Final Report

Clubroot Risk Mitigation Initiative

30-April-2013

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Executive Summary

Variance Report

Table 1. Clubroot Risk Mitigation variance report.

Researcher	***Variance in Budget***	***Variance in Activity***
Strelkov	Addition of Kutcher's project	None other than increase in management with the addition of Kutcher's project
Hwang	None	None
Howard	None	None
Rahman	None	None
Li	None	None
Canola Council Administration	None	None

Activities

This concludes the Clubroot Risk Mitigation Initiative (CRMI). It has been a remarkable success by all counts. Not only were all milestones and objectives met, in many cases they were far exceeded. Below you may read about all the findings – it will take a while. The CRMI produced some of the finest clubroot research in the world, and it has lead Canadian researchers to the forefront of this field internationally. This research has not remained in journals collecting dust - it has been disseminated to the canola industry at large through meetings, video, brochures, and other media, providing much needed tools for the canola industry to continue to grow. This leads us to the milestone that is not recorded – that this clubroot research allowed the canola industry to get ahead of this disease. For the time being, the industry is ahead of clubroot – and that is a remarkable success. But this is a temporary state if we become complacent with our success. The other interesting aspect of the CRMI that surprised the industry was how well the collaborative spirit pervaded the entire scope of this program – which made this file a pleasure to administer. As a result, the CRMI is serving as a model for future multi-lab research aimed at tackling challenges to the canola crop.

Pathology Pillar - Strelkov

1. Development of a Canadian differential set.
    - Current pathotype differentials sets (Some's, Williams', and ECD's) have been useful for broad-based classification of genetic diversity in *P. brassicae*. But these classification systems are cumbersome and not specific enough for the Canadian canola industry.
    - A putative set of 11 *B. napus* and two *B. rapa* genotypes have now been and tested against a collection of *P. brassicae* isolates and populations from Canada and elsewhere. The results from indicate that these hosts may serve as an effective foundation, but a coordinated approach is needed to refine this set and ensure that they are robust and widely accepted..
2. Clubroot resistance stewardship: disease surveillance and pathotype monitoring
    - Clubroot resistance may not be durable. Laboratory screening has shown some erosion of resistance. But so far no major shifts in virulence have been observed in the field.
    - 1064 fields of known clubroot in Alberta. Actual levels estimated to be much higher.
    - 4 fields of clubroot in Saskatchewan and 2 in Manitoba.
3. Mechanisms of resistance
    - Potential markers developed to distinguish Pathotype 6.
    - Spheroid galls look to be a type of resistance.
    - Resistance appears to limit secondary zoospore infection but not primary zoospore infection.
4. Study of *Plasmodiophora brassicae* inoculum dispersal in western Canada
    - *P. brassicae* dispersion due to soil movement and was not an endemic pathogen.
    - *P. brassicae* can move with soil on wind.

- pH has an significant impact on clubroot but that impact is relatively low.

Disease Management Pillar

1. Establishment of a consortium nursery - Hwang
    - Nursery is established and will be available to experimentation in 2012.
2. Integrated control strategies to reduce the impact of clubroot on canola - Hwang
    - Very little effect of seeding date on clubroot severity or gall size.
    - Treatments that delay infection for several weeks after seeding are likely to reduce clubroot severity and have a positive impact on seed yield.
    - Liming soil in general to increase pH is not consistent nor of a degree high enough to reduced clubroot severity. Clubroot conducive conditions, such as high soil moisture, is more important for the development of this disease than pH modification.
    - Calcium cyanamide applications did not reduce clubroot severity in canola.
    - Quintozene and/or high levels of limestone or wood ash can reduce the impact of *P. brassicae* on canola. However, rates that are economically feasible showed little potential to reduce clubroot severity or increase yield in infested commercial fields.
    - Boron, applied at high rates such as 4 kg/ha, does reduce clubroot severity, but it is also phytotoxic to canola plants at these rates.
    - Soil fumigants may help eradicate resting spores from the soil. Vapam testing showed a substantial reduction in clubroot severity when applied prior to planting.
    - Several of the microbial biofungicides reduced clubroot severity when applied as a soil drench, but were not effective when inoculum pressure was high.
    - The fungicides Terrachlor and Ranman demonstrated a reduction in clubroot severity. But these are unlikely to be used by canola farmers due to cost and toxicity concerns.
    - All seed treatments tested have no impact on clubroot severity.
    - Root exudates of both host and non-host plant species significantly stimulated clubroot resting spore germination, suggesting that bait crop can be deployed to stimulate spore germination and to reduce soil inoculum loads in the canola fields.
    - Bait crops may induce resting spore germination. Thus far testing has only showed a small benefit to using a bait crop - too small to be useful in commercial fields.
3. Evaluating Methods for Disinfesting Tools, Machinery and other Hard Surfaces Contaminated with Clubroot Resting Spores - Howard
    - Sanitation is a three step process: 1) bulk soil removal, 2) pressure washing, and 3) disinfection
    - *P. brassicae* infectivity declined sharply as the concentration increased over the range of one-half, one, two and five times the manufacturer's label rate for the disinfectants tested. The five best-performing products were Industrial Bleach, HyperOx, EcoClear, Virkon and SaniDate.

Breeding Pillar

1. Molecular mapping of the clubroot resistance of Rutabaga (*B. napus* var. *rapifera*) - Rahman
    - Two rutabaga genotypes, Rutabaga-BF and Rutabaga-PL, inbred for resistance to multiple pathotypes including pathotype 3, crossed to two clubroot susceptible spring canola lines, A07-29NI and A05-17NI.
    - Most of the F_2 families followed a 3:1 and TC families a 1:1 segregation for resistant and susceptible phenotypes. However, the distribution of the resistant and susceptible plants in F_2 and TC populations of Rutabaga-PL × A05-17NI deviated significantly from simple Mendelian segregation
    - Data suggests that clubroot resistance in Rutabaga-BF is primarily under the control of a major dominant gene; however, it is complex in Rutabaga-PC – more than one loci apparently involved in the control of this trait.
    - Molecular mapping based on the DH population derived from Rutabaga-BF × A07-29NI cross showed that a major gene on the A genome chromosome A8 is involved in the control of resistance. QTL mapping also detected the same genomic region.

2. Mapping clubroot resistance genes in *B. rapa* and developing molecular markers closely linked to these genes - Li
    - The objectives are to construct a high density genetic map in *B. rapa*, make alignment with other published genetic maps and map clubroot resistance genes in five Chinese cabbage cultivars and turnip accessions.
    - A high density genetic map in *B. rapa* has been constructed and more 10,000 molecular markers have been assembled into 10 linkage groups.
    - After testing SRAP markers in five F2 and BC1 populations, all clubroot resistance gene loci belonged to the same locus on A3. A genetic map around the clubroot resistance gene locus on A3 was constructed.
    - Four turnip accessions from the ECD differential set,, ECD1, ECD2, ECD3 and ECD4 with resistance to clubroot were used as donor male parents to cross with a susceptible B. rapa rapeseed, BAR. Data from resulting populations revealed that there were various inheritances of clubroot disease resistance. But, when SRAP markers were used on these populations, the mapped A3 resistance locus was found in, ECD1, ECD2 and ECD4 while the A3 clubroot resistance locus did not exist in ECD3 - where and one or two clubroot resistance loci were located to different chromosomes from A3.
    - Near isogenic lines were developed - each line will contain single clubroot resistance locus in the same genetic background. This may serve to replace the ECD differential set.
    - These four turnip lines, two cabbage and one kale accessions were used to produce *synthetic B. napus*. Seeds from most interspecific crosses have been harvested and crossed to canola. These synthetic *B. napus* combined the resistance genes in *B. rapa* and *B. oleracea*. After preliminary testing, all synthetic *B. napus* were resistant to the Canadian clubroot isolates. With the known genome location and molecular markers linked to the clubroot resistance loci, pyramiding multiple clubroot genes in canola through marker assisted selection is underway.

<u>Surveillance of Clubroot</u> - McLaren

- Clubroot has now been found in over 1,064 canola fields in Alberta, and in several isolated fields in Saskatchewan and Manitoba
- About a total of 500 canola fields were surveyed in all major canola crop regions of Manitoba and Saskatchewan, with several hundreds of soil samples collected for PCR detection of low pathogen inoculum.
- 12 fields from SK and 2 from MB that showed positive in PCR testing for low pathogen inoculum presence in the soil. In SK, clubroot was reported for the first time on canola in two disease-nursery fields in the north-central region.
- Many dust samplers from the field sites in central Alberta tested positive in PCR with quantifiable amounts of inoculum (qPCR). In southern Alberta, eight out of 24 samples were positive in PCR with quantifiable amounts of inoculum. Irrigation water samples collected from southern Alberta did not show detectable amounts of *P. brassicae* inoculum. These findings suggest the potential for the long-range spread of P. brassicae inoculum via wind erosion

<u>Interaction of Soil, Climate, and Biology</u> - Gossen

- The optimum temperature for development of clubroot on canola was near 25°C at each stage of pathogen development, from initial infection of root hairs to symptom development.
- When temperature and moisture were conducive for clubroot, neutral pH (7.0–7.5) reduced but did not eliminate clubroot development, and soil compaction had a much larger effect on clubroot severity than soil type.
- Application of boron (B) slowed the development of clubroot in canola under controlled conditions. In initial field studies, 4 kg ha-1 was the most effective rate that wasn't toxic to the crop.
- Root hair infection was shown to occur in all canola cultivars. The pathogen developed most slowly in root hairs of a moderately resistant cultivar, intermediate in a resistant cultivar and most quickly in a susceptible cultivar. Expression of high levels of resistance was first observed in cortical cells.
- Screening Brassica Germplasm for Resistance

Screening Brassica Germplasm for Resistance - Falk

- A total of 955 *Brassica* accessions, including *B. rapa* (718), *B. napus* (94)*, B. juncea* (93)*, B. oleracea* (30) *, B. carinata* (12) and *B. nigra* (8), were screened against the predominant pathotype 3 of the pathogen.
- Thirty five of the accessions (mostly *B. rapa*) showed >50% less clubroot severity relative to a susceptible control, with 15 of them showing complete resistance (clubroot free).
- One *B. nigra*, two *B. oleracea*, and four *B. rapa* (oriental vegetable) lines maintained a high level of resistance under the high pathogen inoculum pressure, while one *B. nigra* (PI 219576) and two *B. rapa* (turnip) lines showed moderate resistance due largely to genetic heterogeneity which resulted in resistant/susceptible segregation.
- Most of the resistant lines showed consistent resistance against each of the five *P. brassicae* pathotypes identified in Canada, with only the exception of the two turnip and one *B. nigra* lines which varied slightly against different pathotypes. Several promising resistant lines were identified for development of clubroot resistant canola germplasm ().
- Populations segregating for clubroot resistance were developed for several clubroot resistance (CR) sources (*B. rapa*, *B oleracea* and *B. nigra*) by crossing and backcrossing with a clubroot-susceptible (CS) double haploid (DH) line of the same species or amphideploids.
- Two of the *B. rapa* resistance sources (FN, JNC) showed 1:1 segregation ratio in F1 populations, indicating a single major CR gene involved.
- Several molecular markers flanked the CR gene (*Rpb*1) in FN, with the marker sN8591 and sR6340I being the closet from each side at a distance of 0.54 cM and 0.77 cM to *Rpb1*, respectively. The marker sN8591was highly accurate (close to 100%) in identifying *Rpb1* in *B. napus* and *B. rapa* populations, whereas sR6340I showed some errors, especially on *B. napus* samples. These two markers may be used together to maximize the accuracy of MAS. With this tool, introgression of *Rpb1* into five *B. napus,* one *B. rapa,* and one *B. juncea* canola breeding lines is progressing rapidly.

Developing Biocontrol for Clubroot - Peng

- Additional management strategies potentially complimentary to cultivar resistance, including biofungicide seed treatment and crop rotation, was investigated to integrate clubroot-control measures on canola.
- None of the biocontrol treatments reduced clubroot impact, whereas the R cultivar showed consistent disease reduction and substantial yield improvement over that of S cultivar.
- Plots of varying rotations showed a pattern of clubroot pathogen pressure, with those of a 4-year break being 10-fold lower in inoculum loads relative to that in back to back canola.
- Under high disease pressure, the longer rotation reduced above-ground disease impact on the crop and increased the yield significantly relative to a short rotation, even on the R cultivar. A 3- or 4-year rotation, however, will not reduce clubroot impact sufficiently in a heavily infested field to let an S or MS cultivar reach its full yield potential.

Formulation Development for Biocontrol - Hynes

- A seed-coat applied formulation and soil applied granules of *Bacillus subtilis* were developed to facilitate field delivery.
- The *B. subtilis* was microencapsulated by complex coacervation using corn, pea, rice or tapioca starch as filler and protectant, followed by lyophilisation to be applied to canola seed coat. A granular formulation was also developed, as was a granular formulation using corn-cob grits as a low-cost carrier coated with the bacterial fermentation product.
- Reduction in clubroot disease index was inconsistent with treated seed, granules and drench applied *B. subtilis*.

- Fungicides, biofungicide granules or seed treatments were all ineffective against clubroot in field studies.

Publications and Extension

The CRMI produced research that has been published. To date, this is a listing of refereed publications:

- Refereed research papers: 34
- Book chapters and review articles: 5
- Proceedings: 15
- Canadian Plant Disease Survey reports: 12
- Abstracts: 74

Extension:

- AAFC Tech Transfer: 82
- CCC publications: 1
- CCC videos: 6
- CCC grower meetings and tours: 28
- Conferences organised : 4

Overview

Background

Clubroot, caused by the pathogen *Plasmodiophora brassicae*, is a new disease of canola that poses significant risk to Canada's most valuable crop. The threat of this disease is large. Approximately one-quarter of the traditional canola growing area in Alberta is most at risk for clubroot; assuming 25% yield losses (a moderate estimate), then 25% of one-quarter of the provincial canola cash receipts could be lost, equivalent to about $44 million per year in Alberta alone (M. Hartman, personal communication). This figure does not include losses in quality (such as seed oil content) or losses that could occur if clubroot becomes widespread in Saskatchewan and Manitoba.

A collaborative and integrated approach was proposed to mitigate the risk posed by clubroot to western Canadian canola production. Within this framework, three research pillars have been developed: Pathology, Disease Management, and Breeding. Through extensive consultation with academics, government, grower organizations, agronomists, and industry, specific areas of pressing importance have been identified within each pillar; a set of inter-related projects have been developed to address each area and are described below. Moreover, a research team with extensive expertise in clubroot, plant pathology, disease management, and breeding has been assembled and will work closely together on the issues critical to the sustainable risk management of clubroot in canola. This work builds on the strengths of past and current clubroot research, and is complementary to the research effort by the team of AAFC scientists, enabling synergies and rapid, complementary progress on multiple issues. The research has as its principle objective the risk management of clubroot in an integrated and sustainable manner, to secure the vibrant production of canola in Canada by mitigating the risk posed by this disease.

Objectives

Pathology Pillar

1. To identify *Brassica* genotypes that are effective for differentiating pathogen strains
2. To monitor changes in virulence and pathotype composition of *P. brassicae* populations
3. To examine the interactions between host and pathogen and better understand resistance and virulence
4. To better understand the distribution and dispersal of *P. brassicae* in western Canada

Disease Management Pillar

5. To establish a consortium nursery to integrate and facilitate clubroot research efforts
6. To develop effective cultural, chemical and biological clubroot management strategies
7. To develop reliable methods for disinfestation of equipment and for assessing resting spore viability

Breeding Pillar

8. To develop molecular marker(s) for the resistance that has already been identified in rutabaga and is currently being introgressed into Canadian canola; to understand the genetic basis of clubroot resistance in rutabaga; to develop novel spring canola (*B. napus*) germplasm based on rutabaga-resistance.
9. To utilize three Chinese cabbage *(B. rapa*) cultivars with confirmed resistance to Canadian *P. brassicae* isolates to develop mapping populations in this species; to fine-map clubroot resistance genes in *B. rapa* through development of high-density genetic maps; to develop SCAR and/or SNP markers for use in marker assisted selection

Canola Council of Canada Administration Summary

1. Extension activities
    - Clubroot videos on biology, control, sanitation, and the clubroot disease cycle were produced. Clubroot videos can be found at: http://www.youtube.com/playlist?list=PL6A517EB5FF91285F
    - Clubroot sanitation guides produced. An electronic copy can be obtained at: http://www.canolacouncil.org/media/530963/clubroot_sanitation_guide.pdf
    - Clubroot information for grower/agronomist/municipality meetings in:
        - 2010 - Alberta: Leduc
        - 2011 - Alberta: Battle River, Leduc, Lacombe, and Vermilion
            - Saskatchewan: two meetings in Saskatoon, Regina, Lloydminster, Humbolt, Melfort, two meetings in Battleford, Glaslyn, two meetings in Aberdeen, Hooey, Mervin, Kindersley, and St. Walburg.
        - 2012 - Alberta: Beaver County, Leduc, two meetings in Red Deer, and Stettler
            - Saskatchewan: Moose Jaw
        - 2013 – Saskatchewan: Biggar.
    - Field tour training:
        i. Clubroot Galla – 25-July, 2012 in Brooks, AB
        ii. SK Ag Clubroot training – 31-July, 2012 in Brooks, AB
    - Numerous radio interviews were completed over the course of the CRMI
    .
2. Formal Meetings
    - Clubroot Summits (annual meetings of the CRMI) were held at:
        - 10-March, 2010 in Nisku, AB
        - 9-March, 2011 in Saskatoon, SK
        - 7-March, 2012 in Edmonton, AB
        - 19-22 June, 2013 in Edmonton, AB – 2013 International Clubroot Workshop
    - Clubroot research planning meetings:
        - 15-16 December 2010 in Leduc, AB
        - 15-16 December 2011 in Leduc, AB
        - 6-7 December 2012 in Leduc, AB
    - Clubroot Ring Test meetings in Edmonton 23-Januarya 2010 – Meeting to establish consistent clubroot PCR testing methodologies between laboratories
    - Hosted the Alberta Municipality Clubroot Policy Meeting with Alberta Agriculture and Rural Development on 29-March, 2012 in Nisku.

3. Contract Administration
    - 2009
        - Signed Service Agreement AGR-05093 with AAFC on 15-Jan, 2010.
        - Signed research agreement with the U of Alberta for Strelkov and Hwang projects on 11-March, 2010.
        - Signed research agreement with the U of Alberta for Rahman project on 11-March, 2010.
        - Signed research agreement with the U of Manitoba for Li project on 10-February, 2010.
    - 2010
        - Signed research agreement with Pioneer Hi-Bred for their contribution to Strelkov's CRMI project on 15-June, 2010.
        - Amended U of Alberta agreement on Strelkov projects to reflect the additional income from Pioneer Hi-Bred on 10-June, 2010.
        - Purchased mobile clubroot sanitation unit on the behalf of Alberta Agriculture and transferred ownership to ARD on 29-June, 2010.
        - Signed contribution agreement with Alberta Agriculture for Howard project on 7-July, 2010.

- Amended AAFC Service Agreement to accommodate a funding shortfall on 12-August, 2010.

- 2011
  - Amended research agreement with U of Alberta to include Hwang's projects for the final two years of the study period on 28-March, 2011.
  - Amended AAFC Service Agreement to reflect Strelkov taking the lead from Dr. Randy Kutcher (AAFC) on the study of *P. brassicae* inoculum dispersal in Western Canada on 11-October, 2011.
  - Amended research agreement with the U of Alberta to include the addition on Kutcher projects to Strelkov's program on 5-October, 2011.

Financial Summary for CCC Administered Projects

Due to changes in the contracts between CCC and AAFC and with CCC with U of Alberta and AARD as outlined in the CCC Administration report, the original budget (Table 2) was modified various times over the course of the CRMI to the final budget settled in September of 2011 (Table 3). The actual payments and income is shown in Table 4.

Table 2. Original Budget for CRMI from the proposal.

		FY 2009	FY2010	FY2011	FY2012	Total
Income						
	AAFC	$294,622	$546,188	$574,085	$585,105	$2,000,000
	Total	$294,622	$546,188	$574,085	$585,105	$2,000,000
Payments						
	University of Alberta (SS + SFH)	$127,625	$315,850	$325,800	$337,850	$1,107,125
	University of Alberta	$ 71,300	$144,900	$150,621	$148,350	$ 515,171
	University of Manitoba	$ 48,242	$ 49,413	$ 50,589	$ 51,755	$ 199,999
	Alberta Agriculture	$ 40,950	$ 11,025	$ 12,075	$ 13,650	$ 77,700
	CCC admin	$ 6,505	$ 25,000	$ 35,000	$ 33,500	$ 100,005
	Total	$294,622	$546,188	$574,085	$585,105	$2,000,000
Difference		$ -	$ -	$ -	$ -	$ -

Table 3. Final Budget for CRMI after amendments made to contracts with AAFC, U of Alberta, and AARD by August 2011.

		FY 2009	FY2010	FY2011	FY2012	Total
Income						
	AAFC	$ 294,622	$ 476,981	$ 676,292	$ 645,105	$ 2,093,000
	Pioneer Hi-Bred	$ 10,000	$ 10,000	$ 10,000		$ 30,000
	Total	$ 304,622	$ 486,981	$ 686,292	$ 645,105	$ 2,123,000
Payments						
	University of Alberta (SS + SFH)	$ 127,625	$ 325,850	$ 368,800	$ 407,850	$ 1,230,125
	University of Alberta (HR)	$ 71,300	$ 144,900	$ 150,621	$ 148,350	$ 515,171
	University of Manitoba	$ 48,242	$ 49,413	$ 50,589	$ 51,755	$ 199,999
	Alberta Agriculture (RH)	$ 9,150	$ 11,025	$ 12,075	$ 13,650	$ 45,900
	Mobile sanitation unit	$ 33,390				$ 33,390
	CCC admin	$ 6,505		$ 60,000	$ 33,500	$ 100,005
	Total	$ 296,212	$ 531,188	$ 642,085	$ 655,105	$ 2,124,590
Difference		$ 8,410	$ (44,207)	$ 44,207	$ (10,000)	$ (1,590)

Table 4. Actual income and payments for CRMI

		FY 2009	FY2010	FY2011	FY2012	Total
Income						
	AAFC	$ 294,622	$ 476,981	$ 676,292	$ 645,105	$ 2,093,000
	Pioneer Hi-Bred	$ 10,000	$ 10,000	$ 10,000	$ -	$ 30,000
	Alberta Agriculture			$ 2,500	$ -	$ 2,500
	Total	$ 304,622	$ 486,981	$ 688,792	$ 645,105	$ 2,125,500
Payments						
	University of Alberta	$ 127,625	$ 325,850	$ 368,800	$ 407,850	$ 1,230,125
	University of Alberta	$ 71,300	$ 144,900	$ 150,621	$ 148,350	$ 515,171
	University of Manitoba	$ 48,242	$ 49,412	$ 50,589	$ 51,756	$ 199,999
	Alberta Agriculture	$ 9,150	$ 11,025	$ 12,075	$ 13,650	$ 45,900
	CCC mobile sanitation Unit	$ 33,390	$ -	$ -	$ -	$ 33,390
	CCC admin	$ -	$ 449	$ 23,012	$ 79,044	$ 102,504
	Total	$ 289,707	$ 531,636	$ 605,097	$ 700,649	$ 2,127,089
Difference		$ 14,915	$ (44,655)	$ 83,695	$ (55,544)	$ (1,589)

List of publications from CRMI

Refereed Research Papers: 34

Rashid A., H. Ahmed, Q. Xiao, S. F. Hwang and S. E. Strelkov. 2013. Effects of root exudates variable pH and on resting spore germination and root-hair infection of canola (*Brassica napus* L.) by *Plasmodiophora brassicae* (Woronin). Crop Protection 48: 16-23.

