PROJECT DETAILS

- **Title**: Characterization of new strains of the clubroot pathogen in Alberta
- **Funders:** Agriculture and Agri-Food Canada, SaskCanola, Alberta Canola, Manitoba Canola Growers
- **Research program:** Growing Forward 2 and Canola Agronomic Research Program (CARP)
- **Principal investigator:** Steven Strelkov and Sheau-Fang Hwang
- **Collaborators/additional investigators:**
- **Year completed:** 2019

Final report

Abstract

Clubroot, caused by *Plasmodiophora brassicae*, is an important soilborne disease of canola and other crucifers. In Canadian canola cropping systems, the disease is managed primarily by the planting of clubroot resistant (CR) canola varieties. Unfortunately, new strains of *P. brassicae* have emerged recently in Alberta that can overcome the resistance in CR canola. The aim of this project was to identify, characterize, and better understand strains of the clubroot pathogen that are able to overcome clubroot resistance.

Populations of *P. brassicae* representing 151 fields in Alberta were obtained from galled roots of clubroot resistant (CR) canola plants collected in 2014–2017 and characterized for virulence on seven CR canola cultivars. One-hundred and one of these populations could overcome resistance in at least one CR cultivar and were evaluated further by inoculation on 13 Brassica hosts termed the Canadian Clubroot Differential (CCD) Set. The CCD Set included the differentials of Williams and Somé et al., selected hosts of the European Clubroot Differential Set, and the *B. napus* cultivars ‘Brutor’, ‘Mendel’, ‘Westar’ and ‘45H29’. Each unique virulence pattern on the CCD Set represented a distinct pathotype and was identified with a letter. Five reference isolates, obtained prior to the introduction of CR canola, also were assessed. A total of 17 pathotypes were detected using the CCD Set, compared with five pathotypes using the system of Williams and two with the system of Somé et al., suggesting that the CCD Set has a greater differentiating capacity. Pathotype A, a variant of pathotype 3 (as per Williams) which is able to overcome the resistance in CR *B. napus*, was predominant. The original pathotype 3, which is avirulent on CR canola, was classified as CCD pathotype H.

In addition to testing of the virulence phenotypes, restriction site-associated DNA sequencing (RADseq) was used to examine the genetic diversity within *P. brassicae* single-spore and field isolates collected from across Canada. The isolates included individuals that were either capable or incapable of causing disease on clubroot

Find more information on this project and many other relevant canola studies on the Canola Research Hub.
The Canola Research Hub is funded through the substantial support of the Canadian Agricultural Partnership and the canola industry, including Alberta Canola, SaskCanola, Manitoba Canola Growers and the Canola Council of Canada.

resistant canola cultivars. Population analysis indicated that most isolates belonged to one of two distinct populations, corresponding with the ability of isolates to cause disease on resistant cultivars. Finally, a molecular marker also was developed to identify pathotype 5X, which was the first of the resistance-breaking pathotypes of *P. brassicae* to be identified in Canada. A 127-bp product was amplified selectively from all new pathotype 5-like strains following optimized PCR analysis. A TaqMan probe-based quantitative assay also was developed.

This study has improved our understanding of the new strains of *P. brassicae* that have emerged in western Canada in recent years, and has resulted in the development of improved practices for their identification and management. It is clear that an integrated approach, combining other tools in addition to genetic resistance, will be needed for sustainable clubroot control.

Introduction

Clubroot, caused by *Plasmodiophora brassicae*, was first identified in a dozen canola fields in western Canada in 2003. Since then, more than 2700 clubroot-infested fields have been confirmed. While the outbreak is concentrated mainly in central Alberta, clubroot has continued to spread throughout the province, with isolated cases of the disease also found in Saskatchewan, Manitoba and North Dakota.

The clubroot pathogen rapidly multiplies in infected plants and persists in the soil for up to 20 years. Therefore, spore populations can increase rapidly, and yield losses in heavily infested fields can approach 100%.

