospores were as follows.

1. A total of 12 isolates, 6 isolates with mating type 1 (MAT1-1) and 6 isolates with mating type 2 (MAT1-2) were selected to be used in the in vitro crossing method for the production of ascospores.
2. Two trials have been carried out for the production of ascospores. In each trial, a total of 36 crossings, 5 replicates for each crossing were carried out. A third trials is currently ongoing.
3. Once the two agar plugs from each mating type respectively were placed in the V8 medium-containing culture plate, they were transferred to a UV chamber set at 15 degrees for the mycelium and hyphae to grow, merge and mate to producing the asci, harboring the ascospores.
4. The ascospores were harvested and suspended in water and stored for the seedling and field testing

against blackleg.

4.2 These ascospores and pycnidiospores were then used to elucidate the interactions of ascospores and pycnidiospores with commercial canola varieties in controlled environments. A panel of 17 commercial canola lines acquired from seed companies were inoculated with ascospores, pycnidiospores and a combination of the two and assessed for seedling and quantitative resistance, following standard cotyledon inoculation for seedling resistance (Zhang et al. 2016) and petiole inoculation for quantitative resistance (Huang et al. 2014). The experiments were carried out twice in the growth chamber and greenhouse rooms using completely randomized design.

4.3 These ascospores and pycnidiospores were also used to evaluate the efficacy of seed applied fungicides against the infection by ascospores and pycnidiospores influenced by genetic resistance under field conditions. The experiments as detailed in below sections were carried out in Carman MB and Melfort SK for two years 2022 and 2023.

Materials: The follow seed were treated and provided by BASF:

1. cv. Westar –treated with a Base[#]
2. cv. Westar –Base + Fluopyram75
3. cv. InVigor L255PC – Base
4. cv. InVigor L255PC – Base + Fluopyram75

[#] Base: Contains Metalaxyl, Pyraclostrobin, Fluxapyroxad and Clothianidin for control of damping off/root rot and flea beetles.

Treatments

1. Westar - inoculated with Lm ascospores
2. Westar - inoculated with Lm pycnidiospores
3. Westar - inoculated with Lm asco & pycnidial spores
4. InVigor - inoculated with Lm ascospores
5. InVigor - inoculated with Lm pycnidiospores
6. InVigor - inoculated with Lm asco & pycnidial spores
7. Westar (Fluopyram75) - inoculated with Lm ascospores
8. Westar (Fluopyram75) - inoculated with Lm pycnidiospores
9. Westar (Fluopyram75) - inoculated with Lm asco & pycnidial spores
10. InVigor (Fluopyram75) - inoculated with Lm ascospores
11. InVigor (Fluopyram75) - inoculated with Lm pycnidiospores
12. InVigor (Fluopyram75) - inoculated with Lm asco & pycnidial spores

(All seed will be treated with a Base containing Metalaxyl, Pyraclostrobin, Fluxapyroxad and Clothianidin against damping off, root rot and flea beetles. Some will be treated with Base + Fluopyram75).

Field plots

Field trials were carried out at Carman, MB and Melfort, SK, respectively. Each treatment were seeded in meter rows using RCBD with 4 replicates. Each row will be seeded with 30 seeds and thinned to 25 plants at about a

week post >50% emergence, just prior to inoculation.

Inoculation

At about one week post emergence, each lobe of cotyledon were inoculated by wounding with a pair of bent-tip tweezers that were dipped in a suspension of ascospores, pycnidiospores or mixed spores (2 x 10^6/mL) before inoculating each plant. Both cotyledons will be inoculated.

Assessment

At about 10 days post inoculation, infection severity on inoculated cotyledons were rated using a 0-9 scale. Only the most severe infection were recorded for each plant (4 inoculations). At early crop maturity (seeds in lower pods have turned brown), all plants in a row were be uprooted and rated for disease severity using a 0-5 scale. Yield will not be taken.

5. Results – Present and discuss project results, including data, graphs, models, maps, design, and technology development.