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Lahlali, R., Peng, G., , McGregor, L., Gossen, B.D., Hwang, S.F., and McDonald, M.R. 2011. Mechanisms of the biofungicide Serenade (*Bacillus subtilis* QST713) in suppressing clubroot. Bioc. Sci. and Technol. 21: 1351–1362. DOI:10.1080/09583157.2011.618263.

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Peng, G., L. McGregor, R. Lahlali, B.D. Gossen, S.F. Hwang, K.K. Adhikari, S.E. Strelkov and M.R. McDonald. 2011. Potential biological control of clubroot on canola and crucifer vegetable crops : Biocontrol of clubroot. Plant Pathology 60: (3):566-574. http://dx.doi.org/10.1111/j.1365-3059.2010.02400.x

Hwang, S.F., H.U. Ahmed, S.E. Strelkov, B.D. Gossen, G.D. Turnbull, G. Peng and R.J. Howard. 2011. Seedling age and inoculum density affect clubroot severity and seed yield in canola. Can. J. Plant Sci. 91(1): 183-190. http://dx.doi.org/10.4141/cjps10066

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PATHOLOGY PILLAR – Dr. S.E. Strelkov

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Name of Principal Investigator (PI): Stephen Strelkov, University of Alberta
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CLUBROOT RISK MITIGATION INITIATIVE

FINAL REPORT

Pathology Pillar: Clubroot in the Canadian canola crop: 10 years into the outbreak

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Executive Summary:

Clubroot, caused by the obligate parasite *Plasmodiophora brassicae*, is an important soilborne disease of the Brassicaceae. In Canada, clubroot has long been established as a problem in cruciferous vegetables, but was not reported on the Prairie canola (*Brassica napus*) crop until 2003, when 12 clubroot-infested fields were identified in central Alberta. Continued surveillance has shown that the disease is spreading, and as of 2012 there were at least 1,064 fields with confirmed clubroot infestations in the province. While the outbreak is still mainly confined to central Alberta, isolated clubroot infestations and the presence of viable *P. brassicae* inoculum have been confirmed in southern Alberta, Saskatchewan, and Manitoba. Dissemination of the parasite appears to be predominantly through the movement of infested soil on farm and other machinery, although secondary mechanisms of spread, such as via soil tags on seed and wind and water erosion, have also been implicated. Given the significant economic value of the Canadian canola crop, the increased incidence of clubroot on canola has caused major concern and led to the initiation of a large, coordinated research effort aimed at understanding and managing this disease. The purpose of this review is to summarize the extent and nature of the clubroot outbreak in the Canadian canola crop, 10 years after it began, and to provide a context for the research and management strategies that have since been developed.

Keywords: *Brassica*, canola, clubroot, crucifers, disease management, epidemiology, pathotypes, *Plasmodiophora brassicae*, races

Introduction

Clubroot, caused by the obligate parasite *Plasmodiophora brassicae* Woronin, is an important soilborne disease of the Brassicaceae. Infection of susceptible host genotypes by *P. brassicae* results in the formation of large galls or club-shaped swellings on the roots, which hinder water and nutrient uptake by the plant. Severe infections can also result in stunting of the above-ground organs, as well as yellowing, wilting and premature senescence. These symptoms may in turn lead to significant losses in crop yield and quality. On a global scale, losses to clubroot have been conservatively estimated to range from 10 to 15% (Dixon, 2004). The parasite survives in the soil as resting spores, which have a half-life of approximately four years (Wallenhammar, 1996; Hwang *et al.*, 2013a) and can remain viable for up to 20 years. The longevity of the resting spores is a cause for concern, since it makes *P. brassicae* difficult (if not impossible) to eradicate once it becomes established in a field.

Clubroot was most likely introduced to Canada with *P. brassicae*-infected fodder turnips (*Brassica rapa* L. var. *rapa)* brought to this country by European settlers (Howard *et al.*, 2010). The disease was probably well established in parts of Canada by the late 19th or early 20th centuries, as clubroot research was already being conducted in Nova Scotia as early as 1916 (Estey, 1994). Indeed, the occurrence of clubroot on cruciferous vegetables was reported nearly every year in the Maritimes, Quebec and British Columbia, as well as sporadically in Ontario, from the 1920s to the 1950s (Howard *et al.*, 2010). Clubroot outbreaks were also reported occasionally in the 1960s (Creelman, 1965), and in the 1970s the disease remained the most important limiting factor in the production of cabbage and other crucifers in areas where it had become endemic (Howard *et al.*, 2010). Nevertheless, clubroot was not reported outside of the traditional vegetable growing regions in the Maritimes, Quebec, Ontario and British Columbia until the 1970s and 1980s, when unpublished reports emerged of its occurrence in a few home gardens in Alberta and in a market garden in Manitoba.

Clubroot was observed for the first time on the Canadian canola (*Brassica napus* L.) crop in 1997 in Quebec (Morasse *et al.*, 1997). In the Prairie Provinces (Alberta, Saskatchewan and Manitoba), on which more than 98% of the harvested hectares of Canadian canola are grown (Canola Council of Canada, Winnipeg, Manitoba), clubroot was not identified until 2003 (Tewari *et al.*, 2005). In that year, 12 clubroot-infested fields were identified north and northwest of the City of Edmonton, in the central part of Alberta, causing significant concern because of the possible economic impact of clubroot on the multi-billion dollar canola industry. As a consequence, the identification of clubroot on canola in Alberta led to the initiation of a large, coordinated research effort aimed at understanding and managing this disease. The purpose of this review is to summarize the extent and nature of the clubroot outbreak in the Canadian canola crop, 10 years after it began, and to provide a context for the research and management strategies that have since been developed.

Current occurrence of clubroot on canola

In order to obtain a better understanding of the prevalence of the disease, yearly clubroot surveys were initiated in 2004 and have continued to the present. While limited surveying in 2004 revealed no new cases of clubroot in that year, new infestations have been identified every year since, and as of 2012 there were 1,064 fields with confirmed *P. brassicae* infestations in Alberta (Strelkov *et al.*, 2013; Fig. 1). The actual number of infestations is most likely considerably higher, as little or no surveying has been conducted in recent years in some of the most heavily infested areas, such as Sturgeon County, which lie at the heart of the outbreak. The levels of infection in most canola crops range from low to moderate, with infections often occurring in patches within a field. However, a few severely infected canola crops also are found almost every year, sustaining losses of 30% to 100% (Tewari *et al.*, 2005; Strelkov *et al.*, 2007a); these severely infected crops generally represent less than 10 to 15% of all fields surveyed. While the clubroot outbreak is still confined largely to central Alberta, there appears to be a continued dissemination of the disease, with increasing numbers of clubroot-infested fields identified northeast, east and southeast of Edmonton, in areas formerly considered to be peripheral to the epidemic (Strelkov *et al.*, 2013). Isolated cases of clubroot also have been identified in southern Alberta, and in two canola nurseries in north-central Saskatchewan (Dokken-Bouchard *et al.*, 2012). Canola plants with very weak symptoms of clubroot were reported from experimental field plots near Elm Creek, Manitoba, in 2005 (Cao *et al.*, 2009).

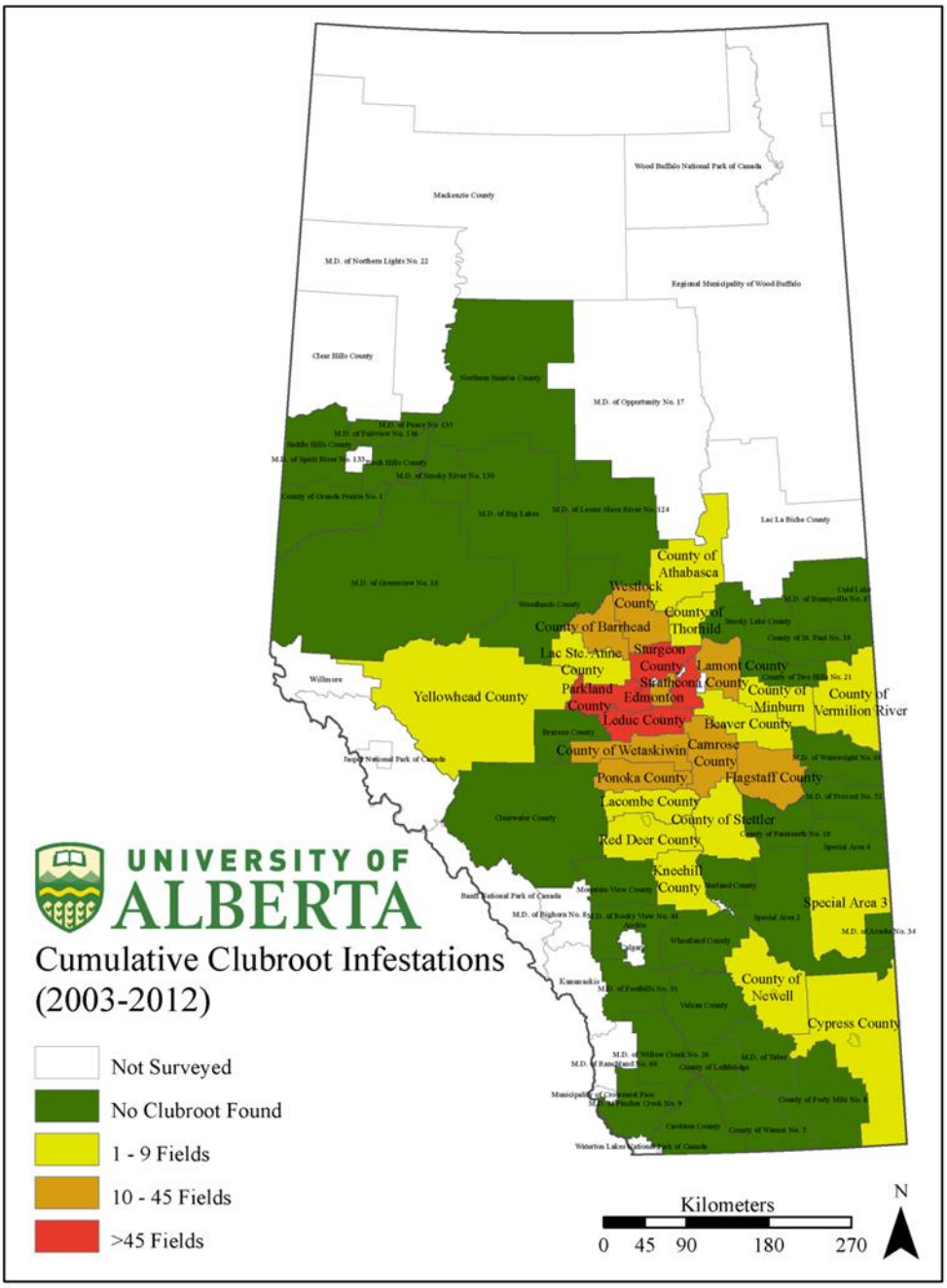

Fig. 1. The distribution of *Plasmodiophora brassicae*-infested fields in Alberta, Canada, as of November 2012. Since 2003, the clubroot pathogen has been confirmed in a total of 1,064 fields representing 24 counties and a rural area of the City of Edmonton.

Concerns regarding the spread of clubroot into Saskatchewan and Manitoba have led to the initiation of clubroot surveillance activities in those provinces. However, while the surveys in Alberta have focused on the visual identification of clubroot symptoms (galls) on the roots of canola plants, surveillance in Saskatchewan and Manitoba has included collection of soil samples for PCR testing (Cao *et al.*, 2007; Rennie *et al.*, 2011), even in the absence of any visible symptoms in a field. Soil samples that test positive for the presence of *P. brassicae* DNA by PCR analysis are then subjected to a bioassay with the universally susceptible Chinese cabbage (*Brassica rapa* L. var. *pekinensis*) 'Granaat', in order to assess the infective potential of the inoculum. In 2012, quantitative PCR analysis of over 200 samples revealed measurable levels of *P. brassicae* resting spores in soil from one field in Saskatchewan and two fields in Manitoba, which caused weak clubroot symptoms in the subsequent bioassays (S.E. Strelkov, *unpublished data*). The original source(s) of this inoculum, or of the inoculum that caused the clubroot symptoms in the cases reported earlier from Saskatchewan and Manitoba (Cao *et al.*, 2009; Dokken-Bouchard *et al.*, 2011), is not clear at this time.

Dispersal of *P. brassicae*

The increasing occurrence of clubroot in the Prairies, especially in Alberta, suggests the fairly rapid spread of *P. brassicae*, with a nearly 90-fold increase in confirmed field infestations over the past decade. It is likely that some of this increase reflects greater clubroot awareness and more intensive disease surveillance. However, there are several lines of evidence that suggest that dissemination of *P. brassicae* is the main reason behind the rising prevalence of clubroot. First, hundreds of canola crops have been surveyed annually for most of the past decade by members of the same research group (Strelkov *et al.*, 2005, 2006a, 2007a, 2008, 2009, 2010, 2011b, 2012, 2013), who presumably would have detected greater numbers of clubroot infestations earlier, if these had existed. Second, re-surveying of a subset of fields, which had been found to be free of clubroot in previous surveys, revealed that a significant proportion had become infested with *P. brassicae* when these were re-sampled in 2012 (Strelkov *et al.*, 2013). Finally, a number of *P. brassicae* dispersal mechanisms have been documented, each of which could contribute to the spread of clubroot in the Prairies. These mechanisms are discussed below.

Since *P. brassicae* is a soilborne parasite, any process that moves infested soil from one point to another could contribute to its dissemination. Therefore, the transportation of machinery from one field to another, such as during routine farming operations, could be expected to transport *P. brassicae* as well, if this machinery was contaminated with infested soil. Indeed, approximately 50 kg and 150 kg of soil could be recovered from a cultivator and tractor, respectively, in farm equipment sanitation trials in southern Alberta (Hwang *et al.*, 2013b), suggesting that the amounts of soil that can be moved on machinery are significant. The important role of machinery in the spread of clubroot was confirmed in a survey that examined the distribution of *P. brassicae*-infected canola plants within clubroot-infested fields (Cao *et al.,* 2009). This study revealed that the highest frequency of infection occurred at the field entrances, and dropped significantly at sampling points 150 m and 300 m from the entrance, consistent with the introduction of *P. brassicae* inoculum in soil carried on contaminated farm machinery. In a similar manner, the movement of heavy equipment associated with activities such as oil and gas well drilling and servicing, earthmoving, and custom agricultural work may also contribute to the dispersal of clubroot (Strelkov *et al.*, 2011a). Nonetheless, while the dissemination of *P. brassicae* on farm and other machinery appears to be its primary means of spread, other mechanisms may also be contributing to clubroot dispersal, although to varying degrees.

A few early studies (for example, Warne, 1943) and numerous anecdotal reports suggested that the transmission of *P. brassicae* as an external contaminant on seeds and tubers also is possible. To evaluate this possibility, particularly within a western Canadian context, a quantitative PCR-based assay was developed to detect and measure the amounts of *P. brassicae* resting spores found on seeds of common field crops and potato tubers grown in clubroot-infested fields (Rennie *et al.*, 2011). Using this assay, quantifiable levels of infestation were found on seven of 46 seed and tuber samples tested, although at amounts that were generally much less than what can be carried on machinery. Nonetheless, the identification of seed- and tuber-borne *P. brassicae* inoculum suggests that clubroot may spread on propagative materials, and that this possibility should not be ignored in the development of clubroot-containment strategies (Rennie *et al.*, 2011). Practices such as commercial seed cleaning and fungicidal seed treatments appear to be highly effective at reducing seed-borne inoculum of *P. brassicae* (Rennie *et al.*, 2011; Hwang *et al.*, 2012), and therefore should be routinely employed by farmers to help prevent clubroot spread.

Anecdotal evidence also suggests that *P. brassicae* can be disseminated through wind- and water-mediated erosion. Indeed, surveys in Alberta have revealed the occurrence of clubroot along water runs and the edges of otherwise disease-free fields, consistent with the movement of resting spores in water or wind-borne dust (Strelkov *et al.*, 2011a). A preliminary study was conducted to assess the significance of wind-borne dispersal of *P. brassicae* in commercial canola production (Rennie *et al.*, 2012). Dust samplers were deployed at five fields in central Alberta and one field in southern Alberta, which were under various tillage regimes. The samplers collected dust at five different heights at four or five different locations at each site, with the accumulated dust collected at biweekly intervals and analyzed for both dust loads and resting spore presence/quantity. The resulting data indicated that *P. brassicae* resting spores can be moved in wind-borne dust, and that dust loads varied according to sampler height (Rennie *et al.*, 2012). Resting spore concentrations as high as 2.2×10^5 spores g^{-1} soil were found in some of the dust samples (S.E. Strelkov, *unpublished data*), but no clear relationships were established between dust quantity, sampler height or position, and resting spores (Rennie *et al.*, 2012). Nonetheless, the results support the suggestion that erosion may contribute to the dissemination of clubroot, with the magnitude of this contribution dependent on the amount of infested dust moved and the distance that it travels. Additional research is needed to fully assess the risk associated with wind-borne spread of *P. brassicae*, as well as with its water-mediated dispersal. Even so, proper stewardship of cultivated fields may be of value not only for soil conservation purposes, but also for reducing the spread of clubroot. The various mechanisms implicated in the dissemination of *P. brassicae* in western Canada are summarized in Fig. 2.

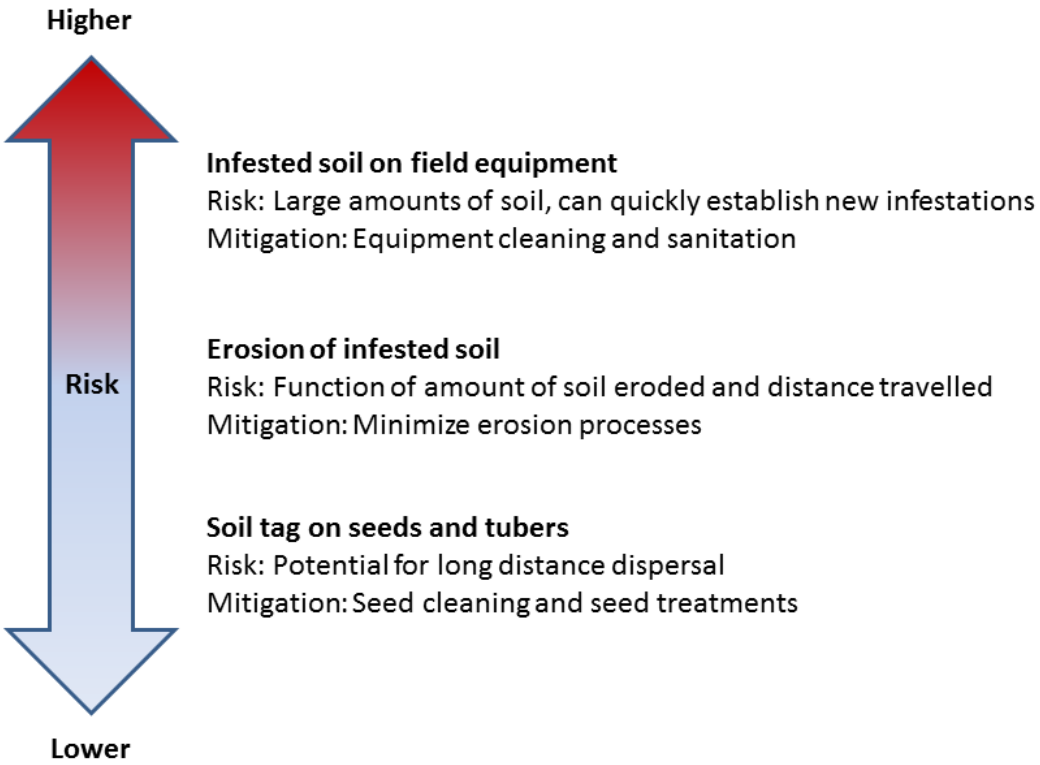

Fig. 2. Possible *Plasmodiophora brassicae* dissemination mechanisms in western Canada. The relative risk and possible mitigation strategies associated with each mechanism are indicated.

Characteristics of clubroot-infested fields

Although the development of clubroot tends to be favored in acidic soils (Karling, 1968), the occurrence of the disease on canola in Alberta has not been restricted to fields with acidic soils (Strelkov *et al.*, 2007a). Indeed, surveys of commercial fields in Alberta from 2005 to 2010 revealed only a small negative correlation (r = -0.33) between clubroot severity and pH, indicating that other factors, such as inoculum load, moisture, soluble calcium, and temperature play an important role in the development of the disease (Gossen *et al.*, 2013). Almost without exception, the most severely infected canola crops in Alberta have been identified in fields that were cropped to canola every year or every second year, suggesting that the frequent cropping of a susceptible crop resulted in a build-up of soil inoculum levels.