Clubroot-resistant canola cultivars first became available to Canadian producers in 2009-10. These cultivars confer a high degree of resistance to the predominant strains of *P. brassicae* (including the highly virulent pathotype 3), and quickly became the most effective and important clubroot management tool for farmers. The availability of clubroot-resistant canola has enabled continued production of this crop in clubroot-infested fields.

Unfortunately, a strain capable overcoming resistance to *P. brassicae* in canola was identified in central Alberta in 2013. This new strain, referred to as pathotype 5X, appears to be highly virulent to all canola cultivars currently on the market. The identification of this new strain, together with preliminary reports of resistance loss or erosion in additional fields of clubroot resistant (CR) canola in 2014, led to the launch of this project in 2015.

Find more information on this project and many other relevant canola studies on the **Canola Research Hub.**
The Canola Research Hub is funded through the substantial support of the Canadian Agricultural Partnership and the canola industry, including Alberta Canola, SaskCanola, Manitoba Canola Growers and the Canola Council of Canada.

Objectives

The primary aim of this project was to mitigate the risk posed to the agricultural industry by the emergence of new strains of *P. brassicae* able to overcome the resistance in "clubroot resistant" canola. Specifically, this project would: (1) monitor spread of this novel clubroot strain through surveys, (2) assess the potential of resistance-defeating pathotypes to reappear in further outbreaks, (3) characterize the pathotypes of *P. brassicae* present where resistance had broken down, (4) multiply inoculum of resistance-defeating pathotype(s) for screening in containers and or securely contained field facilities, and (5) search for molecular markers for novel clubroot pathotypes.

Approach/Methodology

1. Monitoring for clubroot resistance breakdown in clubroot-infested fields where CR canola has been repeatedly grown

- Surveys took place in late summer/early fall, after galls had developed and the crop harvested in order to facilitate field access. Surveillance activities were focused on field entrances where infected plants are typically found (Cao et al. 2009).
- Additional surveys also were carried out on known clubroot 'hotspots' where resistant canola cultivars were grown. The areas near known instances of resistance breakdown also were monitored closely.

- Populations of *P. brassicae* were recovered from diseased plant samples as per our well-established protocols. These were multiplied *in vivo* for further analysis.
- Suspect samples were analyzed for resistance-defeating traits by re-inoculating onto: 1) the same resistant cultivar and onto 2) available commercial resistant cultivars. Pathogen collections made in 2014 and kept in storage were also included in this analysis, along with the collections made in 2015-2017.
- Pathogen resting spores that were multiplied *in vivo* were suspended in water. The roots of the canola plants were dipped into the spore suspension and the seedlings were then transplanted into potting medium and grown in a greenhouse for 6-8 wk, or until clubroot galls could be evaluated. Gall mass, clubroot severity and plant growth parameters were recorded.

2. Pathotype designation for newly isolated strains causing resistance breakdown

- If field populations were capable of causing significant amounts of clubroot disease on a suite of CR canola cultivars, they were evaluated for pathotype designations on the newly developed Canadian Clubroot Differential (CCD) Set. Selected single-spore isolations also were made and will serve as a valuable resource for future work.

Find more information on this project and many other relevant canola studies on the Canola Research Hub.
The Canola Research Hub is funded through the substantial support of the Canadian Agricultural Partnership and the canola industry, including Alberta Canola, SaskCanola, Manitoba Canola Growers and the Canola Council of Canada.

3. Resistance screening to novel pathotypes
    - The disease reaction to the resistance-defeating clubroot strains was compared among commercial cultivars and lines using the procedures in (2) above.
    - Inoculum of resistance-defeating pathotypes was multiplied *in vivo* at the CDC North facility. The inoculum was used to screen new canola cultivars at CDC North. In addition, this inoculum was used to create a secure pathotype 5X field nursery at CDC North. Development of a “mixed nursery” (consisting of a mixture of novel, resistance overcoming pathotypes) is also underway.