5.1 Production of ascospores and pycnidiospores in the lab

Three approaches have been developed from this project to produce ascospores and pycnidiospores from canola stubbles from the Prairie provinces. Following standard fungal isolation procedures (Fernando et al. 2018), pycnidiospores were successfully produced from canola stubbles collected from the field (Figure 1).

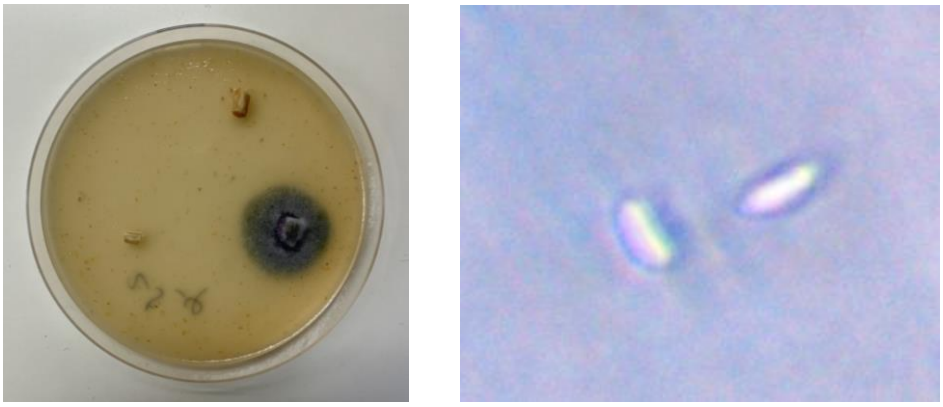

Figure 1. Pycnidiospore production

As compared to the method of in vitro inoculated stubbles and in planta inoculated stubbles to produce ascospores, in vitro crossing of two pycnidiopsore agar plugs with opposite mating types (Figure 2) was the most efficient approach in producing ascospores (Figure 3).

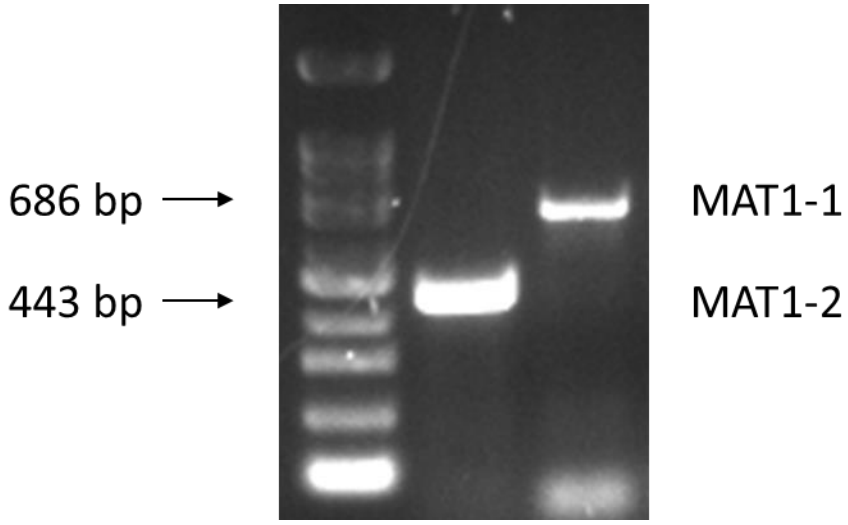

Figure 2 PCR characterization of the two mating types.