Physiologic specialization in Canadian populations of *P. brassicae*

The occurrence of physiologic specialization in *P. brassicae* has long been established (Honig, 1931) and can have important implications for clubroot resistance breeding efforts, since strains of the parasite differ in their ability to infect specific host genotypes. In Canada, work to characterize the strains of *P. brassicae* began in the 1950s, when the virulence patterns of parasite collections from the Maritimes were assessed on a differential set consisting of wild and cultivated crucifers (Ayers, 1957). In recent decades, the differential hosts of Williams (1966), the European Clubroot Differential (ECD) set (Buczacki *et al.*, 1975), and Somé *et al.* (1996) have been most widely used to analyze populations of *P. brassicae*. Until the outbreak of clubroot on canola, however, there had been limited evaluation of the pathotype ('race') composition of Canadian populations of the parasite. Williams (1966) identified pathotypes 2 and 6 in populations of *P. brassicae* from Quebec and British Columbia, respectively, when these were tested on his set of differential hosts. Pathotype 6 also was found to be predominant in cole crops in Ontario (Reyes *et al.*, 1974), while pathotype 3 was most common in an analysis of *P. brassicae* populations from Nova Scotia (Hildebrand & Delbridge, 1995). The identification of clubroot on canola in Alberta led to a concerted effort to characterize the virulence of parasite populations (Strelkov *et al.*, 2006b, 2007; Cao *et al.*, 2009) and single-spore isolates (Xue *et al.*, 2008) from this crop. In this context and throughout this paper, the term 'population' will be used to refer to a collection of resting spores recovered from infested soil or clubs of an infected plant, while 'single-spore isolate' will be used to refer to a population derived from a club inoculated with a single-spore isolate of *P. brassicae* and maintained in isolation (Voorips, 1995; Xue *et al.*, 2008).

The evaluation of more than 50 *P. brassicae* populations and single-spore isolates recovered from canola in central Alberta indicates that pathotype 3, as defined on the differentials of Williams (1966), or pathotype P_2 or ECD 16/15/12, as defined on the differentials of Somé *et al.* (1996) or the ECD set (Buczacki *et al.*, 1975), respectively, is predominant in the region (Strelkov *et al.*, 2006b, 2007; Cao *et al.*, 2009; S.E. Strelkov, *unpublished data*). A single population recovered from soil in Saskatchewan also was classified as pathotype 3 on the differentials of Williams (1966) (S.E. Strelkov, *unpublished data*). In contrast, analysis of two populations from canola in southern Alberta suggests that pathotype 5 or P_3, as classified on the differentials of Williams (1966) or Somé *et al.* (1996), respectively, is most common there (Cao *et al.*, 2009). Testing of a *P. brassicae* population from canola in Manitoba and another from Quebec also revealed the presence of pathotype 5, as defined on the differentials of Williams (1966), but these were classified as P_2 and P_3, respectively, on the differentials of Somé *et al.* (1996) (Cao *et al.*, 2009). Collectively, the results point to the predominance of Williams' (1966) pathotype 3 at the center of the clubroot outbreak in central Alberta, while pathotype 5 has been associated with isolated cases of clubroot on canola elsewhere (Table 1).

Table 1. *Plasmodiophora brassicae pathotype composition in Canada.*

Province	Pathotype(s)[a]		Reference(s)
	Populations[b]	Single-spore isolates[c]	
Alberta	3, 5, 2	3, 8, 2, 6	Strelkov *et al.*, 2006; Strelkov *et al.*, 2007b; Xue *et al.*, 2008; Cao *et al.*, 2009
British Columbia	6	6	Strelkov *et al.*, 2006; Williams, 1966; Xue *et al.*, 2008
Manitoba	5	n/a[d]	Cao *et al.*, 2009
Nova Scotia	3, 1, 2	n/a	Hildebrand & Delbridge, 1995
Ontario	6	3, 5, 8	Reyes *et al.*, 1974; Strelkov *et al.*, 2006; Xue *et al.*, 2008; Cao *et al.*, 2009
Quebec	2, 5	n/a	Williams, 1966; Cao *et al.*, 2009
Saskatchewan	3	n/a	S.E. Strelkov, *unpublished data*

Notes: [a]Pathotype designations are based on the differentials of Williams (1966), since this was the only system used in some of the references. Pathotypes are listed in order from most to least frequently reported in a province; in cases where two pathotypes are equally common, they are listed in numerical order.
[b]A 'population' refers to a collection of resting spores recovered from infested soil or clubs of an infected plant (Voorips, 1995; Xue *et al.*, 2008).
[c]A 'single-spore isolate' refers to a population derived from a club inoculated with a single-spore isolate of *P. brassicae* and maintained in isolation (Voorips, 1995; Xue *et al.*, 2008).
[d]n/a = not available (i.e., single-spore isolates were not characterized).

Although pathotypes 3 and 5 appear to be the most common on canola, additional strains of *P. brassicae* have also been recovered, particularly when single-spore isolates rather than populations of the parasite were examined. Other pathotypes collected from soil and canola in central Alberta include pathotypes 2, 6 and 8, as classified on the differentials of Williams (1966) (Strelkov *et al.*, 2006; Xue *et al.*, 2008; Cao *et al.*, 2009). While pathotype 6 is rare in Alberta, recent studies suggest that it is still the predominant pathotype on cruciferous vegetables in British Columbia and Ontario (Strelkov *et al.*, 2006; Xue *et al.*, 2008; Cao *et al.*, 2009), just as it was decades ago (Williams 1966; Reyes *et al.*, 1974). In Ontario, however, analysis of single-spore isolates versus populations of *P. brassicae* indicates a more complex pathotype composition than previously thought (Xue *et al.*, 2008) (Table 1). The fairly diverse pathotype structure of *P. brassicae* in Canada suggests that clubroot resistance in canola will have to be well-managed, since the virulence of parasite populations can shift quickly in response to selection pressure (Seaman *et al.*, 1963; LeBoldus *et al.*, 2012).

Limitations of the current host differential sets

As noted above, recent studies of physiologic specialization in *P. brassicae* have focused on its virulence on the differentials of Williams (1966), Somé *et al.* (1996), and the ECD set (Buczacki *et al.*, 1975). While these systems have been fairly effective for identifying the predominant strains of the parasite in Canada, each has its own limitations, particularly with respect to the applicability of pathotype designations to the *P. brassicae*/canola pathosystem. The differential set of Williams (1966) consists of two rutabagas (*B. napus* L. var. *napobrassica*) and two cabbages (*B. oleracea* L. var. *capitata*). While the system is straightforward and consists of a small number of hosts (thereby reducing greenhouse space requirements when conducting pathotype assessments), it was originally developed to identify strains of *P. brassicae* from cabbage and rutabaga. Therefore, a pathotype designation based on the system of Williams (1966) may not necessarily reflect virulence patterns on *B. napus* canola. For instance, most of the *P. brassicae* isolates and populations from the Canadian prairies share an identical virulence pattern on many canola cultivars, but are classified as pathotype 3 or 5 based on the distinct reactions of the cabbage 'Jersey Queen' and the rutabaga 'Laurentian', which are two of Williams' differential hosts. Conversely, some parasite populations that are classified into the same pathotype based on this system possess distinct virulence patterns on other hosts (S.E. Strelkov, *unpublished data*). An additional challenge associated with the use of Williams' differential set is the occurrence of host reactions that are inconsistent and (or) intermediate between resistant and susceptible (Kuginuki *et al.*, 1999; Strelkov *et al.*, 2006); in some cases at least, this appears to reflect heterogeneity within the differential genotypes, which are older, open-pollinated cultivars. These intermediate and fluctuating reactions can make the pathotype classification of some *P. brassicae* populations difficult.

Similar limitations have been observed with the differential set of Somé *et al.* (1996). While this set consists of only three *B. napus* hosts that yield clear reactions to the parasite, its capacity to differentiate pathotypes is fairly low, at least as assessed with Canadian populations of *P. brassicae* (Strelkov *et al.*, 2007b; Xue *et al.*, 2008; Cao *et al.*, 2009). The last of the three differential systems used in Canada, the ECD set (Buczacki *et al.*, 1975), consists of three subsets (*B. rapa*, *B. napus* and *B. oleracea*) of five hosts each. This enables comparisons between multiple genera and genotypes, and can provide significant information on the virulence patterns of a particular isolate or population. The large number of hosts, however, results in a requirement for a large amount of greenhouse space when characterizing *P. brassicae* populations. Moreover, not all of the hosts are effective differentials (Toxopeus *et al.*, 1986), and the *B. rapa* subset has not been useful for distinguishing parasite populations from Canada (Strelkov *et al.*, 2006b). The binary notation system of nomenclature used to designate ECD pathotypes also is viewed as cumbersome and confusing by many breeders, farmers, and agricultural extension personnel, resulting in limited acceptance of the ECD system in Canada.

A Canadian clubroot differential set?

The limitations associated with the currently available differential sets have stimulated interest in the development of a novel system(s) to more effectively identify strains of *P. brassicae* in the Canadian canola crop. Any new differential set developed within this context, however, would have to meet four main criteria: (1) it must have a good differentiating capacity; (2) it must have relevance to canola production; (3) it must provide clear and consistent results; and (4) it must consist of plant hosts for which the seeds are readily available. Research aimed at evaluating the suitability of various crucifer genotypes as clubroot differentials is already underway, as described below.

In one recent study, the reactions of five lines from the Rapid Cycling Brassica Collection (RCBC; Wisconsin Fast Plants, Madison, WI) and 84 lines of *Arabidopsis thaliana* (L.) Heynh. were assessed against pathotypes 2, 3, 5 and 6 of the parasite, as classified on the system of Williams' (Sharma *et al.*, 2013). Some of the lines were found to effectively differentiate these four pathotypes, but they could not detect any new virulence patterns among the limited populations tested. While this does not mean that these lines could not be used in the future for development of new methods to distinguish pathotypes of *P. brassicae*, they may be more appropriate for the identification of strains of the parasite from cruciferous vegetables, since they served to confirm selected pathotype designations of Williams (1966). Some of the RCBC and *A. thaliana* lines also may have significant potential as model crops for the study of clubroot, particularly with respect to resistance reactions (Sharma *et al.*, 2013), and

thereby contribute to an improved understanding of this pathosystem.

In a different approach, 11 *B. napus* and two *B. rapa* genotypes were tested for their suitability as potential hosts in a putative Canadian clubroot differential set (S.E. Strelkov, *unpublished data*). These included some of the genotypes from the ECD set (Buczacki *et al.*, 1975), the differentials of Somé *et al.* (1996), and several commercially available Canadian cultivars of canola. The hosts from the ECD set and Somé *et al.* (1996) were selected because, in earlier studies, they gave clear reactions and (or) possessed good differentiating capacity against Canadian populations of the parasite; their inclusion in a new differential system also would allow direct comparisons with data obtained previously using the older systems. Clubroot-resistant canola cultivars also were included as putative differentials because they could help to monitor changes in the virulence profiles of parasite populations from the Canadian canola crop. This collection of hosts was tested against 11 populations and 7 single-spore isolates of *P. brassicae*, representing collections of the parasite from canola in the Prairies, canola in Quebec, and cruciferous vegetables from Canada, the United States and China. The putative differentials were able to distinguish five strains of *P. brassicae*, with some isolates and populations formerly classified as pathotypes 5 and 6 on the differentials of Williams (1966) separated into distinct groups (S.E. Strelkov, *unpublished data*). Several of the hosts gave similar reactions and could be represented by a single genotype, thereby reducing the number of genotypes that would need to be included in a new differential set. The data indicate that these hosts may constitute an effective foundation for the development of a Canadian clubroot differential set, aimed at better characterizing *P. brassicae* populations from this country. Ultimately, a coordinated approach will be needed to ensure that a robust, widely accepted system of differentials is developed. From this perspective, it may also be desirable to explore the development of near-isogenic *B. napus* lines for differentiating strains of the parasite, although these would not likely be available for many years.

Clubroot management in canola

Initially, following the discovery of clubroot on canola in Alberta, few management options were available to farmers. In cases where a field was confirmed to be already infested with *P. brassicae*, the main strategy was rotation out of canola (or other susceptible crops) for four or more years. This was not a popular option, given the higher returns typically associated with canola relative to other cropping options available in many parts of the Prairies (Strelkov *et al.*, 2011a). In cases where a field was free of clubroot, the main management strategies focused on exclusion to prevent introduction of the parasite. However, clubroot exclusion practices rely on proper sanitation of equipment, and while many agricultural and oil and gas companies that regularly enter farmers' fields have adopted rigorous sanitation protocols, the adoption of such practices by farmers themselves has been less common (Strelkov *et al.*, 2011a). The most important clubroot management tool, genetic resistance, did not become available until 2009-2010, when clubroot-resistant canola cultivars were first released into the Canadian market. These cultivars have shown excellent resistance to the predominant pathotypes of *P. brassicae* in Canada (Strelkov *et al.*, 2011a), and have been widely adopted in regions where clubroot is now endemic. Indeed, the cropping of resistant canola cultivars is currently the key clubroot management strategy around which all other strategies revolve. In order to preserve the effectiveness of genetic resistance, however, provincial clubroot management plans recommend a break of three years between resistant canola cultivars on clubroot-infested fields (Alberta Clubroot Management Committee, 2010). While genetic resistance is important, a truly integrated clubroot management approach will have to incorporate other tools, such as soil amendments, fungicides and bait crops, if and when these become available. Clubroot management is a topic of intense interest among those involved in the canola industry, and is discussed in more detail in a number of recent reviews (Strelkov *et al.*, 2011a; Hwang *et al.*, 2013b).

Conclusions

The emergence of clubroot on canola has represented a major challenge to Canadian farmers and the agriculture industry in general. In less than a decade, the disease has become established in central Alberta and has continued to spread. With increasing numbers of clubroot infestations also detected in southern Alberta, Saskatchewan and Manitoba, the disease is now an issue Prairie-wide. A concerted and coordinated research and extension effort, funded largely by farmers, industry and government, has been critical to increasing understanding

of clubroot and developing knowledge-based strategies for its management. While these advances have largely served to protect the canola crop from the worst impacts of clubroot, a proactive approach is still required to stay ahead of the disease. Therefore, research on topics such as the genetics of resistance, mechanisms of parasite virulence and pathogenesis, and disease control in the field, combined with clubroot surveillance and extension activities will continue to serve as the foundation for the sustainable management of clubroot of canola.

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Disease Management Pillar – Dr. S.F. Hwang and R. H. Howard

Official start date of project: 1-April, 2009
Projected end date of project: 31-March, 2013
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Project ID: Disease Management Pillar
Project Title: An Integrated Strategy for the Sustainable Risk Management of Clubroot in Canola
Name of Principal Investigator (PI): Sheau-Fang Hwang (Alberta Agriculture and Rural Development)
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Project ID: An Integrated Strategy for Sustainable Risk Management of Clubroot in Canola – Disease Management Pillar

Project Title: Evaluating Methods for Disinfesting Tools, Machinery and other Hard Surfaces Contaminated with Clubroot Resting Spores
Name of Principal Investigator (PI): R.J. Howard
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oil & gas industry
construction companies

CLUBROOT RISK MITIGATION INITIATIVE

FINAL REPORT

Disease Management Pillar: Management of clubroot (*Plasmodiophora brassicae*) on canola (*Brassica napus*) in western Canada

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N.B.: A version of this report will be submitted for publication in a Special Clubroot Issue of the *Canadian Journal of Plant Pathology*

Executive Summary:

Clubroot has emerged as a serious threat to the canola production industry in Canada. This review focuses on research on the management of clubroot on canola in western Canada since its discovery in Alberta, Canada, in 2003 and summarizes the research results, collected since 2007, on the effects of host plant resistance, crop rotation, baiting crops, seeding date manipulation, seedling age, soil amendments, equipment sanitization, fungicides, fumigants, and biocontrol agents for the management of clubroot in canola. Several options have been put forward for clubroot management in infested fields, including liming the soil to increase soil pH, crop rotation with non-hosts and bait crops to reduce the concentration of pathogen resting spores, manipulating the seeding date, and deployment of resistant cultivars. Research began by assessing treatments developed for the vegetable production industry. Although these treatments provide useful levels of clubroot reduction for intensive production of short-season Brassica vegetable crops, most are not economical for large-scale production of canola, which requires protection over a much longer period of time (seeding to seed harvest). Genetic resistance to clubroot has been shown to be a practical option for the management of clubroot on canola, but resistance stewardship coupled with rotation and cultural practices will be required to maintain the performance and durability of genetic resistance. Efforts to minimize the spread of clubroot have focused largely on the sanitization of field equipment and seed, as pathogen resting spores can be disseminated on infested soil carried on both machinery and seed.

Keywords: baiting crops, biocontrol agents, crop rotation, fumigants, fungicides, resistance, sanitization, seeding date, soil amendments

Introduction

Clubroot, caused by *Plasmodiophora brassicae* Woronin, is an important disease of canola (*Brassica napus* L.) on the Canadian prairies (Hwang *et al*., 2012b). The disease has spread from an original outbreak near Edmonton in 2003 (Tewari *et al*., 2005) to more than 1,000 fields throughout the province of Alberta in 2012, and has also been confirmed in the neighboring provinces of Saskatchewan (Strelkov, 2012) and Manitoba (Cao *et al.*, 2009; S.E. Strelkov, *unpublished data*). This has led to concern that clubroot will spread across the prairies and could have a huge impact on Canadian canola production, since estimated yield losses in severely infected canola crops range from 30% to 100% (Strelkov *et al*., 2007; Hwang *et al*., 2011c). This has resulted in legislation in Alberta for the control of *P. brassicae* under the Agricultural Pests Act (Alberta Clubroot Management Committee, 2010). In response, a coordinated research effort was launched in Canada to develop a better understanding of the management of clubroot in the canola production systems on the prairies.

The pathogen increases rapidly in the presence of susceptible plants via the formation of resting spores, which can persist in the soil for more than 15 years in the absence of a susceptible host (Wallenhammar, 1996). Crop rotations based on standard agronomic recommendations for Canada (one year of canola in four) will not significantly reduce resting spore populations (Strelkov *et al*., 2006), and even substantially longer rotations may not be effective either because several common and endemic weed species such as horseradish (*Armoracia rusticana* P.G. Gaertn., B. Mey. & Scherb (1800)), white mustard (*B. hirta* Moench), wild mustard (*B. kaber* (DC.) L.C. Wheeler), camelina/false flax (*Camelina sativa* L. Crantz), shepherd's purse (*Capsella bursa-pastoris* (L.) Medik.), and stinkweed (*Thlaspi arvense* L.) are also susceptible hosts (Howard *et al*., 2010). All 330 genera and 3700 species of the family Brassicaceae are potential hosts of *P. brassicae* (Dixon, 2009).

The density of viable clubroot inoculum influences the probability that host plants will become infected, the likelihood that galls will develop early in the growing season, and also the probability of multiple infections occurring. High concentrations of resting spores contribute to earlier development of larger galls, which contribute larger numbers of spores to the next generation, and have a more deleterious effect on crop productivity through disruption of water and nutrient flow. Galls that develop later in the plant life cycle were shown to be smaller and less disruptive to the plant. Moreover, older plants tend to be less susceptible to clubroot as demonstrated in Chinese cabbage (*B. rapa* L. subsp. *chinensis* (Rupr.) var. *utilis*) and turnip (*B. rapa* L. var. *rapa*) (Horiuchi and Hori, 1980). If symptoms are allowed to fully develop, each infected plant has the potential to contribute up to 8×10^8 resting spores to the soil in its immediate environment (Hwang *et al*., 2012a), with the half-life of the spores is estimated to be about four years (Wallenhammar, 1996; Hwang *et al.*, 2013a). Forestalling symptom development, whether through early seeding so that the plants have the opportunity to complete more of their life cycle prior to infection, or through the application of fungicidal or pH-altering treatments so that the soil environment is less conducive to spore germination and primary infection, can reduce the impact of disease symptoms on crop production.

The biotrophic nature of *P. brassicae* has presented a challenge for research on management of this pathogen, but several options have been put forward for the management of clubroot in infested fields (Shimotori *et al*., 1996; Donald *et al*., 2001; Mitani *et al*., 2003), including liming of the soil to increase soil pH (Murakami et al., 2002), crop rotation with non-hosts and bait crops to reduce the concentration of pathogen resting spores (Kroll *et al*., 1984; Ikegami, 1985; Murakami *et al*., 2001), manipulating the seeding date (Gossen *et al*., 2009), and the deployment of resistant cultivars (Diederichsen *et al*., 2009). Many of these management options are not practical or cost effective in a canola cropping system (Campbell *et al*., 1985; Myers and Campbell, 1985; Strelkov *et al*., 2011), especially when the levels of resting spores in the soil are high. Crop rotation is complicated by the fact that the pathogen can survive as resting spores in the soil for long periods (Wallenhammar, 1996), and many common Brassica weed species on the Canadian prairies act as hosts for the pathogen. In addition to the development of management strategies for clubroot-infested fields, numerous sanitation practices have been proposed to prevent the initial introduction of *P. brassicae* resting spores to previously un-infested fields (Howard *et al*., 2010). Sanitation of farm machinery is particularly important, since the field-to-field spread of clubroot is usually initiated at the field entrance, and is likely related to the movement of infested soil on farming equipment (Cao *et al*., 2009).

This review focuses on recent studies on clubroot management in canola, including cultural practices, such as

manipulation of seeding dates, application of soil amendments, inclusion of baiting crops, soil fumigation, sanitation, crop rotation, biological control, seed treatments and the deployment of clubroot-resistant cultivars.

Soil amendments

Lime

Given that clubroot development tends to be favoured by acidic soil conditions (Karling, 1968), research has focused on the application of soil amendments that increase soil pH, a technique that has been effectively used in high value horticultural crops (Mukarami *et al*., 2002). Field observations on vegetable Brassica crops have shown that clubroot usually develops in soils where the pH is lower than 7.1 (Myers and Campbell, 1985). Thus, maintaining a pH of 7.2 or higher is considered optimal for clubroot management.

Several clubroot management strategies have been used in high value horticultural crops, including drench application of fungicides and soil amendments with lime to increase its pH. Lime amendments are available in various forms, for instance, agricultural lime (calcium carbonate and calcitic lime), dolomitic lime (calcium and magnesium carbonate), hydrated lime (calcium hydroxide), and quicklime (calcium oxide). Agricultural and dolomitic limes are relatively slow-acting, whereas hydrated lime and quicklime are more reactive and so act much more quickly. Application of slow-acting limes may have to be made in the fall to allow time for the amendments to break down prior to spring planting, and large quantities have to be applied. Fast-acting limes are more suitable for spring application. Finely ground amendments are likely to be more reactive than coarse formulations and alter the pH more rapidly. Annual applications of soil amendments may be required to raise the pH and to maintain it at desirable levels.