4. Search for molecular markers for novel clubroot pathotypes
    - Restriction site-associated DNA sequencing (RADseq) was used to examine the genetic diversity within *P. brassicae* single-spore and field isolates collected from across Canada. The isolates included individuals that were either capable or incapable of causing disease on clubroot resistant canola cultivars.
    - In addition, in order to expedite the identification of new pathotype 5-like strains of *P. brassicae* that can overcome host resistance (pathotype 5X), three primer sets were developed based on the 18S-ITS region of the pathogen.

Results/Discussion

1. Monitoring for clubroot resistance breakdown in clubroot-infested fields where CR canola has been repeatedly grown.

The virulence of 151 *P. brassicae* populations (clubbed roots) was tested on a suite of CR canola cultivars, representing one club from each field in which clubroot was found on a CR canola crop from 2014 to 2017. Under greenhouse conditions, 101 of these populations were able to overcome resistance on at least one CR host, including 15 populations collected in 2014, 24 populations collected in 2015, 22 populations collected in 2016, and 40 populations collected in 2017. The remaining populations were avirulent on the CR hosts evaluated (ID < 20%) and, with the exception of population F41-14 collected in 2014, were not analyzed further. Most of the populations capable of overcoming resistance originated from fields in central Alberta, where the clubroot outbreak is most severe, although isolated cases were found as far south as Newell County and as far north as Athabasca County. As expected, all of the populations were highly virulent on the universal suscept, ECD 05.

Find more information on this project and many other relevant canola studies on the Canola Research Hub.
The Canola Research Hub is funded through the substantial support of the Canadian Agricultural Partnership and the canola industry, including Alberta Canola, SaskCanola, Manitoba Canola Growers and the Canola Council of Canada.

Twelve of the 16 populations characterized from the 2014 collections were virulent on all seven of the CR canola cultivars tested, with IDs ranging from a mean (± SE) of 41.7% ± 10.0% to 100% ± 0% on the various hosts. In contrast, two populations (CDCN#4 and CDCN#6) were virulent on most, but not all, CR hosts: CDCN#4 was virulent on '1960', '6056CR', '74-54RR', 'D3152' and 'L135C', but not on '9558C' (ID = 16.4% ± 4.5%) or '45H29' (ID = 34.7% ± 3.5%), while CDCN#6 was virulent on all hosts except '9558C' (ID = 9.7% ± 2.4%). Another population (CDCN#2) was virulent on only one of the CR hosts ('L135C'), on which it caused an ID of 47.4% ± 3.8%. This was regarded as a susceptible reaction since the 95% confidence interval overlapped a threshold ID of 50%, but the ID obtained was lower than that induced by the other virulent *P. brassicae* populations on the same host. Population F41-14 did not cause a susceptible reaction on any of the CR canola cultivars tested, inducing a highest ID of 42.4% ± 2.6% (on '45H29'). Nonetheless, F41-14 was included in a subsequent assessment of pathotype designation, since in a preliminary evaluation, a subsample of this population appeared to be virulent on '45H29'.

In the 2015 collections, 16 populations were virulent on all of the CR canola cultivars tested, with IDs ranging from 43.8% ± 3.7% to 100% ± 0%. Another population (F1-15) was virulent on all hosts except '9558C' (ID = 24.3% ± 4.2%), while population F6-15 was virulent on all hosts except '9558C' (ID = 29.0% ± 5.5%) and '74-54RR' (ID = 23.6% ± 7.0%). The population F12-15 was virulent on four of the CR canola cultivars ('1960', '45H29', 'D3152' and 'L135C') and avirulent on the other three. One population (F10-15) was virulent only on two CR hosts, namely '45H29' (ID = 57.8% ± 3.3%) and 'L135C' (ID = 53.5% ± 7.6%). Finally, four populations, including F7-15, F9-15, F20-15 and F26-15, were avirulent on all CR canola cultivars except '45H29', on which they induced IDs that ranged from 49.4% ± 4.0% to 58.3% ± 5.7%.