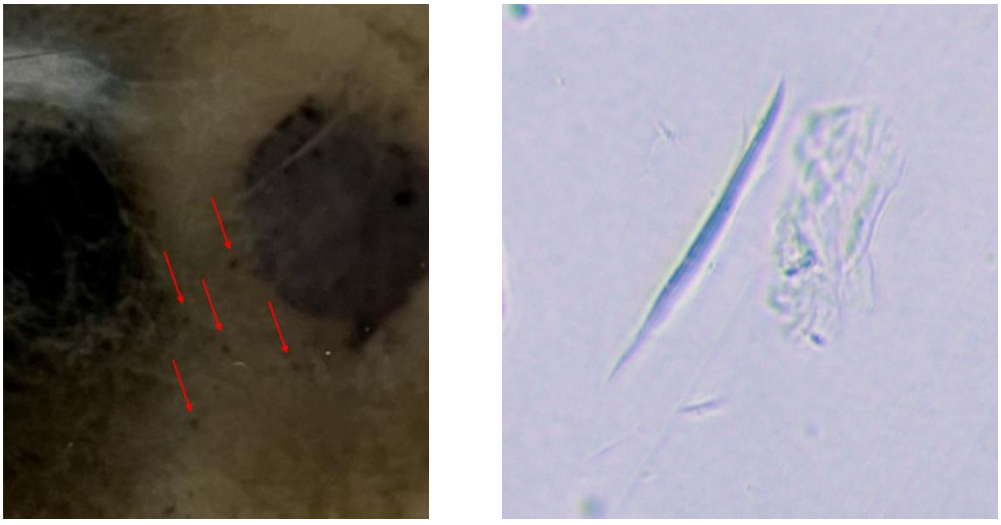

Figure 3 Ascospore production

5.2 Interactions of inoculum types (ascospores and pycnidiospores) with blackleg resistance

Both ascospores and pycnidiospores were harvested for the use of controlled environment and field blackleg evaluations. A panel of 17 commercial canola lines acquired from seed companies were used in the controlled environment experiments, due to MTA requirements, the materials were coded as detailed in Table 1.

Number	Line	Company
1	A1	A
2	A2	A
3	B1	B
4	B2	B
5	B3	B
6	B4	B
7	C1	C
8	C2	C
9	C3	C
10	C4	C
11	C5	C
12	D1	D
13	D2	D
14	D3	D
15	D4	D
16	D5	D
17	D6	D

Table 1 A panel of 17 canola lines used in the controlled environment studies

The controlled-environment studies indicated that, as compared to the typical susceptible reactions in Westar (Figure 4), at the seedling stage, resistant, intermediate and susceptible reactions (Figure 5) were observed in these 17 commercial canola lines when inoculated with ascospores (designated as ASC), pycnidiospores (designated as PYC) and a combination of the two (designated as PYC+ASC).

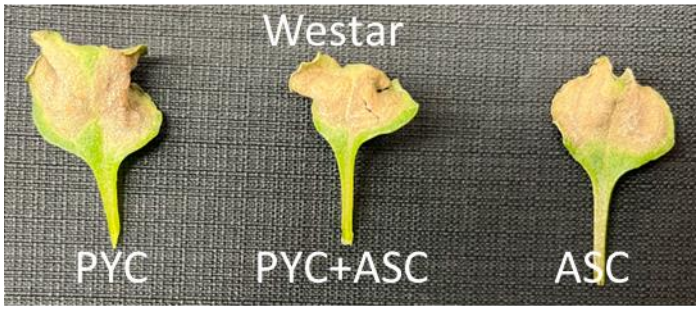

Figure 4 Typical susceptible reactions at seedling stage in Westar

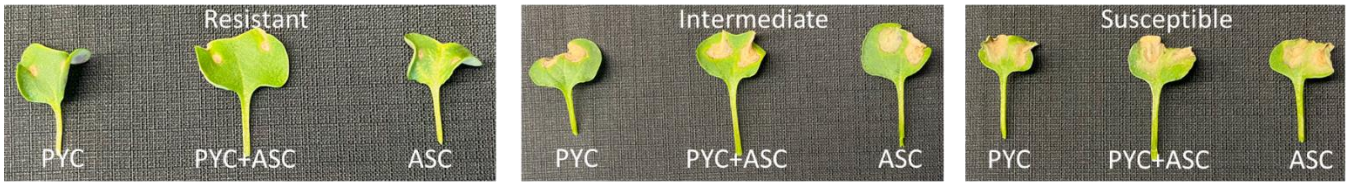

Figure 5 Resistant, intermediate and susceptible reactions at seedling stage in the commercial canola lines