Extensive evaluation of soil amendments, including lime, wood ash and calcium cyanamide, has been carried out on canola in western Canada (Hwang *et al*., 2008). The results of these trials showed that soil amendment with calcium carbonate at 5.0 or 7.5 t/ha or with wood ash at 7.5 t/ha resulted in increased plant cover and height, even under heavy disease pressure (Hwang *et al*., 2008). However, the impact of liming was not consistent across years and soil types and the reduction in clubroot severity, although statistically significant, was not great relative to an untreated control in canola field trials conducted in Alberta. The application of large quantities of lime or wood ash soil amendments in hundreds of fields would be impractical, and their effects on yield would be too small to justify the expense of application (Hwang *et al*., 2011b). Moreover, earlier studies have indicated that liming of acid soils does not always reduce clubroot severity (Webster &Dixon, 1991), and that application of lime may not be effective if temperature and soil moisture content do not limit infection by *P. brassicae* (Colhoun,1953). A study by Kasinathan (2012) supported this conclusion, revealing that while high pH inhibited clubroot infection, the prevalence of clubroot-conducive conditions, especially soil moisture, largely negated this effect.

Calcium cyanamide

Calcium cyanamide ($CaCN_2$) has been used to reduce clubroot on cruciferous vegetables in Europe, Australia and New Zealand (Dixon, 2012). It breaks down into calcium oxide and urea, thus increasing the soil pH and also enriching the soil with nitrogen, and in the process, releases hydrogen cyanide, which is fungitoxic. Calcium cyanamide has also been shown to have beneficial effects on other soil microflora. Soil amendments with calcium cyanamide under western Canadian field conditions did not result in a reduction in clubroot severity relative to control treatments, nor did these amendments cause any changes in seedling emergence or yield (Hwang *et al*., 2008).

Boron

Boron inhibits the change from plasmodium to sporangium during root hair infection (Webster and Dixon 1991). Application of this element has been recommended for the reduction of clubroot on vegetable crops for more than 70 years (O'Brien and Dennis 1936; Dixon 2009), so the application of boron to reduce clubroot severity in canola was investigated in Canada (Deora *et al*., 2011). A significant reduction in clubroot severity on canola was observed when boron was applied at a rate of 4 kg/ha in a field trial. However, higher application rates, while effectively reducing clubroot severity, were also phytotoxic.

Soil fumigants

It has been suggested that by eradicating resting spore populations at a field entrance or in any newly established infection foci in a field, the spread of *P. brassicae* might be contained. Application of Vapam, an aqueous metham-sodium (dithiocarbamate; sodium N-methyldithiocarbamate) product, and several other fumigants have been shown to reduce clubroot severity on cabbage (*B. oleracea* L. var. capitata) (White & Buczacki, 1977). In contact with moist soil, Vapam is largely converted to methyl isothiocyanate, a volatile compound that diffuses as a gaseous fumigant through the soil and has considerable nematicidal, fungicidal and phytocidal activity (Smelt & Leistra, 1974). Preliminary tests have been conducted in Alberta and have resulted in substantial reductions in the severity of clubroot in canola sown in soils that had been fumigated one week prior to planting (Hwang *et al*. 2013b).

Recently, the effect of Vapam fumigant was assessed on primary and secondary infection rates in canola, clubroot severity, and plant growth parameters under controlled environmental conditions in Alberta (S.F. Hwang, *unpublished data*). These preliminary trials resulted in a 12–16 fold reduction of primary and secondary infection and clubroot severity at all of the applications rates (0.4–1.6 mL L^{-1} soil) of Vapam that were assessed. Moreover, after one crop of canola, the residual effect of Vapam at the higher rates (0.8 and 1.6 mL L^{-1} soil) reduced clubroot development and improved plant health in a subsequent crop compared to the non-treated control. The application of Vapam at soil moisture levels in the range of 10–30% had a large effect on both disease symptoms and plant growth parameters. Therefore, it appears that Vapam effectively reduced clubroot severity and has potential for treatment of transplant propagation beds of Brassica vegetables and for the eradication of small infestations of clubroot of canola in commercial fields.

Seed treatments

Warne (1943) provided indirect evidence that the clubroot pathogen can be seedborne, which may contribute to the long distance spread of the disease. A recent study in Alberta revealed that the surfaces of some potato (*Solanum tuberosum* L.) tubers and the seeds of canola and other crops harvested from clubroot-infested fields (Rennie *et al*., 2011) were contaminated with *P. brassicae* resting spores. While seed cleaning seemed to reduce the risk associated with seed-borne dispersal of *P. brassicae* by removing soil lumps, pieces of crop residue and shriveled seed (Rennie *et al*., 2011), a prudent clubroot containment strategy should also include pre-seeding seed sanitization treatments with chemical fungicides, particularly if the seed was harvested from a clubroot-infested field. Moreover, any delay in infection reduces the severity of clubroot symptoms on affected plants, thus supporting the idea that seed treatment with appropriate fungicides may be an effective way to reduce infection from inoculum that is already present in a field (Rod 1992).

Although the fungicidal seed treatments Dynasty 100FS (azoxystrobin), Helix Xtra (thiamethoxam + difenconazole + metalaxyl + fludioxonil), Prosper FX (clothianidin + carbathiin + trifloxystrobin + metalaxyl), Vitavax RS (carbathiin + thiram) and Nebijin (flusulfamide) were shown to reduce infection under greenhouse conditions, and were effective at removing seedborne inoculum of *P. brassicae*, they did not reduce clubroot severity on canola grown in fields that were already infested (Hwang *et al*., 2011b). Therefore, while seed treatments may reduce the risk associated with seed-borne dissemination of *P. brassicae*, it appears that they are insufficient to reduce the impact of clubroot in heavily infested fields.

Fungicides

Reductions in clubroot severity are also possible with the application of fungicides to the soil (Mitani *et al*., 2003). The use of fungicides for the control of clubroot on canola and other crops is discussed in more detail in an accompanying review (Peng *et al.*, 2013). Nevertheless, brief mention is made here of studies undertaken in western Canada to assess the efficacy of chemical fungicides in reducing the severity of clubroot on canola, with the aim of providing farmers with additional disease management options. In field trials conducted in Alberta, pentachloronitrobenzene (PCNB, Terraclor 75% WP) was incorporated into the soil prior to seeding, and found to result in a significant reduction in clubroot severity (Hwang *et al*., 2008). However, health concerns, the persistence of this product in the soil, and the costs associated with its application make it unlikely that PCNB will ever be an option for clubroot management in large-scale production of canola. The efficacy of cyazofamid

(Ranman), which has a direct effect on resting spore germination, root hair infection and the formation of clubroot galls (Mitani *et al*., 2003), was also evaluated in Alberta (Hwang *et al*., 2008), Like PCNB, the application of cyazofamid, when incorporated into the soil prior to seeding, significantly reduced the severity of clubroot in less heavily infested soils. The application rates found to be effective, however, would be prohibitively expensive for treatment of canola crops.

Cultural and biological controls

A range of alternative management strategies have been assessed for their usefulness in canola production, including timing of seeding (Gossen *et al*., 2009; Hwang *et al*., 2012a), biological control (Peng *et al*., 2011), and the use of bait crops (Kroll *et al*., 1983; Ikegami, 1985; Murakami *et al*., 2001; Ahmed *et al*., 2011).

Long crop rotations

The concentration of viable *P. brassicae* resting spores in the soil is an important factor in the subsequent development of a clubroot epidemic (Murakami *et al*. 2002). Reduction of the viable spore density to approximately 1×10^4 to 1×10^5 spores/mL of soil was required to reduce the clubroot disease index to 50% of that observed at 1×10^8 spores/mL (Hwang *et al*., 2011a). Donald and Porter (2009) concluded that extremely long rotations are needed to reduce high inoculum concentrations to a level below a disease-causing threshold. Similarly, Wallenhammar (1996) reported that under conditions in Sweden, 17.3 years were required to reduce the level of infestation in soil from an amount causing 100% infection in plant bioassays to an amount that could not be detected in a bioassay. Resting spores are impervious to most constituents of their soil environment, but following germination, they must quickly establish an infection in a host for continued survival (Suzuki *et al*., 1992; Takahashi, 1994).

Bait crops

In the clubroot disease cycle, the germination of resting spores is the primary event in disease development. Many plants have the ability to induce spore germination without becoming infected and so induction of resting spore germination in the absence of host plants has been proposed as a possible tool in the management of clubroot (Friberg *et al*., 2005). When the germination of resting spores occurs in the absence of a suitable host, the resulting zoospores quickly lose viability (Suzuki *et al*. 1992; Takahashi 1994). Inducing resting spore germination in the absence of host plants to accelerate reduction in spore populations could be used as a component of an integrated clubroot management program (Friberg *et al*., 2006). Host crops, if they are killed before gall formation occurs, could also act as bait crops.

A crop that stimulates resting spore germination (i.e., a bait crop) could be planted and then ploughed down before the pathogen completes it life cycle, thereby reducing resting spore populations in heavily infested fields. Root hair infection has been reported in a wide range of non-Brassica plant species, including common velvet-grass (*Holcus lanatus* L.), perennial ryegrass (*Lolium perenne* L.), Indian Cress (*Tropaeolum majus* L.) (Webb, 1949; MacFarlane, 1952), and *Fragraria magna* (strawberry) (Lugauskas *et al*., 2003); however, cortical infection and the subsequent development of resting spores has been reported in several non-cruciferous plant species, such as *T. majus* and beet (*Beta vulgaris* L.) (Ludwig-Müller *et al*., 1999). Root exudates of perennial ryegrass (Rod & Robak 1994), lettuce (*Lactuca sativa* L.) (Ikegami 1985; Robak 1996), leeks (*Allium ampeloprasum* var. porrum (L.) Gay), rye (*Secale cereale* L.) and red clover (*Trifolium pratense* L.) induce resting spore germination (Friberg *et al*,. 2006). In the absence of host plants, germinated spores likely survive for short periods (Suzuki *et al*., 1992; Takahashi, 1994).

Perennial ryegrass was effective in reducing the resting spore numbers in one study, but was not effective in another study (Robak, 1996). Studies in northern Europe aimed at reducing the soil inoculum concentrations by using bait crops to stimulate germination of resting spores did not result in effective reductions in clubroot (Friberg *et al*., 2005, 2006). Moreover, the benefits of using non-host plants for baiting or in crop rotations was questioned when fallow treatments without bait plants reduced resting spore levels to an extent similar to the most promising non-host crops (Ikegami, 1985; Robak, 1996).

In contrast, a 5-year fallow period or the continuous cultivation of a clubroot-resistant Japanese radish (*Raphanus*

sativus var. *longipinnotu* Bailey) resulted in a substantial decrease in resting spore populations (Ikegami, 1985). The extensive scale of the clubroot outbreak on canola in Alberta necessitates approaches that can be implemented across a large area, and bait crops are one of the few potential management options that meet this requirement. However, given the unique environmental and cultural conditions on the Canadian prairies relative to northern Europe or Japan, information directly applicable to the Canadian context must be obtained before a bait crop approach can be recommended.

In Canada, clubroot incidence and severity on canola were lower following a cruciferous bait crop versus a non-cruciferous host or a cereal; non-cruciferous hosts and cereals had no impact on clubroot in greenhouse assessments (Hwang *et al*., 2013a). Similarly, when sequences of bait crops (canola, ryegrass, fallow) were assessed, clubroot severity in the subsequent canola crop was lowest in the canola-fallow sequence. Clubroot severity was consistently high in the fallow-fallow sequence, but the results of the other sequences with canola were not as consistent across two repetitions of the experiment (Hwang *et al*., 2013a). The assessment of a bait crop at two field sites in Alberta showed only a small impact on resting spore populations and no effect on subsequent clubroot severity. Resting spore concentrations at both sites were about 1×10^6 spores/g soil, so a small reduction in the spore load was difficult to detect and would not be expected to impact clubroot severity. A long-term study may be needed to detect significant reductions in clubroot inoculum and severity in heavily infested field sites. Collectively, and despite the small reductions in resting spore populations observed in some treatments under greenhouse conditions, the Canadian results support the conclusion of Friberg *et al*. (2006) that the impact of bait crops on inoculum potential and clubroot severity is too small and inconsistent to be useful for managing this disease in commercial fields.

Seeding date

Clubroot development is strongly favored by high soil moisture (Karling, 1968) and temperatures near 25˚C (Feng *et al*., 2010; Sharma *et al*., 2011). Hence, a key means of avoiding infection may be through the manipulation of the timing of seeding to reduce the vulnerability of host plants to infection. Cool soil temperatures have been shown to inhibit pathogen development (Hwang *et al*., 2011a). Older plants have been shown to be less susceptible to infection, and the infection that occurs on older plants is less likely to affect yield (Hwang *et al*., 2011c). Early seeding date reduced clubroot severity by 10–50% (only significant at one of two sites) and increased yield by 30–58% (Gossen *et al*., 2012). Similar results were obtained by manipulating seeding date to minimize clubroot in Shanghai pak choy (*B. rapa* subsp. *chinensis* var. *communis* Tsen and Lee) and Chinese flowering cabbage (*B. rapa* subsp. *chinensis* var. *utilis* Tsen and Lee) over several years at a site in central Canada (McDonald & Westerveld, 2008; Gossen *et al*., 2009; Adhikari, 2012). A study by Hwang *et al*. (2012a) reported that early seeding of canola reduced clubroot severity and increased yield, although it also reduced emergence. Previous research has indicated that younger canola seedlings are more susceptible to infection than older seedlings (Hwang *et al.,* 2011a). A resistant cultivar exhibited few or no symptoms of clubroot when inoculated 10–25 days after seeding, but some plants became infected when they were inoculated at 5 days after seeding. The susceptibility of canola roots to infection by *P. brassicae* declines with increasing age, perhaps as a result of the thickening of cell walls (Mellano *et al*., 1970) and formation of other barriers that limit pathogen colonization.

The manipulation of seeding dates alone is not sufficient, however, to manage clubroot on its own. Although additional research in this area is still required, it is likely that early seeding may be a useful tool for clubroot management on canola when used along with other crop management options, including host plant resistance, soil amendment and fungicide application.

Sanitation **(see also *Appendix 1*, p. 40 of this report)**

As a soil-borne pathogen, the intra- and inter-field spread of *P. brassicae* has generally been regarded as slow. Clubroot is a monocyclic disease, and zoospore movement in the soil is limited (Howard *et al*., 2010). However, any activity that results in transport of soil from one point to another has the potential to disseminate the pathogen (Strelkov *et al.*, 2011). A study of the distribution of infected plants within clubroot-infested fields showed that disease incidence was highest at the field entrances, and significantly lower at distances of 150 m and 300 m from the entrance (Cao *et al*., 2009), suggesting the introduction of pathogen resting spores in infested soil on machinery.

Sanitation practices are meant to slow the spread of the disease from field to field. To prevent transfer of contaminated soil to new sites, field equipment moving from infested to clubroot-free sites should be cleaned and sanitized prior to use. Sanitation involves three key steps: i) removing bulk soil and crop debris, ii) pressure washing, scrubbing or using compressed air to remove any remaining residues, and iii) applying disinfectant to the cleaned surfaces and allowing at least 20 minutes of contact time to ensure that any remaining spores are killed (Howard *et al*., 2010). Unfortunately, many farmers find rigorous sanitation protocols to be excessively time-consuming and labour-intensive, since field equipment typically exposes hundreds of surfaces directly to soil, or to spore-bearing dust. In contrast, rigorous sanitation protocols have been adopted by agricultural consultants and the oil and gas industry, which regularly enter farmers' fields with their equipment. While chlorine has been shown to be useful for disinfesting water containing *P. brassicae* resting spores (Datnoff *et al*., 1987), it is highly corrosive and therefore not desirable for routine application onto field equipment. None of the commercial disinfectants that have been evaluated to date completely eliminated clubroot resting spores (Donald *et al*., 2002; Howard *et al*., 2010). Other sources of contamination to be considered in sanitation procedures include seeds and tubers harvested from infested fields, which may serve as vectors for the dispersal of this pathogen (Rennie *et al*., 2011). Preliminary assessments of seed-to-seedling transmission of clubroot under field conditions from resting spores inoculated onto canola seeds have not resulted in any detectable levels of clubroot in a susceptible canola crop or on a susceptible canola cultivar sown on the same site the following year (McDonald and Gossen, unpublished). The successful of transmission of clubroot under greenhouse conditions (Rennie *et al*., submitted), however, still raises concerns that dissemination of the pathogen on seed may represent a viable mechanism for *P. brassicae* dispersal. Therefore, farmers should avoid planting of common, untreated seeds harvested from clubroot-infested fields.

Irrigation water that has had contact with infested soils could infect large areas. Water should not be applied to plantings of cruciferous vegetables if it has been drawn from bodies of water contaminated with clubroot spores resulting from runoff from infested fields (Howard *et al*., 2010).

Clubroot spores can survive passage through livestock, so the use of raw manure on fields from animals that have been fed or pastured on clubroot-contaminated fodder should be avoided. Although research on the effect of the composting process is limited, temperature and moisture content were shown to be important for the successful eradication of *P. brassicae* resting spores from composted residues infested with clubroot (Fayolle *et al*., 2006; Howard *et al*., 2010).

Genetic resistance to clubroot

With the exception of *B. juncea* (L.) Czern. and *B. carinata* Braun, genotypes with resistance to one or more of the pathotypes of *P. brassicae* can be found in all of the major Brassica crops (Diederichsen *et al*., 2009). Both qualitative (Crute *et al*., 1986; Wit and van de Weg, 1964) and quantitative (Chiang and Crête, 1970; Figdore *et al*., 1993; Grandclément and Thomas, 1996; Voorrips *et al*., 1997) types of resistance have been reported. Most of these sources of resistance, however, are pathotype-specific.

In *B. napus*, most studies have reported oligogenic control of resistance to *P. brassicae* (Crute *et al*., 1986). This would make the pyramiding of resistance genes in *B. napus* genotypes more practical than in other species. Gustafsson and Fält (1986) proposed models based on three, four and five resistance genes, where the most favoured model was based on four genes. A complex type of inheritance, with dominant genes from *B. rapa* and recessive genes from *B. oleracea*, can be expected in a re-synthesized *B. napus*, with resistance from both ancestral species (Diederichsen *et al*., 1996). Segregation analysis suggested that resistance in re-synthesized *B. napus* was controlled by at least two dominant and unlinked genes (Diederichsen and Sacristán, 1996). Manzanares-Dauleux *et al.* (2000) located one major gene (Pb-Bn1) for resistance against two *P. brassicae* isolates on chromosome N03, as well one additional minor QTL for each isolate on chromosomes N12 and N19. Previous reports indicate that clubroot resistance in canola is controlled by a combination of major genes and quantitative trait loci (Matsumoto *et al*., 1998; Piao *et al*., 2009; Hirai *et al*., 2004, Suwabe *et al*., 2003, 2006).

Accessions of *B. oleracea* L. with pathotype-independent resistance to *P. brassicae* have also been reported

(Voorrips, 1996). Most studies on the C genome indicate that clubroot resistance in *B. oleracea* is quantitative and under the polygenic control of one or two major QTLs and some QTLs with minor effects (Landry *et al*., 1992; Figdore *et al*., 1993; Grandclément and Thomas, 1996; Voorrips *et al*., 1997; Moriguchi *et al*., 1999; Rocherieux *et al*., 2004; Nomura *et al*., 2005). A few studies, however, indicated that clubroot resistance in *B. oleracea* is qualitative and controlled by either dominant (Chiang & Crête, 1983) or recessive (Yoshikawa, 1993) genes. It is possible that both quantitative and qualitative resistance mechanisms may be at play in this species.

Resistance genes from stubble turnips (*B. rapa*) have been used in resistance breeding of various Brassica crops, including Chinese cabbage, oilseed rape, and *B. oleracea*. Although most turnip lines carry more than one resistance gene, cultivars of the other Brassica crops with resistance derived from turnip generally carry a single, dominant resistance gene that is pathotype-specific. While important to clubroot management, genetic resistance has generally been race specific (Diederichsen *et al*., 2009) and can break down when virulent races increase in the pathogen population. Therefore, genetic resistance should be carefully managed in combination with other methods of clubroot control.

Root hair studies

Infection of root hairs by *P. brassicae* occurs in a broad range of plants and has been observed in resistant (Diederichsen *et al*., 2009) and non-host plants (Feng *et al*., 2012). These root hairs not only serve as a niche for pathogen survival, but also provide an opportunity for close interaction between host and pathogen, which facilitates the evolution of mechanisms by which *P. brassicae* can break down basal resistance to cortical infection. In susceptible plants, both root hair infection and clubroot severity increase with higher inoculum density (Hwang *et al*., 2011a, b).

Root hair infection occurs more frequently with a compatible isolate (susceptible reaction) compared to an incompatible isolate (resistant reaction) and, subsequently, fewer cells within the root become infected when the host is resistant (Tanaka *et al*., 2006). The compatible isolate forms secondary plasmodia with many nuclei and, eventually, resting spores in the host root tissue, whereas plasmodia formed by the incompatible isolate remain immature with only a small number of nuclei and do not produce resting spores (Hwang *et al*., 2011a). These results suggest that resistance in host species is associated with suppression of infection and subsequent plasmodial development during both primary and secondary colonization. The main difference between the resistant and susceptible host reactions is that in the resistant hosts, the secondary thickening of the cell walls in the xylem are not degraded and there are fewer cell wall breakages (Donald *et al*., 2008).

Previously, the influence of cultivar resistance on root hair infection in resistant and susceptible cultivars was studied, and a qPCR protocol was developed to estimate *P. brassicae* populations in the soil (Hwang *et al*., 2011a). In that study, however, only two cultivars (one resistant and one susceptible) were compared over a range of inoculum densities. In a comparison of root hair infection in two resistant and three susceptible canola cultivars, infection and disease severity were greater, while plant height was reduced, in the susceptible cultivars relative to the resistant cultivars. Root hair infection and the amount of *P. brassicae* DNA rose over time. The increases in infection and pathogen DNA, however, were greater in the susceptible cultivars than in the resistant ones. Moreover, there were strong linear relationships between percent root infection and the amount of *P. brassicae* DNA in the root hairs. The slow increase in the amount of pathogen DNA and root hair infection observed in the resistant cultivars, and the sharp increases observed at 4-6 days and 8-10 days in the susceptible cultivars, suggest the occurrence of secondary infection and pathogen development in the susceptible cultivars. It is evident that successful management of *P. brassicae* through the deployment of genetically resistant cultivars will require careful control of susceptible weeds and canola volunteers, in order to maximize the impact of resistant cultivars on resting spore levels in the soil.

Resistant plants showed reduced growth and delayed development when inoculated with resting spores of an avirulent pathotype of *P. brassicae* (Hwang *et al*., 2012a), which indicates that resistance is an active process. The rate of infection and pathogen development are most rapid in susceptible canola lines (Deora *et al*., 2012). In at least one vegetable Brassica genotype, symptomless plants have been observed where microscopic analysis has revealed low levels of pathogen infection in inoculated roots (Gludovacz *et al*., 2013). The mechanism for this response has not yet been determined, but it is possible that a gene for tolerance to *P. brassicae* is involved.