All 22 populations collected in 2016 were virulent on the entire suite of CR cultivars evaluated, with IDs ranging from 51.4% ± 0.8% to 100% ± 0%. These populations were generally more aggressive than the 2014 and 2015 collections, and produced the highest IDs on the suite of CR canola cultivars. The lowest ID, when averaged across all of the populations tested each year, was observed on '9558C', although these values were still > 50% in 2014 (52.2% ± 6.1%), 2015 (62.8% ± 6.4%) and 2016 (70.5% ± 4.0%). When the populations were grouped into pathotypes, as classified below, it was clear that the CR canola cultivars were not effective differentials. These hosts were, in general, susceptible to the CCD pathotypes A, B, C, D, E, O and P. The response to pathotypes D and G was more variable, especially amongst the 2015 collections, with the range of ID values occasionally crossing the 50% threshold between a resistant and a susceptible reaction. Most of the CR cultivars were resistant to the pathogen populations classified as pathotypes H and I, an observation consistent with the finding that these shared pathotype designations with some of the 'old' *P. brassicae* strains. Interestingly, however, those populations classified as H and I collected from fields planted to CR canola

Find more information on this project and many other relevant canola studies on the **Canola Research Hub.**
The Canola Research Hub is funded through the substantial support of the Canadian Agricultural Partnership and the canola industry, including Alberta Canola, SaskCanola, Manitoba Canola Growers and the Canola Council of Canada.

generally induced higher IDs than those recovered prior to the introduction of the CR trait, even if the ID values were still < 50%.

Amongst the 2017 collections, all 40 populations were virulent on the entire suite of CR canola genotypes. On most of these CR hosts, the pathogen populations induced IDs of >90%. The exception was '9558C', which developed IDs of 30.4% ± 2.10% to 95.1% ± 0.88% in response to inoculation.

2. Pathotype designation for newly isolated strains causing resistance breakdown

The virulence pattern of each of the *P. brassicae* populations collected from 2014-2016 and able to overcome resistance on at least one of the CR canola cultivars was assessed on 13 Brassica hosts representing the CCD Set. All of the populations were virulent on the universally susceptible check, ECD 05, with IDs that ranged from 86.8% ± 1.7% to 100% ± 0%. Similarly, all of the populations were virulent on the *B. napus* cultivars 'Brutor' (IDs of 54.4% ± 4.0% to 100% ± 0%) and 'Westar' (52.1% ± 1.3% to 100% ± 0%), and all but one (exception F10-15) were virulent on ECD 08. Most populations also were virulent on the differentials ECD 06 and ECD 09. The exceptions were CDCN#6, CDCS, F9-15, F10-15, F11-15 and F12-15, which were avirulent on both ECD 06 and ECD 09, and F381-16/C.C. and F395-16/C.C., which were avirulent only on ECD 06. The number of pathogen populations virulent on the rutabaga 'Laurentian' was high and appeared to increase over the years, with susceptibility to 12 of 16 populations in 2014, 21 of 24 populations in 2015, and all of the populations collected in 2016. Likewise, the cabbage ECD 13 was susceptible to 9 of 16 populations from 2014, 20 of 24 populations from 2015, and 21 of the 22 populations from 2016. In contrast, only one *P. brassicae* population, F183-14, was virulent on ECD 11, causing an ID of 50.0% ± 2.0%, while all others were avirulent (IDs ranging from 0% ± 0% to 38.2% ± 3.1%). No population was virulent on the rutabaga ECD 10, which developed a highest ID of only 35.4% ± 5.0% after inoculation with F23-15. Similarly, ECD 02 was resistant to all of the populations tested, and in general developed the lowest IDs (0% ± 0% to 15.3% ± 1.4%) of any of the differentials. Neither of the two CR *B. napus* cultivars, 'Mendel' or '45H29', was resistant to all of the *P. brassicae* populations evaluated. Seven of 16 populations tested from 2014 and 15 of the 24 populations from 2015 were virulent on 'Mendel,' but this proportion increased to 21 of 22 from 2016. All but three pathogen populations collected in 2014 were virulent on '45H29', and this host was susceptible to all of the populations collected in 2015 and 2016.