At the adult plant stage, quantitative resistance was present in all the 17 commercial canola lines when inoculated with ascospores, pycnidiospores and a combination of the two, with varied level of quantitative resistance observed across the panel (Figure 6). Similarly to seedling resistance, when interacting with these quantitative resistance, a mixture of ascospores and pycnidiospores has shown the greatest aggressiveness, followed by ascospores and pycnidiospores, respectively (Figure 6).

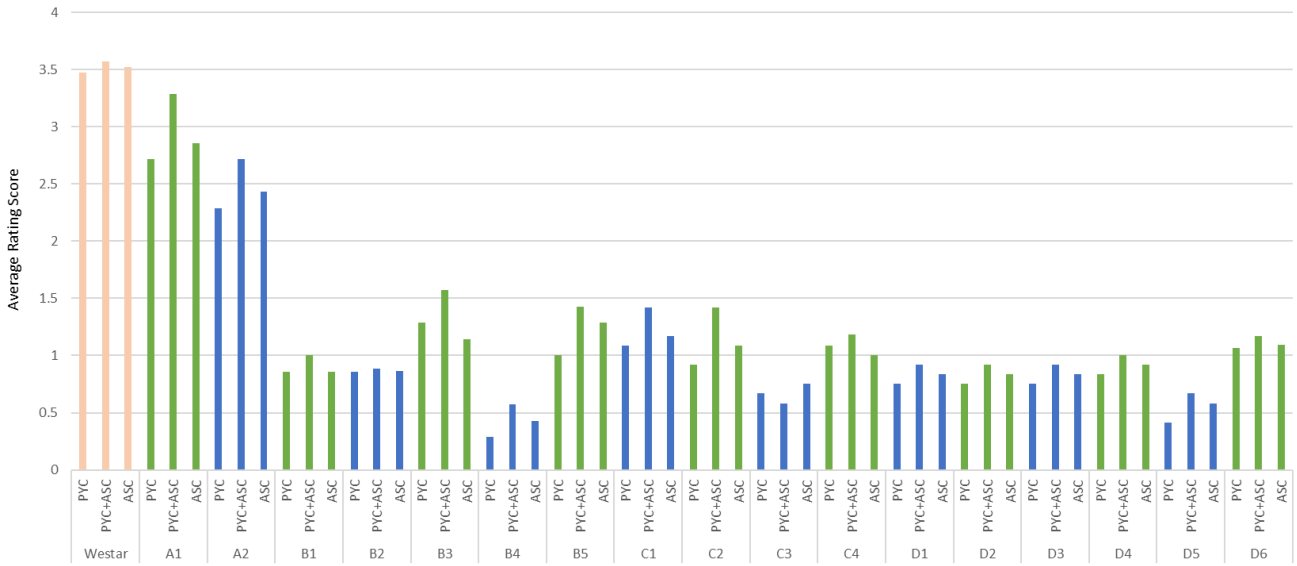

Figure 6 Quantitative resistance evaluation in the commercial canola lines

5.3 Efficacy of seed applied fungicides against the infection by ascospores and pycnidiospores influenced by genetic resistance

Data from the two-year (2022 and 2023) field trials set in Carman MB (Figure 7) and Melfort SK showed that, a similar trend of the aggressiveness of these spores (PYC+ASC > ASC > PYC) was also found in both Westar and InVigor L255PC at seedling and adult plant stages, when these inoculums were directly applied to freshly pricked wounds. A reduction in cotyledon rating score at seedling stage and disease severity at adult plant resistance stage was detected when Westar and InVigor L255PC were applied with Fluopyram fungicide seed treatment. As compared to Westar, the quantitative resistance in InVigor L255PC was effective in lowering disease severity index at adult plant resistance stage (Figure 8 and Figure 9).