Commercial use of resistance

The cropping of genetically resistant canola hybrids has been the predominant component of the clubroot management strategy in western Canada since the introduction of the first clubroot-resistant cultivar, Pioneer '45H29', into the market in 2009, which was followed quickly by the introduction by several companies of other clubroot-resistant cultivars (Strelkov *et al.*, 2011). The durability of this resistance is not known, however, and due to proprietary considerations, the genetic basis for the resistance also is unknown. This has complicated efforts aimed at resistance stewardship, since it is not possible to develop rational strategies for the rotation of resistance sources in infested fields. The number of resistance genes that are available to breeders at the present time is limited (Hirai, 2006), and a recent trial indicated that there were no substantial differences in pathotype resistance among clubroot-resistant canola cultivars available in western Canada (Deora *et al*., 2012, 2013), suggesting that resistance genes in all of these lines originate from a similar source. In contrast, another recent study revealed that pathogen populations that had been repeatedly cycled on and become adapted to one Brassica host did not exhibit a similar adaptation to other hosts, when the cycled populations were inoculated on the latter, indicating that distinct sources of resistance may occur in at least some genotypes (LeBoldus *et al.*, 2012). Nevertheless, there is substantial genetic and pathotype variation present in the *P. brassicae* populations in western Canada (Strelkov *et al*., 2006; Xue *et al*., 2008; Cao *et al*., 2009), and the deployment of a cultivar with single-gene resistance against a genetically diverse pathogen on a large scale imposes a strong selection pressure for pathogen genotypes that are able to overcome this resistance.

Resistance breakdown

Resistance to *P. brassicae* in cruciferous crops, including oilseed rape, has broken down in the past (Seaman *et al*., 1963; Kuginuki *et al*., 1999; Oxley 2007; Diederichsen *et al*., 2009). While clubroot-resistant cultivars of *B. napus, B. oleracea* and *B. rapa* are available, their extensive use is often limited by the short durability and race or pathotype-specificity of the resistance (Voorrips, 1995; Diederichsen *et al*., 2009).It was noted by LeBoldus *et al*. (2012) that many of the currently available resistant cultivars develop at least small galls under high inoculum pressure, and that repeated cycles of inoculation result in a rapid decline in the level of resistance of some hosts, further highlighting the need to integrate the deployment of clubroot-resistant cultivars with other tools for disease control (Diederichsen *et al*., 2009; Donald & Porter, 2009). Regardless of the nature of the resistance, it will be prudent to ensure that it is carefully managed in order to maintain its durability.

Spore populations

Ideally, the cropping of resistant cultivars should be accompanied by a reduction in the viable *P. brassicae* resting spore populations in the soil. However, clubroot resistant crops are not necessarily free of susceptible plants. Susceptible volunteers will continue to be present in infested fields for many years. As noted above, susceptible weed species may also be present, and there may be a small percentage of susceptible, off-type canola plants associated with hybrid production. The cropping of resistant cultivars may stimulate resting spore germination, thereby reducing the viable spore population in the soil, while no (or few) viable resting spores will be produced on the resistant plants to contribute to soil inoculum loads. However, these benefits from the cropping of a resistant cultivar may be offset by the presence of susceptible hosts within the crop. Furthermore, the deployment of resistant cultivars may create selection pressure on the pathogen to co-evolve and erode or overcome resistance (Hwang *et al.*, 2011b; LeBoldus *et al*., 2012).

A Canadian study provided evidence for the presence of increased soil inoculum levels following the cultivation of both resistant and susceptible canola cultivars in heavily infested soil, although the increase was greater after the cropping of the susceptible cultivar (Hwang *et al*., 2012a). In moderately infested Swedish soils, oilseed turnip rape showed a slight decrease in disease in a bioassay conducted after cultivation of partially resistant cultivars of the crop (Wallenhammar *et al*., 1999). Collectively, these results suggest that, while a resistant host may serve to ameliorate the disease situation in lightly or moderately infested soils, the cropping of resistant cultivars will not serve to reduce clubroot infestations in heavily infested fields. Further research, however, is required to confirm these trends.

Experiments were conducted to assess the effects of growing resistant and susceptible canola genotypes on *P. brassicae* resting spore populations under greenhouse, mini-plot and field conditions (Hwang *et al*. 2012a). One

crop of susceptible canola contributed 1.4×10^8 resting spores mL^{-1} soil in a mini-plot experiment, and 1.0×10^{10} spores g^{-1} gall under field conditions. Repeated cropping of susceptible canola over four, 6-week cycles resulted in increasing gall mass and clubroot severity over time, while disease pressure declined slightly in continuously fallow soil and where a resistant cultivar was repeatedly cultivated. Soil inoculum loads, however, were similar after continuous cropping of resistant canola versus continuous fallow, suggesting that while the resistant cultivar does not increase soil inoculum loads, it also does not act as a bait crop (i.e., it does not increase the germination of resting spores).

Earlier studies revealed that colonization and club formation were significantly reduced when susceptible and resistant radish (*Raphanus sativus* L.) cultivars were grown together, relative to treatments using only susceptible plants (Kroll *et al*., 1984). Yamagishi *et al*. (1986) reported that resistant crucifers reduced the numbers of residual resting spores after continuous cultivation over 4 years, although such consistent reductions in *P. brassicae* populations have not been achieved under Canadian field conditions. A Canadian study (Hwang *et al.*, 2013a) showed that cropping mixtures of susceptible and resistant cultivars, with the susceptible cultivar used to model the influence of susceptible volunteers and weeds, resulted in reduced clubroot severity in a subsequent susceptible canola crop, relative to clubroot severity following the cropping of only a susceptible cultivar. However, clubroot severity following a mixture of resistant and susceptible cultivars was higher than after the cropping of only a resistant cultivar or after a fallow treatment. This suggests that susceptible volunteer canola plants play a significant role in maintaining inoculum levels in infested soil. No empirical data are available, however, on the number of resting spores contributed by volunteers in infested fields where resistant canola is grown.

Conclusions

Since clubroot was first identified on canola in Alberta in 2003, intensive research has been conducted into the management and containment of this disease. Large-scale agriculture and scattered land tenure, which necessitates that individual producers move farm equipment over significant distances, and tight crop rotation schedules by producers have promoted the proliferation and dispersal of clubroot. Several clubroot management strategies have been used in high value horticultural crops, but were found to be impractical for large-scale agricultural usage. Soil amendments to increase soil pH may reduce disease severity by a small amount, but not enough to have a significant impact on clubroot proliferation. The required application rates, environmental concerns and costs for soil-applied fungicides make them impractical and unfeasible for use in the large-scale production of field crops. Crop rotations reduce spore populations to a limited extent, but to have a significant effect on spore populations, an interval of a decade or more would be required between canola crops. Moreover, bait crops are unlikely to be an important component of an IPM program for clubroot of canola. Treatments with soil fumigants, such as Vapam, have shown promise for elimination or the substantial reduction of small-scale infestations near field entrances. Further research is required to perfect application of these control procedures. Resistant cultivars have become the predominant means of clubroot management, but few clubroot resistance genes are available for deployment, and single gene resistance to clubroot has broken down quickly in the past. Also, substantial genetic and pathotype variation exists within the *P. brassicae* population in western Canada. Production of canola lines with single-gene resistance against a genetically diverse pathogen on a large acreage will surely impose strong selection pressure for pathogen genotypes that are able to overcome this resistance, and so the eventual breakdown of this resistance seems likely. Therefore, genetic resistance should be considered as just one component in the management of clubroot in canola on the Canadian prairies. In clubroot-infested fields, at least within a western Canadian context, we conclude that cropping of clubroot-susceptible canola cultivars will result in an increase in *P. brassicae* resting spore populations in the soil over time, while cropping of clubroot-resistant cultivars will not contribute significantly to inoculum build-up, and would be equivalent to leaving the land fallow. However, it is likely that susceptible off-types, susceptible canola volunteers and cruciferous weeds would continue to increase resting spore populations if they are not well controlled.

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Appendix 1: Sanitization

Introduction

Plasmodiophora brassicae is principally soil-borne, and the between- and within-field spread of this pathogen has generally been regarded as relatively slow (Strelkov et al., 2011). Clubroot is a monocyclic disease, and zoospore movement in the soil is limited (Howard et al., 2010). However, any activity that results in transport of infested soil or infected crop residues from one point to another has the potential to disseminate the pathogen (Strelkov, 2011). A study on the distribution of infected plants within clubroot-infested fields by Cao et al. (2009) showed that incidence was highest at the field entrances, and significantly lower at distances of 150 m and 300 m from the entrance, suggesting that tillage equipment laden with *P. brassicae*-infested soil and crop debris was likely responsible for introducing the pathogen.

Other sources of contamination to be considered in spreading clubroot include seeds and tubers harvested from infested fields, which may serve as avenues for the dispersal of this pathogen (Rennie et al., 2011). Preliminary assessments of seed-to-seedling transmission of clubroot under field conditions from resting spores inoculated onto canola seeds have not resulted in any detectable levels of clubroot in a susceptible canola crop or on a susceptible canola cultivar sown on the same site the following year (McDonald and Gossen, unpublished). The success of transmission under greenhouse conditions (Rennie et al., submitted), however, still raises concerns that dissemination of the pathogen on seed may represent a viable mechanism for dispersal of clubroot.

While Rennie et al. (2011) provided direct evidence for the occurrence of significant numbers of *P. brassicae* resting spores on seeds and tubers of various crops harvested from clubroot-infested fields, seedborne transmission is most likely a secondary mechanism of spread relative to the movement of infested soil on farm equipment and other machinery (Cao et al., 2009).The amount of soil, and therefore number of resting spores, carried on equipment far exceeds that found on even the most heavily infested of seedlots. Moreover, the distribution of infected plants within clubroot-infested fields of canola in Alberta, Canada showed that incidence was often highest at the field entrances, where contaminated farm machinery would normally begin tillage operations (Cao et al., 2009). Nevertheless, transmission of resting spores on seeds and tubers could lead to the dissemination of *P. brassicae* over longer distances than might typically be associated with the movement of farm machinery, and could also result in the introduction of novel races or pathotypes to particular regions. As such, the possibility of seedborne transmission should not be ignored in the development of clubroot-containment strategies, and farmers should avoid planting of common, untreated seeds harvested from clubroot-infested fields (Alberta Clubroot Management Committee, 2010).

Irrigation water pumped from canals, creeks and reservoirs contaminated with runoff from clubroot-infested fields could subsequently infest large areas of non-infested fields. Contaminated water should not be applied to plantings of cruciferous vegetables (Howard et al., 2010) or other susceptible crops such as canola. In fact, it could be argued that this practice should be discouraged for all agricultural fields given the longevity of clubroot resting spores in soil and the possibility that susceptible rotational crops could eventually be grown in these fields.

Clubroot spores can survive passage through livestock, so the use of raw manure on fields from animals that have been fed or pastured on clubroot-contaminated fodder should be avoided (Howard et al., 2010). Although research on the effect of the composting process is limited, temperature and moisture content were shown to be important for the successful eradication of *P. brassicae* resting spores from composted residues infested with clubroot (Fayolle et al., 2006; Nobel & Roberts, 2004; Wichuk et al., 2011).

The importance of sanitization in clubroot management

Sanitization (sanitation) is the process of cleaning and disinfecting or otherwise decontaminating hard surfaces (machinery, equipment, vehicles, tools, footwear), seed, plant materials, water and/or soil infested with pathogens. In a plant agriculture setting, such practices are meant to eradicate pathogens or to eliminate the risk of introducing them or to slow its spread from infested to non-infested fields or beyond localized areas within already infested fields. To prevent the transfer of clubroot-contaminated soil and infected crop residues to new sites, field equipment, tools, vehicles and the like should be cleaned and sanitized prior to moving them from infested to

clubroot-free fields. This process has been recommended for use on vegetable farms for many years (Donald, 2006; Miller et al., 1996; Tremblay et al., 1999), but its use in canola production systems has been a relatively recent application. Sanitization involves three key steps: i) rough cleaning using scraping, brushing or blowing to remove bulk soil and crop debris from contaminated surfaces; ii) fine cleaning using pressure washing, scrubbing or compressed air to remove any remaining residues; and iii) disinfection by applying an effective biocide to the cleaned surfaces and allowing at least 20 minutes of contact time to ensure that any remaining spores are killed (Canola Council of Canada, 2011). Unfortunately, many farmers find rigorous sanitation protocols to be excessively time-consuming and labour-intensive, since field equipment typically has many internal and external surfaces that may be exposed to soil- or to spore-bearing dust and infected crop residues. Similar challenges are faced by the petroleum, construction and transportation companies, which often work in clubroot-infested fields in areas such as central Alberta. The Canadian Association of Petroleum Producers (CAPP) has recognized the challenges faced by its members and has produced a plan for mitigating the spread of clubroot through petroleum industry-related activities (CAPP, 2008).

The most critical steps in sanitizing clubroot-infested farm machinery, equipment and vehicles are the rough and fine cleaning, which should aim to remove up to 99% of the clubroot contamination (Canola Council of Canada, 2011). At this point, the surfaces should be free of visible soil and plant material. The application of a disinfectant, such as 1-2% sodium hypochlorite solution, on pre-cleaned surfaces will serve to kill or inactivate residual resting spores. A contact time of 20-30 minutes is required to achieve adequate spore mortality. In on-farm demonstration trials in southern Alberta, it took at least 2 hours to clean and disinfect a 12 m wide field cultivator and up to 4 hours for a large tractor (Howard et al., unpublished). This work was done using a Mobile Sanitation Unit that contained a commercial pressure washer, air compressor and other equipment that would typically be used by farmers for cleaning machinery, field equipment and vehicles such as farm trucks.

Tractors and tillage equipment generally carry the heaviest load of infested soil (Canola Council of Canada, 2011). For example, a 12 m wide cultivator that had worked in a moist, clubroot-infested field in southern Alberta had ca. 50 kg of soil on the shovels, shanks and tires, whereas the large tractor used to pull it had ca. 150 kg of soil on the tires and frame. Heavy soil loads were also observed on a double disc unit working in the same field. At harvest in an infested canola field, swathers and combines were observed to carry large amounts of crop debris in the form of straw, chaff and seed (Howard et al., unpublished). Like soil, infested crop debris can be transported long distances on machinery, vehicles and equipment and may serve to spread clubroot into new areas.

Disinfectants

While chlorine has been shown to be useful for disinfesting water containing *P. brassicae* resting spores (Datnoff et al., 1987), it is highly corrosive and therefore may not be desirable for repeated applications to bare metal on tools and field equipment. Likewise, sensitive electronic equipment, electrical systems and panels may also be damaged by water and/or corrosive disinfectants, thus compromising equipment warranties and longevity. In such cases, it is recommended to use brushes or compressed air to remove loose dust and debris (Canola Council of Canada, 2011).

A few studies have compared at the relative efficacy of chemical disinfectants against the resting spores of *P. brassicae*. Donald et al. (2002) reported that none of the nine commercial disinfectants they tested completely inactivated clubroot resting spores and, in fact, most were ineffective. Hypochlorite was the only treatment that resulted in a significant reduction in clubroot severity when treated spores were used to inoculate broccoli seedlings. Howard et al. (unpublished) evaluated the efficacy of ten commercial disinfectants (Table 1) against clubroot resting spores obtained from diseased canola roots. Aqueous suspensions of each product were prepared at 0.5X, 1X, 2X and 5X, where X = the manufacturer's recommended concentration for the general disinfection of hard surfaces, and clubroot resting spores were added to each mixture. After exposure times of 10-20 minutes, the resting spores were collected by filtration and used to inoculated seedlings of a clubroot-susceptible canola variety in a bioassay test. Infectivity declined sharply as disinfectant concentration increased over the range of dosages tested for General Storage Disinfectant, HyperOx, KleenGrow, EcoClear, SaniDate, Thymox, Virkon, and Industrial Bleach; however, this same trend was not seen for EO Anode Water and Dutrion. Overall, the best-performing products were Bleach, HyperOx, EcoClear, Virkon and Sanidate.

Table 1. Disinfectants evaluated for efficacy against resting spores of *Plasmodiophora brassicae* in *in vitro* laboratory trials at the Crop Diversification Centre South, Brooks, Alberta.

Biocide	Supplier	Active ingredients	Recommended Concentration[a]
Dutrion	Dutrion, Ferintosh, AB	Chlorine dioxide	50 ppm
EcoClear	Ecoval Corp., Toronto, ON	Acetic acid 25%	1:3 or 1:2.25[b]
EO Anode Water	Biostel, Olds, AB	Hypochlorous acid and other biocidal ions	90:10 mixture of anolyte: catholyte
General Storage Disinfectant	Ag- Services Inc., Brampton, ON	Alkyl dimethyl benzyl ammonium chloride 10.0%	0.6% v/v
HyperOx	Vetoquinol, Lavaltrie, QC	Peracetic acid 5% + hydrogen peroxide 25%	0.78% v/v
Industrial Bleach	Brenntag, Winnipeg, MB	Sodium hypochlorite 10.8%	1% w/v
KleenGrow	Pace Chemicals, Burnaby, BC	Didecyl dimethyl ammonium chloride 7.5%	0.8% v/v
SaniDate	Brenntag, Winnipeg, MB	Hydrogen peroxide 27.11% Peracetic acid 2.0%	2% v/v
Thymox	Laboratoire M2, Sherbrooke, QC	Thymol 23%	1% v/v
Virkon	Vetoquinol, Lavaltrie, QC	Potassium peroxomonosulphate 21.4%	1% w/v

Note: [a]Manufacturer's recommendation for general disinfection of hard surfaces.
[b]Product registered for weed control. Use 1:3 for actively growing young weeds or 1:2.25 for control of larger weeds and suppression of perennial weeds. In this study 0, 10, 20, 40 and 100% were used.

Thermal treatments

Donald et al. (2002) noted that clubroot resting spores subjected to pressurized heat (autoclave 121°C , 20 min) and dry heat (oven 80°C, 12 hr) induced gross changes in their pathogenic activity and reduced disease severity in broccoli and Chinese cabbage seedlings inoculated with the treated spores. However, neither treatment resulted in 100% inactivation of the resting spores. Howard et al. (unpublished) subjected aqueous suspensions of resting spores to seven temperature regimes (40, 50, 60, 70, 80, 90 and 100°C) and for various durations and then inoculated canola seedlings with the treated spores to determine if they were still infective. All treatments showed declining infectivity in direct proportion to the exposure times, which ranged from 30 min to 72 hr in increments of 0.5 or 1.0 hr. The sharpest declines occurred for 80, 90 and 100°C, where most spores were rendered non-viable after as little as 30 min of treatment. At 40 and 50°C, spores remained infective for up to 48 hr of thermal treatment.

Other sanitization protocols

Donald et al. (2002) investigated the use of ultraviolet light and calcium, boron and potassium salts for their ability to inactivate clubroot spores in water solutions. Exposure to UV light resulted in reduced pathogenic activity, which was correlated (r = 0.80) with reductions in the severity of root galling on vegetable seedlings that had been inoculated with treated spores. By contrast, the ionic treatments resulted in relatively small reductions in pathogenic activity and there was no correlation with the degree of symptom expression.

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Breeding Pillar – Dr. H. Rahman and G. Y. Li

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Project Title: An integrated strategy for sustainable risk management of clubroot in canola – Breeding Pillar
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Project ID:
Project Title: Mapping clubroot resistance genes in *B. rapa* and developing molecular markers closely linked to these genes
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CLUBROOT RISK MITIGATION INITIATIVE

FINAL REPORT

Breeding Pillar: An integrated strategy for sustainable risk management of clubroot in canola

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Executive Summary

Clubroot disease caused by *Plasmodiophora brassicae* is one of the emerging threats to canola (*Brassica napus*) production in Canada. Several pathotypes of this pathogen have been identified in canola fields of which pathotype 3 is the most prevalent and the virulent one in Canada. Resistance to this pathotype was identified in different *Brassica* germplasm. The objective of this research was to study the genetic basis of clubroot resistance in rutabaga (*B. napus* var. *napobrassica*) and map the resistance gene(s) to identify molecular markers for use in marker assisted breeding. Two rutabaga genotypes, Rutabaga-BF and Rutabaga-PL, inbred for resistance to multiple pathotypes including pathotype 3, crossed to two clubroot susceptible spring canola lines, A07-29NI and A05-17NI, and F_1 plants were produced. The F_2 and testcross (TC) populations produced through self-pollination and crossing of the F_1 plants to their susceptible parent, respectively; and doubled haploid (DH) populations produced from the F_1 plants through microspore culture. Parents, F_1, F_2, TC and DH populations were evaluated for resistance to the pathotype 2, 3, 5, 6 and 8. In case of the cross Rutabaga-BF × A07-29NI, most of the F_2 families followed a 3:1 and TC families a 1:1 segregation for resistant and susceptible phenotypes. However, the distribution of the resistant and susceptible plants in F_2 and TC populations of Rutabaga-PL × A05-17NI deviated significantly from simple Mendelian segregation. In the case of DH population, a bi-modal distribution with 1:1 segregation for resistance and susceptibility was found in both cases. Data suggests that clubroot resistance in Rutabaga-BF is primarily under the control of a major dominant gene; however, it is complex in Rutabaga-PC – more than one loci apparently involved in the control of this trait. Molecular mapping based on the DH population derived from Rutabaga-BF × A07-29NI cross showed that a major gene on the A genome chromosome A8 is involved in the control of resistance to pathotype 3. A strong correlation between resistance to pathotype 3 and to other pathotypes, such as, pathotype 2, 5, 6 and 8 was found in the DH population of this cross. QTL mapping also detected the same genomic region is involved in the control of resistance to these pathotype.

Objectives:

The primary objective of this research is to mitigate the risk of clubroot in an integrated and sustainable manner of disease management. The specific objectives of this part of the research are the followings:

• To understand the genetic basis of clubroot resistance in rutabaga.
• To develop molecular marker(s) for the rutabaga resistance for use in marker-assisted breeding.
• To develop novel spring canola (*B. napus*) germplasm based on rutabaga-resistance.