Each unique virulence pattern on the hosts of the CCD Set was regarded as a distinct pathotype and assigned an identifying letter. In 2014, a total of nine distinct virulence patterns were observed. The most frequently identified was pathotype A, which represented 5 of 16 *P. brassicae* populations tested from that year. Populations classified as pathotype A are virulent on all of the differential hosts except ECD 02, ECD 10, and ECD 11. Two populations each were classified as pathotypes E, G, and H. It is worth noting that pathotype H

Find more information on this project and many other relevant canola studies on the **Canola Research Hub.**
The Canola Research Hub is funded through the substantial support of the Canadian Agricultural Partnership and the canola industry, including Alberta Canola, SaskCanola, Manitoba Canola Growers and the Canola Council of Canada.

was avirulent on both of the CR *B. napus* cultivars 'Mendel' and '45H29'. This indicates that it does not represent strains capable of overcoming existing clubroot resistance in the suite of CR cultivars, a fact confirmed by testing of the 'original' pathotypes (see section below). A single population (F183-14) was classified as pathotype B, which is distinguished from pathotype A by its virulence on ECD 11. One population each of pathotypes C, D, I, and P also was identified in 2014. Like pathotype H, pathotype I is avirulent on 'Mendel' and '45H29', but is distinguished from H by its avirulence on ECD 13 and 'Laurentian'.

Pathotype A remained predominant among the *P. brassicae* populations collected in 2015 and 2016. Fifteen of the 24 collections from 2015 were classified as pathotype A. The second most frequently detected pathotype in 2015 was D, with 5 populations identified, followed by pathotypes G (2 populations), and J and K with one each. Of a total of five virulence patterns identified in 2015, three also had been found in 2014 (A, D and G), while two were novel (J and K). The vast majority of the populations collected in 2016 were classified as pathotype A (19 of 22 populations), with two populations classified as pathotype O and one as P. Pathotype O represented a new virulence pattern identified in 2016. The virulence patterns of the first *P. brassicae* populations found to be capable of overcoming clubroot resistance in canola, detected in 2013 (populations L-G1, L-G2, L-G3), were not observed amongst the collections made in 2014-2016. Therefore, these populations were given a distinct pathotype classification and designated as pathotype X, in keeping with common usage. Another unique virulence phenotype found in the 2013 collection (population D-G3) was designated as pathotype L.

The greatest number of pathotypes was identified in central Alberta, where pathotype A was most common. Populations capable of overcoming clubroot resistance, but identified further away from the main outbreak, tended be other pathotypes. For example, population CDCS collected from Newell County was classified as pathotype G, while population F175-14 from Athabasca County was classified as pathotype C. In a few instances, clusters of fields included distinct pathotypes, such as the populations representing pathotype O found in Westlock County.

3. Resistance screening to novel pathotypes

The host reaction to the resistance-defeating novel strains of *P. brassicae* was compared among commercial cultivars and lines. Six representative novel strains of the pathogen and one pathotype common in western Canada since 2004 were inoculated onto two susceptible canola cultivars, one resistant line, and six CR cultivars. Although all cultivars/lines exhibited a susceptible response to inoculation with the new strains of *P. brassicae*, the severity of clubroot, root hair infection rates, and the amount of *P. brassicae* DNA present in each canola genotype varied depending on the strain. In addition, the effect of inoculum concentration on

Find more information on this project and many other relevant canola studies on the **Canola Research Hub.**
The Canola Research Hub is funded through the substantial support of the Canadian Agricultural Partnership and the canola industry, including Alberta Canola, SaskCanola, Manitoba Canola Growers and the Canola Council of Canada.

disease severity and gall formation was recorded for one of these new strains across one susceptible canola cultivar, a universally susceptible cabbage line, eight resistant canola cultivars, and two resistant canola lines. Although clubroot galls were observed at inoculum concentrations of 1×10^3 spores/mL of soil, clear differentiation of susceptible and resistant reactions among canola cultivars/lines was not observed until inoculum concentration reached 1×10^5 spores/mL. At spore concentrations of 1×10^6 spores/mL and above, all cultivars/lines developed susceptible reactions, although there was some differentiation in the degree of reaction.