Figure 7 Field trials at Carman

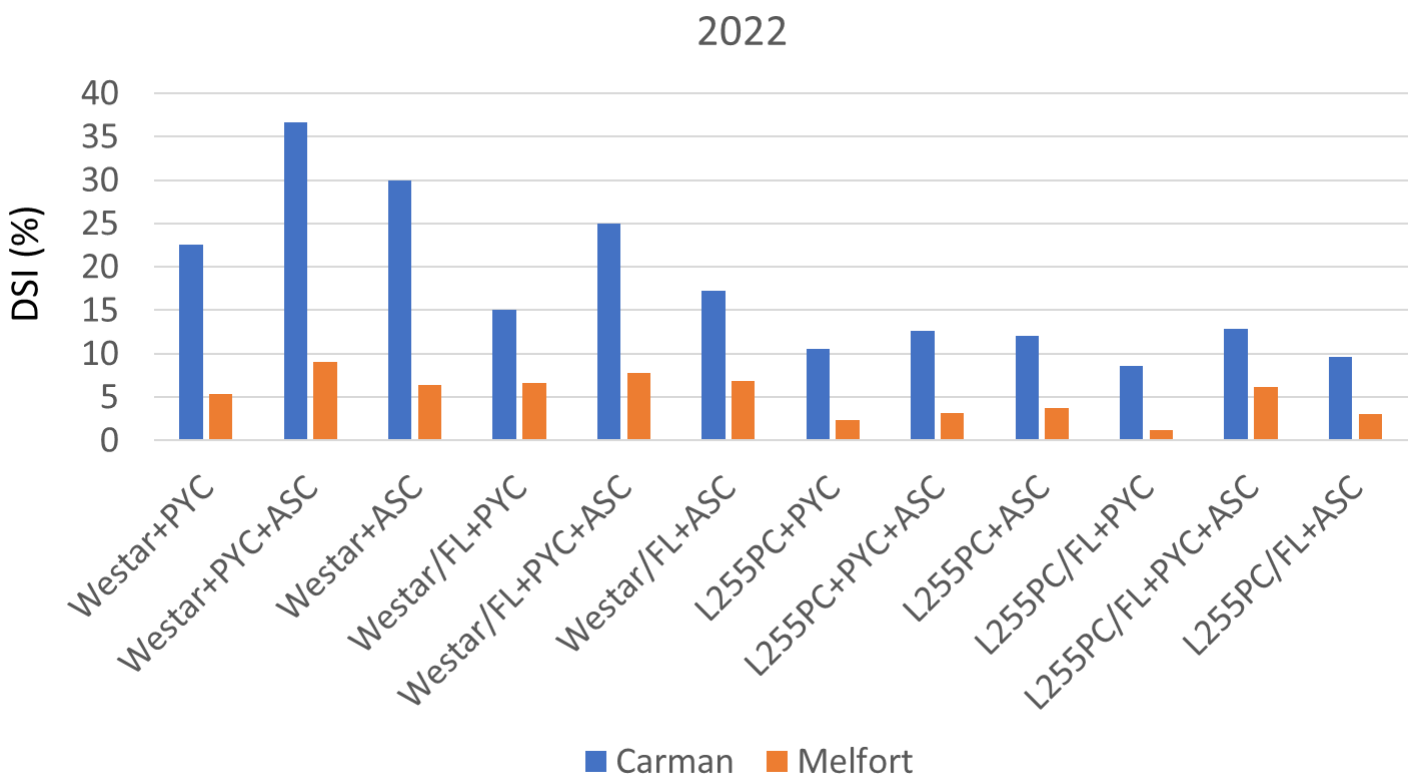

Figure 8 Disease severity in Carman and Melfort 2022

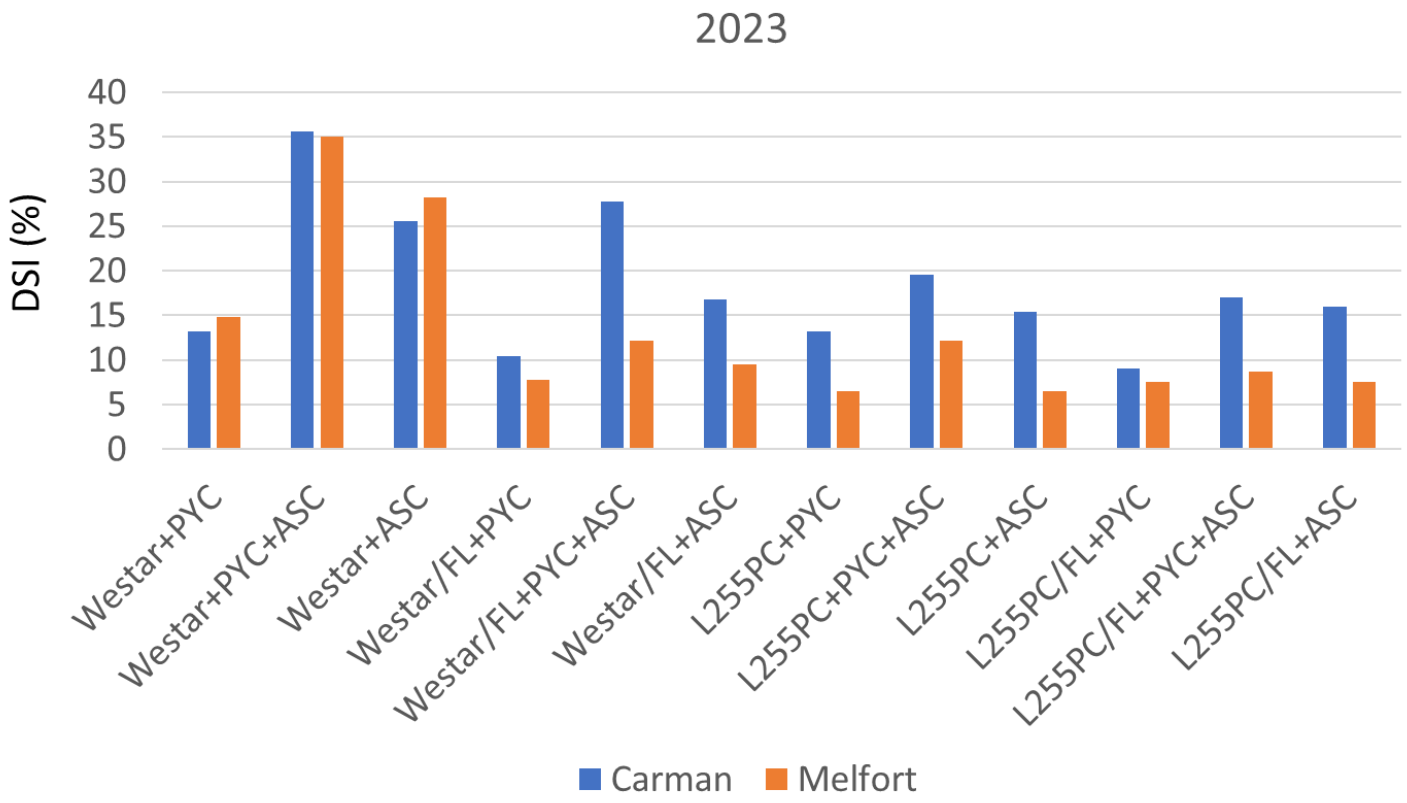

Figure 9 Disease severity in Carman and Melfort 2023

6. Conclusions and Recommendations – Highlight significant conclusions based on the discussion and analysis provided in the previous section with emphasis on the project objectives specified above; also provide recommendations for the application and adoption of the project results and identify any further research, development, and communication needs, if applicable.