Materials and Methods:

Plant materials

Two clubroot resistant rutabaga inbred lines (Rutabaga-BF and Rutabaga-PC) used in this research project developed at the U of A through self-pollination of single plants from two rutabaga populations received from Dr. Dean Spaner, Department of Agricultural, Food and Nutritional Science, University of Alberta (U of A). These two lines were crossed to two U of A spring canola lines (A05-17NI and A07-29NI, both susceptible to clubroot) and F_1 seeds were produced. The F_1 plants were evaluated for resistance to *P. brassicae* pathotype 3 prior to use for production of doubled haploid (DH) lines, self-pollination for F_2 seeds, and backcrossing to the susceptible parents for testcross (TC) seeds. The F_2 and TC seeds harvested from different F_1 plants were maintained as separate families. Microspore culture technique was applied following the protocol described by Kebede et al. (2010) and Coventry et al. (1988) for production of DH lines. For this, immature flower buds were harvested from the F_1 plants, microspores were isolated and cultured in vitro, and plantlets were generated.

Screening for clubroot resistance

Single spore isolates of *Plasmodiophora brassicae*, which are most pre-dominant in Alberta as well as most virulent on canola cultivars (Xue et al. 2008, Strelkov et al. 2006) were used for inoculation of the DH lines, and F_1, F_2 and TC populations. Isolates were obtained from Dr. Stephen Strelkov, U of A. Resting spores of *P. brassicae* were extracted following the technique described by Strelkov et al. (2007). Briefly, 2.0–2.5 g dried clubroot root galls were grounded in a mortar with a pestle by adding 50 ml sterile deionized water (sdH_2O), and the homogenate was filtered through eight layers of cheesecloth (American Fiber & Finishing Inc., Albemarle, NC, USA). The concentration of resting spores in the effluent was quantified with a haemocytometer (VWR, Mississauga, ON Canada) and suspensions of 1.0×10^7 resting spores ml^{-1} was used for inoculation.

Inoculation was done by root-dip method (Nieuwhof and Wiering 1961), where one-week old seedlings, germinated on moistened Whatman filter paper No. 1 in petri dish, were inoculated by dipping the roots in spore suspension for 10 seconds. Inoculated seedlings were planted in 72 cells tray filled with Sunshine professional Growing Mix (Sunshine Horticulture, 15831 N.E. Bellevue, USA). The seedlings were grown in a greenhouse set at 21 ± 2°C temperature with 16 hour photoperiod.

Seedlings were evaluated 45 days after inoculation for resistance. For this, roots were washed with tap water, visually examined for galls and rated on a 0 to 3 scale as described by Kuginuki et al. (1999), where 0 = no galling, 1 = a few small galls, 2 = moderate galling, and 3 = severe galling. Resistance was estimated in three different ways as appropriate to the materials used, viz. qualitative resistance, average disease score and index of disease (ID). In case of qualitative assessment, classification was done in two ways: (i) a plant with no visible gall (zero score) was classified as resistant, while all other plants were classified as susceptible, and (ii) plants with 0 and 1 scores were classified as resistant, while the plants with 2 and 3 scores were classified as susceptible. Average disease score was calculated using the following formula:

$$\text{Average score} = \sum (n \times 0 + n \times 1 + n \times 2 + n \times 3)/N$$

where, n is the number of plants in each class and 0, 1, 2 and 3 are the disease symptom severity classes and N is the total number of plants.

Index of disease (ID) was calculated using the method of Horiuchi and Hori (1980) as modified by Strelkov et al. (2006):

$$ID\,(\%) = \frac{\sum(\mathrm{n}\times 0 + \mathrm{n}\times 1 + \mathrm{n}\times 2 + \mathrm{n}\times 3)}{\mathrm{N}\times 3}\times 100\%$$

where, n is the number of plants in each class, N is the total number of plants and 0, 1, 2 and 3 are the disease symptom severity classes. An ID of zero indicate absence of visible gall in any of the plants, i.e. highly resistant, while ID of 100 indicate severe galling in all plants, i.e. extreme susceptibility.

Molecular mapping

Genomic DNA was extracted from leaf samples of the DH lines and their parents using a SIGMA DNA extraction Kit (Sigma-Aldrich, St Louis, USA) and following manufacturer's instructions. Simple sequence repeat (SSR) markers obtained from Agriculture and Agri-Food Canada, Saskatoon through a material transfer agreement and SSR markers published by various research groups were used. In addition to these markers, AFLP markers and markers published by different researchers found to be linked to clubroot resistance genes were also used in this research project.

Polymerase chain reaction (PCR) was carried out in a total volume of 13 µL containing 1x Taq buffer, 2.0 mM MgCl2, 200 µM dNTPs, 0.4 µM of each primer, 0.2 µM M13 dye, and 1.25U Taq polymerase (Promega Corp., Madison, WI, USA). The reactions were carried out in an MJ Research PTC-200 DNA Engine Thermal Cycler (Bio-Rad laboratories, Hercules, CA, USA); amplifications consisted of an initial denaturation step of 5 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at optimum annealing temperature of each primer, and 1.5 min at 72 °C, followed by a final extension of 30 min at 72 °C. Amplified DNA fragments were detected using fluorescent dyes, including FAM (blue), VIC (green), NED (yellow), PET (red), and LIZ (orange) (Life Technologies), on a 3700 Genetic Analyzer (Life Technologies).

The DH lines were genotyped with polymorphic markers, and genetic linkage map was constructed by use of the computer software JoinMap4 (Van Ooijen 2006). Mapping of clubroot resistance was done following composite interval mapping (CIM) procedure as described by Zeng (1994) with QTL Cartographer (QTLCart) v2.5 software (N.C. State University, Bioinformatics Research Center 2010).

Results

Parental line development, production of F_1, and evaluation for resistance

The two rutabaga populations were initially screened for resistance to a mixed culture of *Plasmodiophora brassicae* pathotypes prevalent in Canada from where resistant plants were selected and vernalized for 8 weeks to induce flowering. The resistant plants were self-pollinated and S_1 seeds were harvested. A total of 72 S_1 plants were evaluated for resistance to *P. brassicae* pathotype 3 where 71 plants were resistant and 1 plant showed slight disease symptom (score 1 in 0-3 scale; 3 = susceptible). Five S_1 plants from two families, which showed high resistance to pathotype 3, were crossed on individual plant basis to the susceptible spring *B. napus* canola lines A05-17NI and A07-29NI and F_1 seeds were harvested. All S_1 plants used in crossings were also self-pollinated for S_2 seeds.

A total of five S_2 families together with their corresponding six F_1's were evaluated for resistance to pathotype 3 to identify the homozygous S_2 families and their corresponding F_1's (Table 1). Based on clubroot resistance in S_2's and their corresponding F_1's, two single-plant derived crosses which designated as Rutabaga-BF × A07-29NI and A05-17NI × Rutabaga-PC were selected for this research. The corresponding rutabaga S_2 families were designated as Rutabaga-BF and Rutabaga-PC. The F_1 plants were vernalized and used for production of DH lines. The same F_1 plants were also self-pollinated for F_2 seeds, and backcrossed to the respective susceptible parent for TC seeds.

Table 1. Evaluation of the rutabaga S_2 and the F_1's of rutabaga × canola (*Brassica napus*) crosses for resistance to *Plasmodiophora brassicae* pathotype 3.

Genotype	No. plants tested	No. highly resistant*	No. with slight disease**	Index of disease (ID)*
Rutabaga-BF (3 fam)	45	45	0	0.00
Rutabaga-PC (2 fam)	45	45	0	0.00
Rutabaga-BF × A07-29NI (3 cross)[1]	117	86	31	18.2
A05-17NI × Rutabaga-PC (2 cross)	62	59	3	1.61

*No disease symptom, totally clean
**Very small gall, mostly scored as 1 in 0-3 scale.
**ID value of 100 = highly susceptible, 0 = highly resistant (no visible gall).
[1]Data from reciprocal crosses

Production of doubled haploid (DH) lines from F_1's of rutabaga × canola crosses

Development of DH lines through microspore culture is routinely practiced in *B. napus*. Microspore embryogenesis is highly dependent on genotype of the donor plant, where wide variation between genotypes for embryo yield generally found (Chuong et al. 1988, Zhang and Takahata 2001). Therefore, both Rutabaga-BF × A07-29NI and A05-17NI × Rutabaga-PC crosses were used to produce DH lines. Based on number of DH lines obtained, the DH population of Rutabaga-BF × A07-29NI was used for molecular mapping of the resistance.

A total of 1,856 flower buds from 15 F_1 plants of the three crosses were used for isolation of microspores from where 7,193 embryos were obtained (Table 2). Germinated embryos are transferred in greenhouse for production of DH lines. A total of 142 DH lines were produced from these crosses (Table 2).

Table 2. Microspore culture for the production of doubled haploid (DH) lines from rutabaga x spring canola *Brassica napus* crosses.

Cross	No. F_1 plants	Total no. buds	Total no. embryos	No. DH harvested
Rutabaga-BF × A07-29NI[1]	8	1,099	4,328	106
A05-17NI × Rutabaga-PC	7	757	2,865	36
Total	**15**	**1,856**	**7,193**	**142**

[1]Data from reciprocal crosses

Inheritance of resistance to pathotype 3 in F_2, testcross (TC) and doubled haploid (DH) populations

***F_2 and TC*:**

Evaluation of the F_2 and TC populations was done at two time, which designated as Test 1 and Test 2. In Test 1, 36 to 82 F_2 and 33 to 45 TC plants from four families of Rutabaga-BF × A07-29NI cross, and 44 F_2 and 45 to 46 TC plants from two families of A05-17NI × Rutabaga-PC cross were evaluated. In case of Test 2, 129 to 141 F_2 and 68 to 72 TC plants of Rutabaga-BF × A07-29NI, and 203 to 306 F_2 and 203 to 233 TC plants of A05-17NI × Rutabaga-PC were evaluated. Thus, a total of 806 F_2 and 445 TC plants of Rutabaga-BF × A07-29NI, and 597 F_2 and 549 TC plants of A05-17NI × Rutabaga-PC were tested in the two tests. In all cases, a clear bi-modal distribution for resistance and susceptibility was found (Fig. 1 and 2).

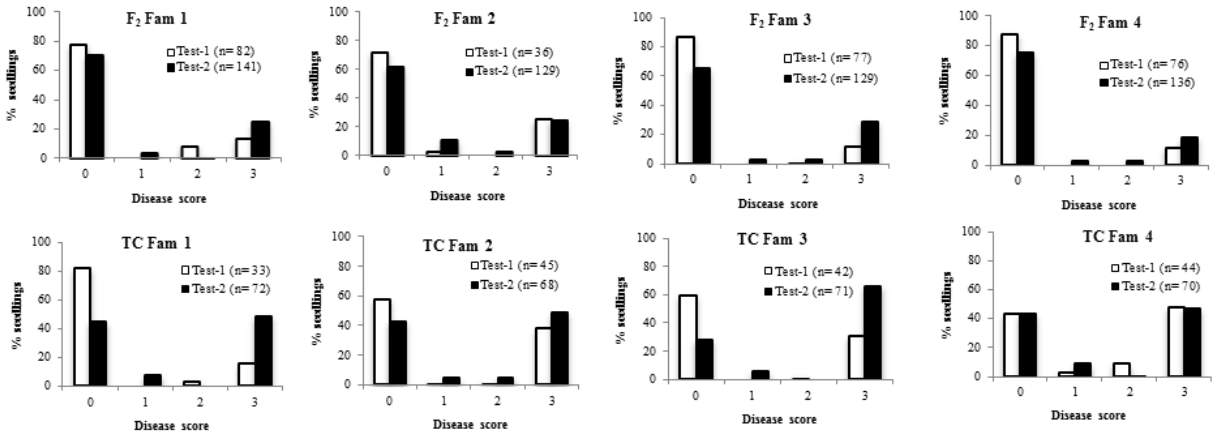

Fig 1: Distribution of F_2 and testcross (TC) populations of Rutabaga-BF × A07-29NI cross of *Brassica napus* for resistance to *Plasmodiophora brassicae* pathotype 3 (n = number of seedlings evaluated).

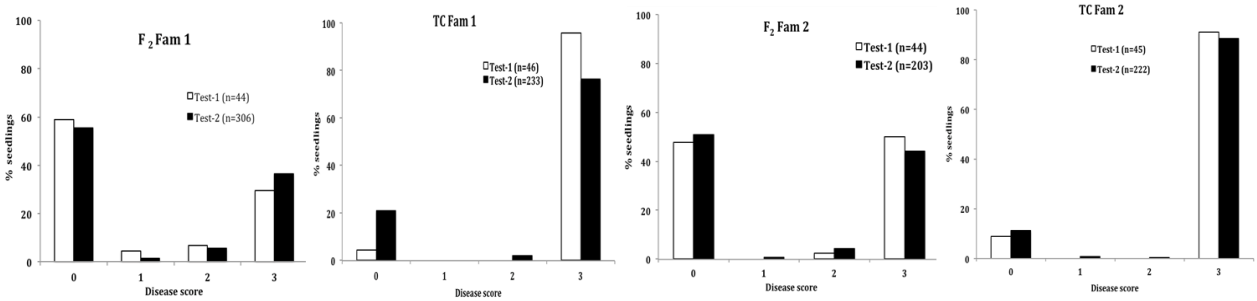

Fig 2: Distribution of F_2 and testcross (TC) populations of A05-17NI × Rutabaga-PC cross of *Brassica napus* for resistance to *Plasmodiophora brassicae* pathotype 3 (n = number of seedlings evaluated).

Table 3. Segregation for clubroot resistance in F_2 and testcross (TC) populations of Rutabaga-BF × A07-29NI and A05-17NI × Rutabaga-PC crosses of *Brassica napus* evaluated in two tests at different time. Plants with disease score 0 was considered as resistant (R) and those with scores 1, 2 and 3 were considered as susceptible (S).

Gen.	Test	No. fam.	No. plants		Ratio	Homogeneity		Segregation (R:S)	
			R (Score 0)	S (Score 1, 2 & 3)		χ^2	p	χ^2	p
Rutabaga BF x A07-29NI:									
F_2	1	4	224	47	3:1	5.03	0.17	8.47	<0.05
	2	4	367	168	3:1	11.22	<0.05	11.69	<0.05
	1+2	**4+4**	**591**	**215**	**3:1**	**35.22**	**<0.05**	**1.22**	**0.27**
TC	1	4	97	67	1:1	11.30	<0.05	5.49	<0.05
	2	4	111	170	1:1	4.94	<0.05	12.39	<0.05
	1+2	**4+4**	**208**	**237**	**1:1**	**32.22**	**<0.05**	**1.89**	**0.17**
A05-17NI x Rutabaga-PC:									
F_2	1	2	47	41	3:1	1.52	0.22	21.88	<0.05
	2	2	324	284	3:1	98.94	<0.05	152.84	<0.05
	1+2	**2+2**	**371**	**325**	**3:1**	**100.46**	**<0.05**	**174.46**	**<0.05**
TC	1	2	6	85	1:1	0.08	0.78	68.85	<0.05
	2	2	74	381	1:1	31.09	<0.05	238.23	<0.05
	1+2	**2+2**	**80**	**466**	**1:1**	**34.19**	**<0.05**	**272.89**	**<0.05**

*Bulk culture of microspores from six F_1 plants

Table 4. Segregation for clubroot resistance in F_2 and testcross (TC) populations of Rutabaga-BF × A07-29NI and A05-17NI × Rutabaga-PC crosses of *Brassica napus* evaluated in two tests at different time. Plants with disease score 0 and 1 were considered as resistant (R) and those with scores 2 and 3 were considered as susceptible (S).

Gen.	Test	No. fam.	No. plants		Ratio	Homogeneity		Segregation (R:S)	
			R (Score 0 & 1)	S (Score 2 & 3)		χ^2	p	χ^2	p
Rutabaga BF x A07-29NI:									
F_2	1	4	225	46	3:1	3.04	0.39	9.31	<0.05
	2	4	394	141	3:1	4.67	0.20	0.52	0.47
	1+2	**4+4**	**619**	**187**	**3:1**	**16.16**	**0.02**	**1.39**	**0.24**
TC	1	4	99	65	1:1	10.00	<0.05	7.05	<0.05
	2	4	129	152	1:1	5.92	0.12	1.88	0.17
	1+2	**4+4**	**228**	**217**	**1:1**	**24.58**	**<0.05**	**0.27**	**0.81**
A05-17NI x Rutabaga-PC:									
F_2	1	2	49	39	3:1	2.54	0.11	16.19	<0.05
	2	2	332	276	3:1	105.42	<0.05	134.88	<0.05
	1+2	**2+2**	**381**	**315**	**3:1**	**108.08**	**<0.05**	**150.95**	**<0.05**
TC	1	2	6	85	1:1	0.19	0.66	68.58	<0.05
	2	2	77	378	1:1	33.81	<0.05	199.12	<0.05
	1+2	**2+2**	**83**	**463**	**1:1**	**37.23**	**<0.05**	**264.47**	**<0.05**

*Bulk culture of microspores from six F_1 plants

Chi-square test for fit to 3:1 segregation in F_2 and 1:1 segregation in TC populations was done. For this, two types of classifications for resistance and susceptibility were followed: (i) plants with disease score 0 as resistant and with score 1, 2 and 3 as susceptible, and (ii) plants with disease score 0 and 1 as resistant and with score 2 and 3 as

susceptible. The F_2 and TC populations derived from more than one F_1 plant; therefore, a homogeneity χ^2 test was done to check whether segregation pattern among the families was similar. Chi-square test for fit to segregation ratio was done on pooled data from the families. In case of the cross Rutabaga-BF × A07-29NI, segregation for resistance and susceptibility fit to 3:1 in F_2 and 1:1 in TC, especially when disease score 0 and 1 considered as resistant and scores 2 and 3 as susceptible (Table 4 and 3). Deviation from this segregation was observed in some of the families of this cross, e.g., Family 3 in Test 1 (Fig. 1). However, in case of the cross A05-17NI × Rutabaga-PC, deviation from simple Mendelian segregation was found in all cases. In this case, about 50% F_2 plants were resistant and 50% susceptible, while in TC, only 15% were resistant and 85% were susceptible (Fig 2, Table 3, 4). This suggests that genetic control of clubroot resistance in Rutabaga-PC is more complex as compared to Rutabaga-BF.

Doubled haploid (DH):

A total of 96 DH lines from reciprocal crossess of Rutabaga-BF × A07-29NI and 34 lines of A05-17NI × Rutabaga-PC were produced. The distribution of the lines for resistance phenotype was clearly a bi-modal (Fig. 3).

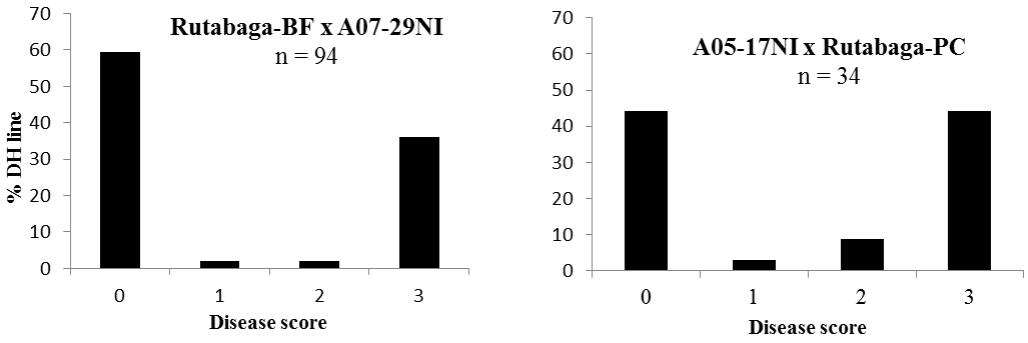

Fig 3: Distribution of doubled haploid (DH) lines of Rutabaga-BF × A07-29NI and A05-17NI × Rutabaga-PC crosses of *Brassica napus* for resistance to *Plasmodiophora brassicae* pathotype 3 (n = number of DH lines evaluated).

The DH lines of Rutabaga-BF × A07-29NI were produced separately from five F_1 plants, while DH lines of A05-17NI × Rutabaga-PC were produced bulk culture of microspores from six plants. Therefore, a homogeneity χ^2 test was done for the DH lines derived from the different F_1 plants of Rutabaga-BF × A07-29NI. Homogeneity χ^2 value was well within the acceptable level that pooling data could be justified (Table 5 and 6). Chi-square test for fit to 1:1 segregation for resistance and susceptibility agreed in DH populations of both crosses (Fig. 3, Table 5, 6) despite the F_2 and TC populations of A05-17NI × Rutabaga-PC cross deviated significantly from the simple Mendelian segregation.

Thus, based on segregation in F_2, TC and DH population of the two crosses, it is apparent that resistance to *P. brassicae* pathotype 3 in Rutabaga-BF is primarily controlled by a dominant genes, while the genetic control of resistance in Rutabaga-PC is more complex.

Table 5. Segregation for clubroot resistance in doubled haploid (DH) populations of Rutabaga-BF × A07-29NI and A05-17NI × Rutabaga-PC crosses of *Brassica napus*. Plants with disease score 0 was considered as resistant (R) and those with scores 1, 2 and 3 were considered as susceptible (S).

Cross	No. fam.	No. plants/lines		Ratio	Homogeneity		Segregation (R:S)	
		R (Score 0)	S (Score 1, 2 & 3)		χ^2	p	χ^2	p
Rutabaga-BF × A07-29NI	5	56	38	1:1	1.75	0.78	3.45	0.06
A05-17NI × Rutabaga-PC	*	15	19	1:1	-	-	0.47	0.49

*Bulk culture of microspores from six F_1 plants

Table 6. Segregation for clubroot resistance in doubled haploid (DH) populations of Rutabaga-BF × A07-29NI and A05-17NI × Rutabaga-PC crosses of *Brassica napus*. Plants with disease score 0 and 1 were considered as resistant and those with scores 2 and 3 were considered as susceptible (S).

Cross	No. fam.	No. plants/lines		Ratio	Homogeneity		Segregation (R:S)	
		R (Score 0 & 1)	S (Score 2 & 3)		χ^2	p	χ^2	p
Rutabaga-BF × A07-29NI	5	59	35	1:1	1.30	0.86	6.13	<0.05
A05-17NI × Rutabaga-PC	*	16	18	1:1	-	-	0.12	0.73

*Bulk culture of microspores from six F_1 plants

Evaluation of the DH lines of Rutabaga-BF x A07-29NI for resistance to other pathotypes

In addition to pathotype 3, the 97 DH lines from Rutabaga-BF x A07-29NI cross were also evaluated for resistance to the pathotypes 2, 5, 6 and 8 (Table 7). In general, most of the DH lines showing resistance pathotype 3 also showed resistance to the other pathotypes. Of the 68 DH lines considered to be resistant to pathotype 3 (score 0 and 1), 61 lines showed resistance to the other pathotypes (2, 5, 6 and 8) as well. This is also evident from strong correlation of resistance to pathotype 3 and resistance to other pathotypes (Table 8).