P. brassicae populations representing the key pathotypes have been made available to private and public breeders (subject to appropriate biosafety considerations) for screening purposes, in order to assist with the identification of effective resistance sources and the development of new CR canola products. Pathotypes 3A and 2B are of particular interest in the development of the next generation of clubroot resistant cultivars, since 3A is the most common of the new strains and 2B can overcome resistance in one host (ECD 11, *B. oleracea* 'Badger Shipper') resistant to 3A. These new pathotypes are being used to complement screening with other, older strains, under greenhouse conditions. The first of the new pathotypes identified, '5X', also remains of interest and continues to be included in many resistance tests. This includes greenhouse "tub screening", in which plants are grown in tubs of naturally infested soil to more closely resemble the field situation. A pathotype 5X field nursery was established in a biosecure location at CDC-N and in 2017 underwent final validation (by planting a susceptible host genotype and monitoring disease development) to ensure it is ready for use by stakeholders in 2018. Work also is underway on another biosecure nursery which will include a mixture of the some of the most relevant new pathotypes including 3A and 2B.

4. Search for molecular markers for novel clubroot pathotypes

Over 8750 variants were identified through RADseq. Population analysis indicated that most isolates belonged to one of two distinct populations, corresponding with the ability of isolates to cause disease on resistant cultivars. Within each population, there were low levels of genetic diversity. One thousand and fifty of the genetic variants that distinguished the two populations were nonsynonymous, altering the coding sequences of genes. The identification of two distinct populations of *P. brassicae* in Canada suggests multiple introductions of the pathogen into the country. The genetic variation found here will be important for future research and monitoring of the pathogen, including development and validation of additional markers specific for the new pathotypes.

In parallel work, a more targeted approach was taken to develop molecular markers specific to the resistance-breaking pathotype 5X. Three primer sets were developed based on the 18S-ITS region of the pathogen. With

Find more information on this project and many other relevant canola studies on the Canola Research Hub.
The Canola Research Hub is funded through the substantial support of the Canadian Agricultural Partnership and the canola industry, including Alberta Canola, SaskCanola, Manitoba Canola Growers and the Canola Council of Canada.

the primers P5XF3 and P5XR3, a 127-bp product was amplified from all new pathotype 5-like strains following optimized PCR analysis. A TaqMan probe-based quantitative assay also was developed. These protocols could be used to detect as little as ≤ 500 fg *P. brassicae* DNA, and as few as 1×10^4/mL pathogen resting spores; infection of host tissues could be detected as soon as 4 days after inoculation. The PCR and qPCR assays developed as part of this project represent useful tools for the rapid and reliable diagnosis and quantification of new pathotype 5-like strains of *P. brassicae*.

Key milestone

The development of the Canadian Clubroot Differential (CCD) Set represents a significant milestone in this project and in Canadian clubroot research. The CCD Set is an improved system for the identification and classification of new strains of *P. brassicae*. The CCD Set has a greater differentiating capacity than the systems that were previously in use, and has allowed the identification of many new pathotypes of *P. brassicae* that would have otherwise gone undetected. This system also includes the differential hosts of Williams and Somé et al., allowing users to obtain pathotype designations according to those systems as well. The CCD Set is an important new tool for agronomists, breeders and researchers.

Future Related Opportunities

Continued monitoring will be required to track the continued shifts in *P. brassicae* populations and mitigate the negative impacts these shifts could have on clubroot management. The tools developed as part of this project should be very helpful for this purpose. The CCD Set will serve as an effective method to identify novel pathotypes and quickly determine their ability to overcome certain key sources of resistance. The genomic information obtained and analyses conducted provide an excellent resource for quickly identifying potential molecular markers, and for comparing genetic similarities or differences between different populations of the pathogen. The molecular marker for pathotype 5X will facilitate screening of larger numbers of samples for the presence of this pathotype, and together with the genomic information could serve as the basis for development of additional markers for new pathotypes identified in this study and in the future.

The Canola Research Hub is funded through the substantial support of the Canadian Agricultural Partnership and the canola industry, including Alberta Canola, SaskCanola, Manitoba Canola Growers and the Canola Council of Canada.