In conclusion, this is the first investigation into the infection by pycnidio- and asco-spores, as well as a mixture via wounds under the influence of cultivar resistance and fungicide seed treatment for blackleg disease in canola. The three objectives set for this project were successfully achieved, which developed approaches to efficiently produce ascospore and pycnidiospore inoculum, assessed potential interactions of inoculum types (ascospores and pycnidiospores) with blackleg resistance in commercial canola lines, and evaluated the efficacy of seed applied fungicides against the infection by ascospores and pycnidiospores influenced by genetic resistance. The results from this project indicated that, the mixture of ascospores and pycnidiospores exhibited the greatest aggressiveness level, followed by ascospores and pycnidiospores respectively, in both in-door and field conditions. When the inoculums were directly applied through fresh wounds, the efficacy of Fluopyram seed treatment improves, this corroborates with previous findings (Peng et al. 2020; Huang et al. 2023), but not when canola plants were infected with natural inoculums (Huang et al. 2024). Canadian canola lines showed varied levels of quantitative resistance against different spore types. The findings from this study suggested that, genetic resistance is the most promising strategy for canola growers to adopt in dealing with blackleg disease, and another key discovery from this study is, as a mixture of ascospores and pycnidiospores have shown the greatest aggressiveness against blackleg infections, the optimization of canola residue management becomes more important as the presence of both ascospores and pycnidiospores is most likely the case in western Canada. However, more future research is required to generate new data and provide data-driven recommendation about the presence of both ascospores and pycnidiospores, and what are the composition of these two types of spores across the prairie provinces where canola is intensively grown, in the face of blackleg challenges (Canadian Plant Disease Survey, 2023).

7. Extension and communication activities: (e.g. extension meetings, extension publications, peer-reviewed publications, conference presentations, photos, etc).

1. **Huang S, Peng G, Fernando WGD. Ascospore and pycnidiospore: which is the culprit for blackleg disease in Canada? Canadian Phytopathological Society Manitoba Regional Meeting, Winnipeg. December 13, 2023.**
2. **Dr. Shuanglong Huang communicated the objectives and some of the results from this project at Carman Field Tour on July 27, 2023. See Figure 10 below.**

Figure 10 Extension activity for this project at Carman Field Tour

8. Acknowledgements – Include actions taken to acknowledge support by the Funders.

During all the communications and extensions, we have always acknowledged the funders for their strong support on this project.

9. Literature Cited

Huang S, Peng G, Fernando WGD. December 13, 2023. Ascospore and pycnidiospore: which is the culprit for blackleg disease in Canada? (CPS Manitoba Regional Meeting, Winnipeg)

10. Other Administrative Aspects: HQP personnel (PhD and/or MSc students) trained and involved; equipment bought; project materials developed

One of the Co-investigators for this project, Dr. Shuanglong Huang, Research Associate, was well trained in plant pathology research and project management.

11. Appendices - If necessary, include any materials supporting the previous sections, e.g. detailed data tables, maps, graphs, specifications.

Primer sequences used for *L. maculans* mating type characterizations in this study.

MAT1-1-F: CTCGATGCAATGTACTTGG

MAT1-2-F: AGCCGGCGGTGAAGTTGAAGCCG

MAT1-R: TGGCGAATTAAGGGATTGCTG

12. Financial (to be provided to each Funding Agency (at the addresses indicated in 11.2)

a. Comprehensive Financial Statement that summarizes the total income and expenditures to date attributable to the Funders' Funding.
b. Explanation of variances from budget which are greater than 10%.
c. Invoice

13. Final Report Posting Do you consent to a version of this Final Report (with sensitive information removed) to be posted on the funder's website?	☑ Yes - this version can be posted ☐ Yes - a modified version will be sent ☐ No
14. Research Abstract Posting Do you consent to the 2-3 Research Abstract submitted with this Final Report to be posted on the funders and the Canola Council of Canada's website?	☑ Yes ☐ No

Please send an electronic copy of this completed document to:

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