Table 7: Resistance reactions of double haploid (DH) lines from Rutabaga-BF × A07-29NI crosses of *Brassica napus* against Canadian *Plasmodiophora brassiceae* pathotypes

Pathotype	No. resistant DH lines		No. susceptible DH lines	
	Score 0	Score 0 & 1	Score 1, 2 & 3	Score 2 & 3
Pathotype 2	64	64	43	43
Pathotype 3	65	68	42	39
Pathotype 5	66	67	41	40
Pathotype 6	65	65	42	42
Pathotype 8	64	64	43	43
All Pathotypes	60	61	37	35

Table 8: Coefficient of correlation of resistance reactions of the doubled haploid (DH) populations from Rutabaga-BF × A07-29NI cross of *Brassica napus* against different *Plasmodiophora brassiceae* pathotypes.

	Pathotype 3	Pathotype 5	Pathotype 6	Pathotype 8
Pathotype 2	0.947	0.957	0.942	0.922
Pathotype 3		0.899	0.946	0.887
Pathotype 5			0.896	0.878
Pathotype 6				0.903

In all cases, $p < 0.0001$

Screening of the parents for polymorphic markers

A total of 1,088 SSR primer pairs, which have been assigned to the 19 linkage group (LG) of *B. napus* genome by various researchers, were evaluated for polymorphism between the resistant and susceptible parents. Of the tested primers, 247(23%) failed PCR amplification. In case of the cross Rutabaga-BF × A07-29NI, total of 501 SSR markers were identified polymorphic between the parents that can be detected by the use of an ABI sequencer (Table 9). Of the 501 markers, about 66% markers produced polymorphic fragments with more than 4 bp difference.

Initially, we undertook the approach of bulk segregant analysis (BSA) followed by genotyping of the mapping population for identification of molecular markers associated with resistance. For this, we choose the TC families of Rutabaga-BF × A07-29NI cross, where BSA identified few markers to be associated with resistance to pathotype-3. The molecular marker work with TC population, instead of DH population, was undertaken as backup approach of mapping the resistance genes. This was primarily for the reason that production of DH lines can be uncertain in some crosses (Chuong et al. 1988, Zhang and Takahata 2001). However, with the success of DH line development from Rutabaga-BF × A07-29NI cross, we focused molecular marker work on this DH population. The DH population is advantageous for several reasons, such as the same line (genotype) can be tested in replicated trials as well as against multiple pathotype, which is not possible with pedigree population, such as TC. Furthermore, we also focused on construction of a genetic linkage map based on this DH population which would allow us to scan the whole genome for resistance gene.

Table 9. Evaluation of simple sequence repeat (SSR, microsatellite) markers for polymorphism between the clubroot resistant rutabaga (*Brassica napus* ssp. *napobrassica*) parents Rutabaga-BF and Rutabaga-PC, and clubroot susceptible spring canola (*B. napus*) parents A07-29NI and A05-17NI.

Linkage group	No. SSR marker tested	No. polymorphic SSR marker								
		Rutabaga-BF × A07.29NI					Rutabaga-PC × A05.17NI			
		Total	%	>4 base	%	No. primer for LG constr.	Total	%	>4 base	%
LG-1	54	29	53.70	23	42.59	10	23	42.59	14	25.93
LG-2	46	18	39.13	14	30.43	7	22	47.83	14	30.43
LG-3	55	31	56.36	22	40.00	7	29	52.73	24	43.64
LG-4	53	21	39.62	18	33.96	7	24	45.28	19	35.85
LG-5	32	19	59.38	13	40.63	8	13	40.63	11	34.38
LG-6	64	30	46.88	23	35.94	9	29	45.31	21	32.81
LG-7	38	23	60.53	13	34.21	6	24	63.16	15	39.47
LG-8	72	34	47.22	19	26.39	12	35	48.61	22	30.56
LG-9	127	84	66.14	44	34.65	15	88	69.29	60	47.24
LG-10	40	24	60.00	15	37.50	5	24	60.00	15	37.50
LG-11	79	24	30.38	9	11.39	6	21	26.58	14	17.72
LG-12	63	23	36.51	17	26.98	8	28	44.44	21	33.33
LG-13	40	19	47.50	14	35.00	7	19	47.50	15	37.50
LG-14	54	28	51.85	21	38.89	6	28	51.85	24	44.44
LG-15	58	21	36.21	13	22.41	4	18	31.03	14	24.14
LG-16	49	21	42.86	10	20.41	4	20	40.82	14	28.57
LG-17	95	15	15.79	10	10.53	5	17	17.89	13	13.68
LG-18	32	15	46.88	15	46.88	9	9	28.13	7	21.88
LG-19	37	22	59.46	18	48.65	7	16	43.24	13	35.14
Total	**1,088**	**501**		**331**		**142**	**487**		**350**	

Linkage map construction and QTL mapping

For construction of a genetic linkage map, the DH mapping population (94 lines) of Rutabaga-BF × A07-29NI was genotyped with 142 of the 331 polymorphic SSR markers. Of the 142 SSRs, 130 produced only two fragments – either of the A or the C genome, while 12 produced more than two fragments - probably amplifying both A and C genomes. Thus, genotyping data of the 130 (142-12=130) SSRs (Table 9) were included for construction of a framework genetic map. This mapping population (94 DH lines) was also genotyped with 32 AFLP primer combinations; this gave 299 polymorphic markers to be included in the linkage map.

Based on segregation of the F_2, TC and DH populations, it was obvious that a major gene involved in the control of resistance to *P. brassicae* pathotype 3 in Rutabaga-BF. Single marker analysis on the DH population of Rutabaga-BF × A07-29NI identified a genomic region of A8 to be involved in the control of resistance. By using genotyping data from all markers of the chromosome A8, we constructed a linkage map for this chromosome and identified the genomic region, which reside around the SSR marker sS1702, confer resistance to pathotype 3 (Fig 4). This genomic region also confers resistance to the pathotypes 2, 5, 6 and 8 as well (Fig 4 *lower*).

Thus evaluation of the DH lines for resistance to pathotype 2, 3, 5, 6 and 8, and QTL mapping suggests that the same genomic region of the chromosome A8 confer resistance to multiple pathotypes.

Construction of a genetic linkage map for all 19 chromosomes is in progress. QTL mapping based on this complete linkage map will enable to detect any minor locus/loci involved in the control of resistance to these pathotypes. This work expected to be completed within six months

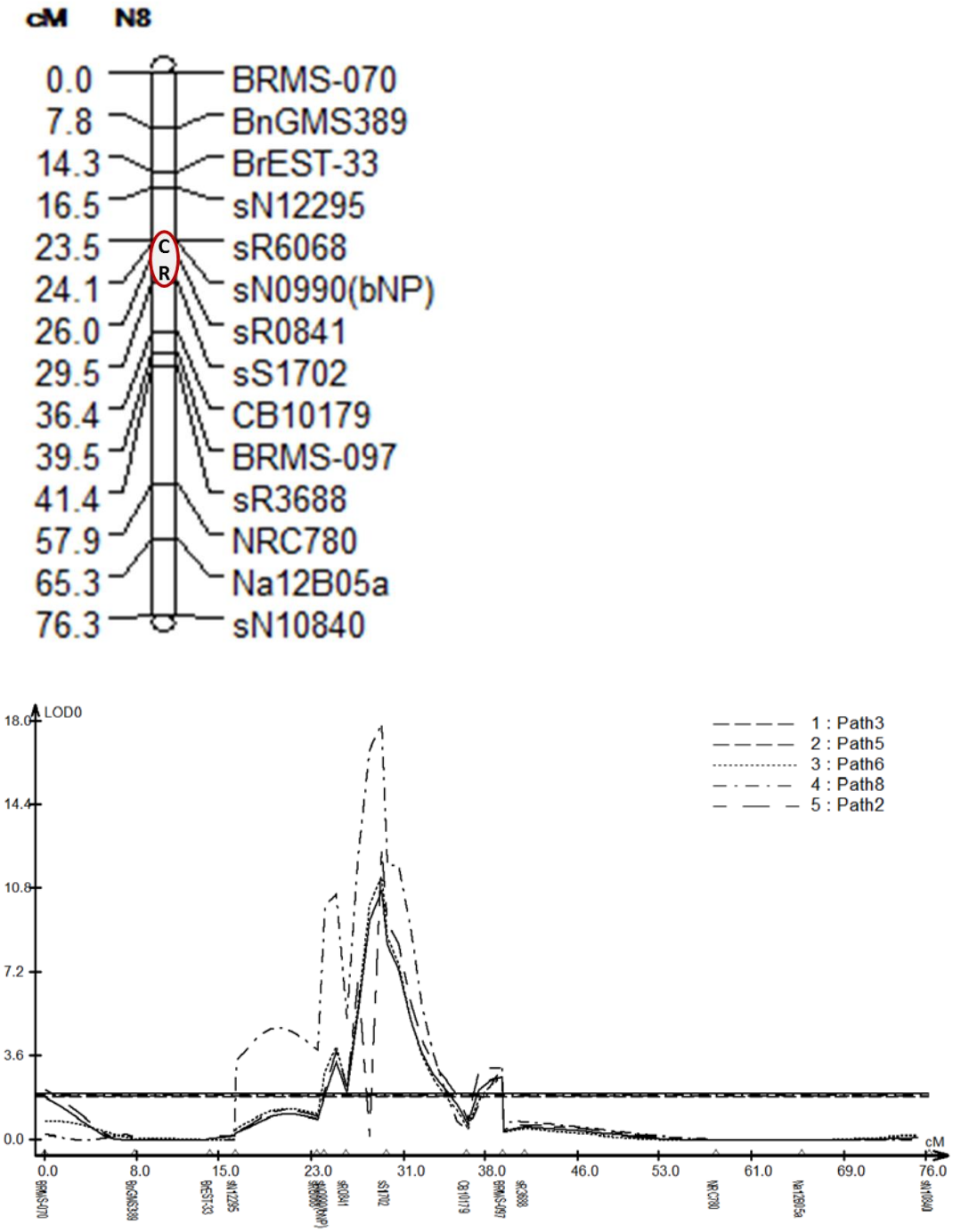

Fig 4: *Upper*: A genetic linkage map of the chromosome A8 constructed based on a doubled haploid (DH) population derived from F_1 plants of Rutabaga-BF × A07-29NI of *Brassica napus* and by use of simple sequence repeat markers (SSRs). The genomic region involved in the control of clubroot resistance indicated on the chromosome by 'CR'. *Lower*: LOD score profile from QTL mapping. QTL detected on the chromosome A8 confer resistance to *Plasmodiophora brassicae* pathotypes 2, 3, 5, 6 and 8.

Test of the markers identified in Rutabaga-BF × A07-29NI cross in the DH population of A05-17-NI × Rutabaga-PC

Single marker analysis as well as QTL mapping identified a genomic region of A8 associated with resistance to pathotype 3 in the DH population of Rutabaga-BF × A07-29NI cross. Two markers from this genomic region tested on the 34 DH lines developed from A05-17-NI × Rutabaga-PC. These markers failed to amplify most of the resistant DH lines (Table 10, 11) despite these markers produced polymorphic fragments between the two parents. The result indicates that the genetic control of clubroot resistance in Rutabaga-PC is different from the genetic control of resistance in Rutabaga-BF.

Table 10. Test of the simple sequence repeat (SSR) marker alleles of A8, identified in the mapping population of Rutabaga-BF × A07-29NI, for co-segregation with resistance to *Plasmodiophora brassicae* pathotype 3 in the doubled haploid population of A05-17NI × Rutabaga-PC cross of *Brassica napus*. Plants with disease score 0 was considered as resistant and those with scores 1, 2 and 3 were considered as susceptible.

Marker used	LG	Total DH	No. resistant DH (Score 0)				No. susceptible DH (Score 1, 2 & 3)			
			Total	No. DH amplified by marker	No. DH + allele	No. DH – allele	Total	No. DH amplified	No. DH + allele	No. DH – allele
sN0990(bNP)	N8	34	15	3	3	0	19	1	1	0
sS1702	N8	34	15	1	1	0	19	15	0	15

+ sign indicate presence of marker allele from the resistant parent; - sign indicate marker allele from susceptible parent

Table 11. Test of the simple sequence repeat (SSR) marker alleles of A8, identified in the mapping population of Rutabaga-BF × A07-29NI, for co-segregation with resistance to *Plasmodiophora brassicae* pathotype 3 in the doubled haploid population of A05-17NI × Rutabaga-PC cross of *Brassica napus*. Plants with disease score 0 and 1 were considered as resistant and those with scores 2 and 3 were considered as susceptible.

Marker used	LG	Total DH	No. resistant DH (Score 0 & 1)				No. susceptible DH (Score 2 & 3)			
			Total DH	No. DH amplified by marker	No. DH + allele	No. DH – allele	Total DH	No. DH amplified	No. DH + allele	No. DH – allele
sN0990(bNP)	N8	34	16	3	3	0	18	1	1	0
sS1702	N8	34	16	1	1	0	18	14	0	14

+ sign indicate presence of marker allele from the resistant parent; - sign indicate marker allele from susceptible parent

Development of canola quality spring type lines from rutabaga × spring canola *B. napus* crosses

Development of canola lines from Rutabaga × Canola crosses started with funding from Alberta Crop Industry Development Fund (ACIDF) and Alberta Canola Producers Commission (ACPC) under the Project No. 2007F066R. This project started in 2007 and ended in 2012. In this project, pedigree breeding technique was applied from where several spring type canola quality advanced generation lines were developed. Data from different generation populations from different crosses tested up to 2011 field trials reported to ACIDF and ACPC in the Final Report (submitted in June 2012). These lines were tested in 2012 field trials, i.e. in last year of the project funded by AAFC through CCC. Data of 249 F_6 generation lines developed from a 3-way cross, tested in field nursery plots in 2012, presented in Table 6 as an example.

About 85% of these F_6 lines had <0.5% erucic acid; while seed glucosinolate content in these lines varied from 10.1 to 60.9 µmol/g seed with a mean of 19.9 µmol/g seed (Table 6). This suggests that majority of the F_6 lines possess canola quality property. The F_6 lines were, on an average, two days late in flowering and six days late in maturity as compared to the spring canola parent A07-26NR. However, some of the lines were as early as A07-26NR for these two traits. Significant variation for oil, protein and saturated fatty acid contents existed in this population; this suggest the possibility of extracting clubroot resistant spring *B. napus* canola lines with high oil and low saturated fatty acid contents from this population.

Table 6. Example of advanced generation canola quality families developed from rutabaga × spring canola *Brassica napus* crosses through pedigree breeding.

		BN	DTF	DTM	Oil (%)	Protein (%)	GLS (µ mol/g)	Sat (% fatty acids)
F_6 (n=249)	Range	3 - 8	48 - 58	96 - 111	42.8 - 53.0	20.7 - 29.0	10.1 - 60.9	6.0 - 7.5
	Mean	6.5	50.6	103.1	47.6	24.3	19.9	6.6
A07-26NR (n=6)	Range	6 - 7	48 - 49	96 - 99	48.2 – 49.1	24.6 – 25.7	14.7 – 15.5	6.7 – 6.7
	Mean	6.7	48.4	97.4	48.6	25.2	15.1	6.7

Note: Families were tested in 2012 in nursery plots (one replication, 3 m^2 plot). BN = general impression of breeder before harvest (1 = poor, 9 = excellent), DTF = days to flower, DTM = days to maturity, GLS = total glucosinolate content in seed, Sat = total saturated fatty acids.

QTL mapping of flowering time gene

The DH lines developed from Rutabaga × Canola crosses were used for mapping of the clubroot resistance gene. Development of DH lines and construction of a genetic linkage map require substantial amount of effort and resources. To get maximum benefit from resources invested in this research project, some additional studies undertaken to maximize the use of the materials and data generated in this project. The DH mapping population, in addition to clubroot resistance, also segregating for some important traits, such as, vernalization requirement and days to flowering. Therefore, phenotypic evaluation of the mapping population was done for molecular mapping of these traits. Knowledge of genetic control and associated molecular markers for these traits will be valuable to the canola breeders for use in breeding for the development of early flowering and maturing canola cultivars for the Canadian prairies. With this view, the DH mapping population of Rutabaga × Canola evaluated in field trial in 2012 in two experiments, vernalized and non-vernalized. In case of the vernalized experiment, the DH lines and the parents seeded in greenhouse and vernalized at 4ºC for eight weeks. After vernalization, the lines acclimatized at 10ºC for two weeks and transplanted to field plots in spring 2012. In the case on non-vernalized experiment, the DH lines and the parents seeded directly in field plots in spring 2012. Each experiment was replicated. Data for days to flowering from these two experiments presented in Fig. 5 and 6.

In case of the vernalized experiment, all DH lines flowered. Variation for days to flower was continuous where few lines flowered earlier than the spring canola parent A07-29NI while few lines flowered later than the rutabaga parent (Rutabaga-BF), i.e. transgressive segregation occurred in both directions. In case of the non-vernalized experiment, about 40% DH lines failed to flower without vernalization, as was found in the case of the rutabaga parent – an effect of the vernalization gene was clearly evident. In this experiment also, few DH lines flowered earlier than the spring canola parent.

The non-vernalized experiment will be repeated in 2013, and QTL mapping will be done to identify the genomic regions involved in the control of vernalization requirement and days to flowering.

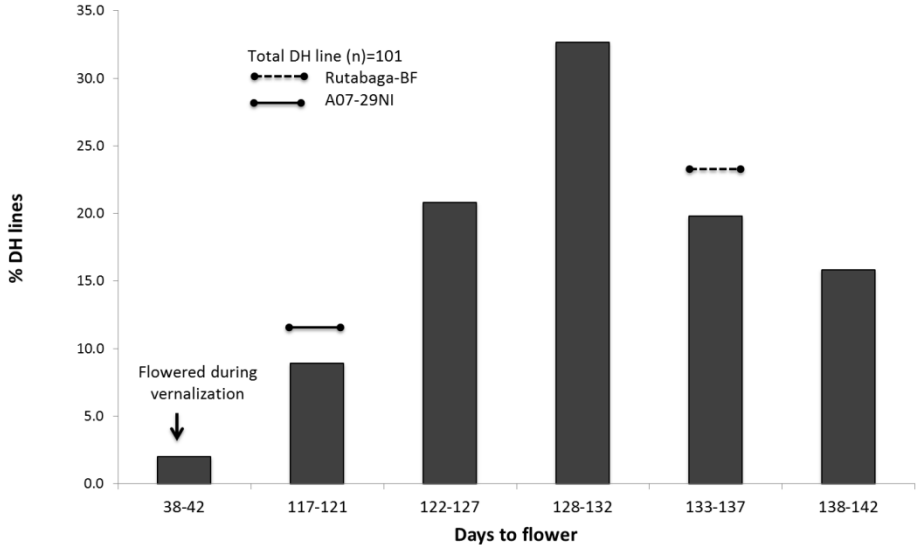

Fig. 5. Distribution of the doubled haploid (DH) lines, derived from Rutabaga-BF × A07-29NI cross of *Brassica napus*, for days to flower in vernalized experiment. In this experiment, the lines were grown in greenhouse for 30 days (day 1-30), vernalized at 4ºC for 60 days (day 31-90), acclimatized at 10ºC for 14 days (day 91-104), and transplanted to field in spring 2012 (day 105).

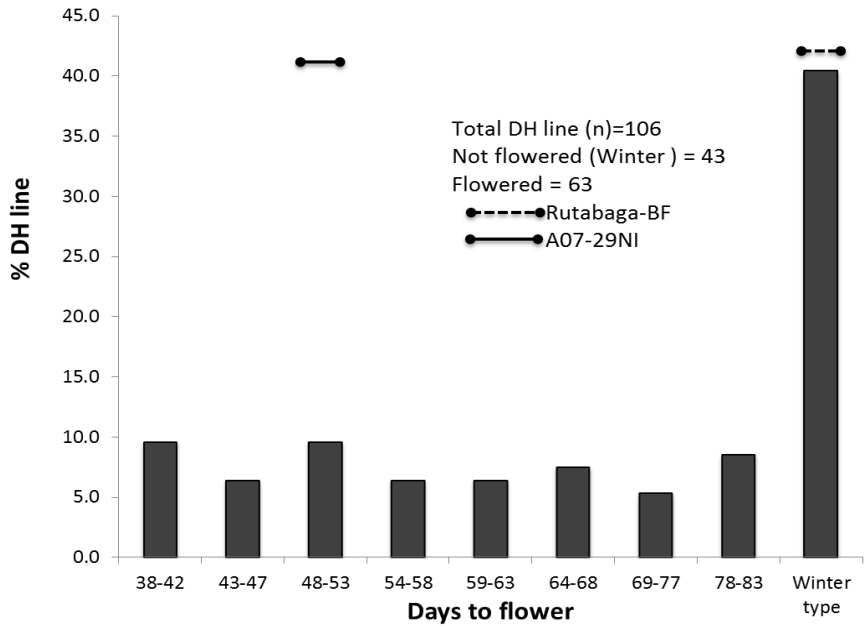

Fig. 6. Distribution of the doubled haploid (DH) lines, derived from Rutabaga-BF × A07-29NI cross of *Brassica napus*, for days to flower in non-vernalized experiment. In this experiment, the lines were seeded in field in spring 2012 without vernalization.

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CLUBROOT RISK MITIGATION INITIATIVE

FINAL REPORT

Breeding Pillar: Mapping clubroot resistance genes in *B. rapa* and developing molecular markers closely linked to these genes

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Introduction, Literature review, Objectives and Milestones

Clubroot disease resistance genes in *B. rapa* such as Chinese cabbage and turnip offers some opportunities to transfer clubroot resistant genes in *B. rapa* into canola and produce clubroot resistant canola cultivars. On the other hand, gene introgression from species to species is necessary and multiple genes are required for good resistance.

In interspecific gene transfer, the flanking regions of resistance genes are often incorporated into the new species, which results in introgression of some unbeneficial traits due to linkage drag. These unwanted traits become problematic, especially in the region where few recombination events occur. Moreover when multiple resistance genes are included, it is not easy to distinguish the phenotypic differences among individuals with one, two or more resistance genes and difficult to transfer these genes through conventional breeding. Therefore, molecular marker assisted selection (MAS) becomes mandatory in order to transfer multiple resistance genes simultaneously. To achieve effective and efficient MAS, gene effects and gene locations in genomes have to be well characterized. Meanwhile, easily detected and closely linked molecular markers should be developed to implement MAS in breeding.

The primary objective of this project is to map the clubroot resistance genes in *B. rapa*. After gene mapping, it becomes known that the clubroot resistant genes in different Chinese cultivars and turnip accessions are the same or different. If the clubroot resistant genes in different accessions are the same, it is good enough to transfer one gene from *B. rapa* accession into canola. If more than one gene is mapped, all clubroot resistant genes can be introduced into canola to develop a multiple gene resistance.

Molecular markers are necessary to perform marker-assisted selection. After gene mapping, the markers flanking clubroot resistant genes on genetic maps can be used in marker assisted selection. Moreover, if the markers on the *B. rapa* genetic maps are not polymorphic in canola or not efficient due to the two genome composition in canola, new molecular markers will be developed with the known gene positions and known marker sequences in *B. rapa* and those markers will be useful in canola. Therefore, clubroot gene mapping in *B. rapa* will establish a foundation for clubroot resistant gene transfer.

The milestones in this project are to construct a high density genetic map in *B. rapa*, make alignment with other published genetic maps and map clubroot resistance genes in five Chinese cabbage cultivars and turnip accessions.

Approach/Methodology

Development of mapping populations:

Highly resistant and highly susceptible cultivars and lines in *B. rapa* are selected for developing segregating marker mapping populations. To handle hundreds of individuals in the mapping populations, an indoor testing method are implemented. For this purpose, a modified small pot testing method as described by Voorrips and Visser (1993) may be used. Several plants in each pot will be inoculated by adding *P. brassicae* spores that are collected from clubroot galls. After plants are grown for six weeks, their roots are examined and disease symptoms that are scored on a zero to three scale.

Mapping population development:

Mapping populations in *B. rapa* are produced by crossing the most resistant and most susceptible cultivars and lines. selfing and backcrossing populations are produced and used for gene mapping.
Map construction:

The SRAP protocol are used to construct a high density genetic map and clubroot mapping populations are analyzed with the same set of primer combinations in the genetic map construction. These primer combinations are selected from the list used in the construction of the previous ultradense *B. napus* genetic map (Li and Quiros 2001; Sun et al. 2007).

In each mapping population, approximately 2000 SRAP molecular markers are scored and used to map each individual clubroot resistant gene in mapping populations. Additionally, 50-100 SSR markers will be developed to align a new genetic map with those previously reported genetic maps and used for clubroot gene mapping in *B. napus* and *B. rapa*.

Results and Discussion

Gene mapping in Chinese cabbage

After screening of over forty Chinese cultivar and breeding DH lines, five commercial Chinese cabbage *(B. rapa*) cultivars from China that contain clubroot resistance genes were identified . Five Chinese cabbage cultivars with clubroot resistance were crossed to a *B. rapa* accession that is susceptible to clubroot. In total, five F2 and BC1 mapping populations were produced. After indoor screening, we found that a 3:1 ratio of resistant individuals to susceptible ones in two F2 populations and a 1:1 ratio in all three BC1 populations, suggesting that one Mendelian gene control the clubroot resistance in all five Chinese cabbage cultivars (Table 1).

Table 1. Backcross and selfing mapping populations developed with five Chinese cabbage cultivars and a susceptible *B. rapa* rapeseed*

Mapping populations	**Clubroot symptom scoring***				**Expected ratio****	**X2 test (p value)**
	0	1	2	3		
BC1a	29	1	7	30	1:1	0.39 ns
BC1b	28	0	9	23	1:1	0.61 ns
BC1c	41	0	7	39	1:1	0.59 ns
BC1d	38	1	2	11	3:1	1 ns
F2a	44		2	16	3:1	0.46 ns
CK	0	0	0	44		

*Scorings; 0, no galls on roots; 1, with a few small galls on secondary roots; 2, with a few small galls on both primary and secondary roots; 3 with lots of big galls on both primary and secondary roots.
**Testing a 1:1 ratio of resistant and susceptible individuals in backcross populations, and 3:1, in selfing populations.

Construction of a high density genetic map in B. rapa

A high density genetic map in *B. rapa* has been constructed and more 10,000 molecular markers have been assembled into 10 linkage groups (Table 2). With this high-density map, the fine mapping of clubroot resistance genes is conducted.

Table 2. A genetic map with 10,999 markers including 9,216 SRAP, 46 SSR and 1737 Solexa sequence molecular markers were assembled into 10 linkage groups for clubroot resistance gene mapping and gene cloning.

Linkage group of *B. rapa*	No. of bin	No. of SRAP	No. of SSR	No. of Solexa sequence	Length (cM)	No. of SRAP marker with sequence
R01	39	908	13	169	132.6	16
R02	49	938	1	136	164	19
R03	60	1303	4	248	205.4	16
R04	33	575	2	128	117.3	5
R05	46	729	2	178	141.5	15
R06	43	949	2	181	152.8	15
R07	50	880	9	129	158.6	7
R08	40	798	5	148	124.5	17
R09	66	1568	2	301	167.4	20
R10	39	568	6	119	131.5	13
total	465	9216	46	1737	1495.6	143

Mapping of a locus on chromosome A3

The high density genetic map in *B. rapa* with over 10,000 SARP markers was used to map genes resistant to clubroot on linkage groups. These SRAP molecular markers on the genetic map were used to identify clubroot resistance genes in five mapping populations. Ninety-six primer pairs were used in the initial screening and two SRAP markers were found to co-segregate with the resistance gene in the plants tested. By comparing these two SRAP markers with the SRAP molecular markers on the ultra-dense genetic recombination map, it was found these two markers located on R3 linkage group. After testing SRAP markers in five F2 and BC1 populations, all clubroot resistance gene loci belonged to the same locus on A3.

Fine mapping and identification of candidate clubroot resistance genes

BAC clone sequences on linkage group R3 (http://www.brassica-rapa.org/BRGP/geneticMap.jsp) were selected to develop more molecular markers for clubroot resistant gene. In total, nine BAC clones were selected and primers were designed in accordance with the BAC sequences to develop SNP and SCAR markers. Therefore, a genetic map around the clubroot resistance gene locus on A3 was constructed (Figure 1). Two flanking markers were used to find 219 recombinants from 8,000 BC2 individuals.

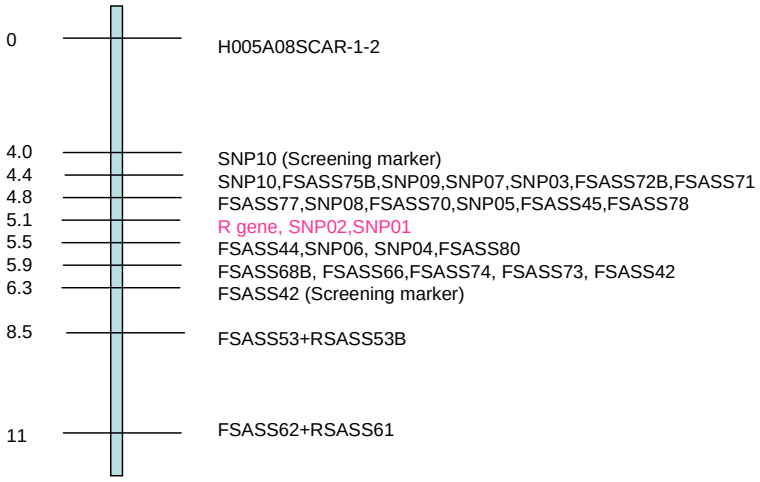


Figure 1. A fine map for a clubroot resistance gene on linkage group 3 in *B. rapa* (prefix FSASS represents SCAR markers and SNP, SNP markers).

These recombinants selected previously were analyzed using molecular markers located within the FSASS75B and FSASS68B that was used to screen the large BC2 population. In total, 18 SSR, SCAR and SNP molecular markers were developed and used to find the recombination breakpoint in each recombinant. Currently, there are four recombinants that were used to pinpoint the gene locus in a 50 kb region. Finally, the clubroot resistant genes were narrowed into a 50-kb region.

To clone the clubroot genes on R3, a BAC library was constructed using BC2 plants that showed resistant. Over 200,000 BAC clones were produced, covering approximate 20 times of the whole genome. Two closely linked molecular markers were used to screen the BAC library and seven positive BAC clones anchoring the molecular markers were selected.

Application of fine mapped genes

In order to check the effectiveness of the fine mapped clubroot resistance genes, DNA of breeding materials from Monsanto were analyzed with the molecular markers developed during gene cloning. Interestingly, it was found that the molecular markers perfectly matched with phenotypes that were derived from one clubroot resistant source and did not match in the progeny of another resistant source. These results indicated that the resistance genes in Monsanto's breeding materials are diverse and gene pyramiding can be performed with two different resistant sources. Therefore, canola cultivars with multiple clubroot resistance gene loci will be developed soon in Monsanto. This strongly demonstrated that gene fine mapping and cloning will help seed companies to develop new canola cultivars with improved clubroot resistance.

Sequencing BAC clones and making new constructs for further confirmation of the resistance genes

Since there are multiple genes in tandem repeats on chromosome A3, the complementary testing results showed that the used candidate gene in the transformation might not be the best one or the high level of clubroot resistance in the original parent requires more than one resistance gene. To further confirm what gene components confer the best resistance, more complementary constructs have been made to produce new transgenes.

Two BAC clones anchoring the candidate genes of clubroot resistance were selected for BAC sequencing. Ten times of sequence coverage for each BAC clone have been produced and used to assemble sequence for two selected BAC clones. New constructs containing individual clubroot resistance gene are being made to produce transgenic plants. Meanwhile, double gene constructs are also being made and used to do plant transformation. These new transgenic plants with single and double clubroot resistance genes will be used to confirm the function of these clubroot resistance genes.

Extended mapping of clubroot resistance genes in ECD source

The European clubroot differential (ECD) set was obtained from Warwick HRI's Vegetable Genebank, UK. The ECD set includes 5 accessions of *B. rapa*, five accessions of *B. napus* and 5 accessions of *B. oleracea.* These fifteen accessions were evaluated to identify clubroot disease resistance against the Canadian field isolates. Among these 15 ECD accessions, four turnip accessions, two cabbage lines and one kale are resistant and one *B. napus* is partially resistant (Table 3). After evaluation, all four turnips, ECD1, ECD2, ECD3 and ECD4 with resistance to clubroot were used as donor male parents to cross with a susceptible *B. rapa* rapeseed, BAR. Four F_1 crosses, BARxECD1, BARxECD2, BARxECD3, and BARxECD4 were backcrossed to the susceptible parent and five F_1 individuals from each cross were used to produce BC_1 segregating populations. Seeds of all populations and parental lines were produced in greenhouses at the University of Manitoba while all testing for clubroot disease resistance was performed by Monsanto Canada at the University of Guelph.

Table 3. Clubroot resistance evaluation on ECD sets (ECD1-15) by Canadian clubroot field isolates.

ECD accessions	Clubroot ratings			
	0	**1**	**2**	**3**
ECD1	35	0	0	0
ECD2	43	0	0	0
ECD3	39	0	0	0
ECD4	43	0	0	0
ECD5	0	2	2	43
ECD6	4	0	2	35
ECD7	0	2	2	29
ECD8	2	0	1	29
ECD9	9	3	3	21
ECD10	24	0	0	4
ECD11	41	0	0	0
ECD12	22	0	0	0
ECD13	12	0	6	13
ECD14	0	2	2	16
ECD15	18	2	4	9

Evaluation of clubroot resistance disease in BC_1 populations of B. rapa

The following analysis focuses on four turnip accessions of the ECD set. Since all four turnips, ECD1, ECD2, ECD3 and ECD4 showed totally resistant to the Canadian clubroot isolates, four mapping populations were developed by crossing these four turnips with a susceptible *B. rapa* rapeseed, BAR. All four BC_1 segregating populations were phenotyped and genetic analysis of phenotypes in all four segregating populations illustrated that single and two gene models explained well the segregation patterns of clubroot disease resistance. In the population derived from ECD1, two genes segregated in one BC_1 line derived from one original individual plant while one gene segregated in another BC1 line derived from another ECD1 individual plant, suggesting that the ECD1 accession contained

mixed plants, of which some had one clubroot resistance locus and others had two clubroot resistance genes (Table 4). In the mapping populations developed with ECD2 and ECD4, all four BC1 lines showed a segregation ratio of 3:1, suggesting that all ECD2 and ECD4 plants contained two clubroot resistance loci (Table 4). In the mapping population derived from ECD3, seven BC_1 families were tested, of which three fitted for single gene segregation of clubroot resistance and four showed segregation of two clubroot resistance genes (Table 4), suggesting that the ECD3 accession contained a mixture of individual plants with one or two clubroot resistance loci. Therefore, these data revealed that there were various inheritances of clubroot disease resistance in all turnips of the ECD set.

Table 4. Segregation of clubroot disease in BC_1 populations of a cross between ECD (1-4) and susceptible parent (BAR) in *B. rapa*.

BC_1 Populations	Clubroot phenotypes		χ^2	P value	DF	Ratio
	Resistance	Susceptible				
BARx(BARxECD1)	49	10	2.04	0.15	1	3:1
BARx(BARxECD2)	32	15	1.20	0.27	1	3:1
BARx(BARxECD3)	40	10	0.67	0.41	1	3:1
BARx(BARxECD4)	63	23	0.14	0.71	1	3:1
Total			4.04		4	3:1
Goodness of fit χ^2	184	58	0.14	0.71	1	3:1
Homogeneity χ^2 (Total- Pooled data)			3.91	0.27	3	3:1
BARx(BARxECD1-4)	24	22	0.09	0.77	1	1:1
BARx(BARxECD3-2)	101	90	0.63	0.43	1	1:1
Total			0.72		2	1:1
Goodness of fit χ^2	125	112	0.71	0.40	1	1:1
Homogeneity χ^2 (Total- Pooled data)			0.01	0.92	1	1:1

Confirmation of the A3 clubroot locus in EDC accessions

Fine mapping of clubroot resistance gene in Chinese cabbage was carried out and molecular markers linked to the gene on linkage group A3 were developed. These molecular markers linked to the A3 clubroot resistance locus were employed to determine haplotypes of resistance genes in the CDE mapping populations on linkage group A3. Four SSR markers, FSASS72b-RSASS72b, FSASS79b-RSASS79b and FSASS77b-RSASS77 were used to select clubroot resistance individual plants with clubroot resistance genes on linkage group A3. These SSR markers were analyzed in all BC_1 segregating populations and PCR products were separated on ABI 3130xl Genetic Analyzer.

To identify the presence of the clubroot resistance locus on chromosome A3, all these four previously developed SSR markers, FSASS72b-RSASS72b, FSASS79b-RSASS79b and FSASS77b-RSASS77 were used to genotype all four BC1segregating populations developed from these four ECD turnips. In the BC1 populations from ECD1, all SSR co-segregated with clubroot resistance in the line where clubroot resistant and susceptible plants segregated at a ratio of 1:1. In the segregating line at a ratio of 3:1 for clubroot resistance, all SSR markers linked to the A3 clubroot resistance locus showed susceptible alleles in all susceptible individual plants while in resistant plants, all SSR markers showed a 2:1 ratio of heterozygous and homozygous genotypes as those expected in a two gene segregation model. In the two segregating populations derived from ECD2 and ECD4, all SSR markers linked to the A3 clubroot resistance locus showed the same genotype as the susceptible parent whereas in the resistant plants, all SSR markers showed segregation at a ratio of 2:1 for heterozygous and homozygous genotypes as those expected in two gene segregation model (Table). In the BC1 population derived from ECD3, all SSR

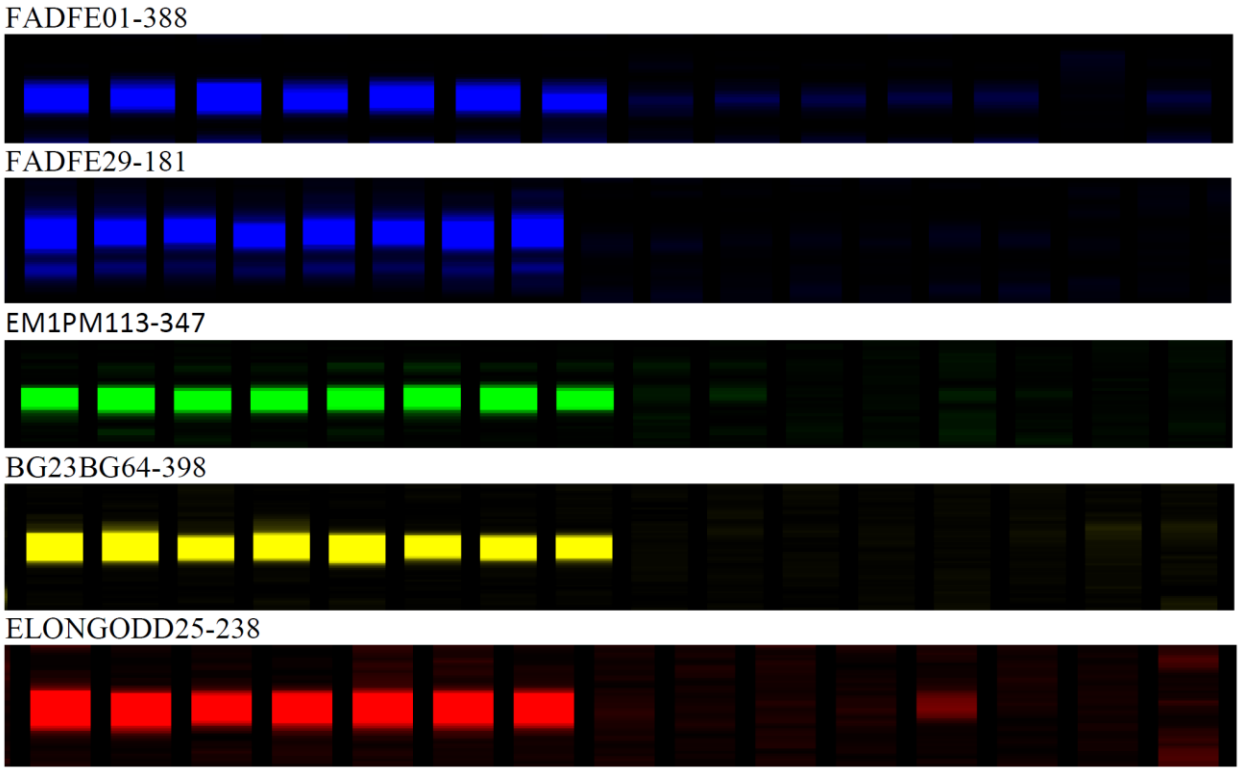
markers showed no linkage to clubroot resistance. The results displayed that the mapped A3 resistance locus and another resistance locus existed in three turnip accessions, ECD1, ECD2 and ECD4 while the A3 clubroot resistance locus did not exist in ECD3 and one or two clubroot resistance loci were located to different chromosomes from the A3 or different regions from the mapped A3 clubroot resistance locus.

Identification of SRAP markers linked to new clubroot resistance loci

To map additional clubroot resistance loci in the segregating BC_1 populations, individual plants which did not have segregation of the A3 molecular markers, but showed segregation of resistant and susceptible phenotypes were selected to perform further analysis. Eight resistance plants and eight susceptible plants were used for bulk segregant analysis using SRAP molecular markers. SRAP PCR was performed using standard protocol described by Li and Quiros (2001).

To map clubroot resistance loci on the previous ultradense genetic map, 150 SRAP primer pairs which were used in the construction of the map were selected to identify SRAP markers linked to clubroot resistance loci. After eight clubroot resistance lines and eight susceptible lines from BC_1 populations were tested, four SRAP markers, FADFE01-388, FADFE29-180, FADFE39-285, and FADFE49-255 were found to be linked to a new clubroot resistance (Fig. 2). These four SRAP markers were then tested on all clubroot resistance individual plants which did not carry the A3 resistance locus to confirm these SRAP markers which were really linked to the new clubroot resistance locus.

Since the SRAP primers and one parental line used in the mapping of new clubroot resistance loci were the same as those used in the construction of ultradense genetic map in *B. rapa* (Li et al., 2011), it was feasible to identify the linkage groups of the genetic map which were anchored by the SRAP markers linked to clubroot resistance loci. After these four SRAP markers linked to the new clubroot resistance locus were compared with the SRAP markers on the ultradense genetic map, they were found to correspond to the SRAP markers located on linkage group A8 and two of these four SRAP markers were found to the same loci in the bins, R08b29 and R08b30 of ultra-high density genetic map spanning about 5.6 cM genetic distance of *B. rapa* genome (Li et al., 2011). Fortunately, a large numbers of SRAP markers were sequenced and the sequence information of SRAP markers was used to blast *B. rapa* genome sequence to find the region containing the new clubroot resistance locus (http://brassicadb.org/brad/). Eventually sequence information of the mapped SRAP markers allowed identifying a region on linkage A8 where the new clubroot resistance locus was located.



F**ig. 2.** SRAP molecular marker association with clubroot resistance genes in BC_1 populations of *B. rapa*. Left side bands represent clubroot resistance individual and right side absence of band represent clubroot susceptible individual in BC_1 populations of *B. rapa*.

Development of microsatellite markers for the new clubroot resistance locus on chromosome A8

The A8 sequence region anchoring the clubroot resistance gene in the BC1 individual populations were used to design SSR markers. In total, thirty three primer pairs for SSR markers were selected and these SRRs covered a 20 Mb physical region (Table 4). Among these 34 SSR primer pairs, 19 (57%) produced polymorphic loci between parental lines and were used for further association analysis with clubroot disease resistance in all four BC_1 populations. Twelve out of 19 primer pairs (63%) were found to be linked to the clubroot resistance locus. These twelve primer pairs produced 12 SSR markers were used for clubroot resistance gene mapping in all BC_1 segregating populations (Fig. 3). These all SSR markers were tested on the BC_1 progenies and these SSR markers were found to be linked to the new clubroot resistance locus. Currently, we mapped two clubroot resistance genes in the ECD turnips and a few more genes segregating are to be identified in some of the BC_1 families derived from resistance sources ECDs. Since all the ECD accessions are open pollinated lines, they belong to a mixture of individual plants with one or two different clubroot resistance loci. We will continue to map all clubroot resistance loci in the ECD turnips and at the same time, we have developed and will develop all near isogenic lines and each line will contain single clubroot resistance locus in the same genetic background. This set of near isogenic lines would be better that the mixture of multiple clubroot resistance loci as in the open pollinated populations such as the European ECD turnips when pathotypes of clubroot pathogen are investigated. the resistance spectra of each individual clubroot resistance locus can be precisely determined using the near isogenic line set.

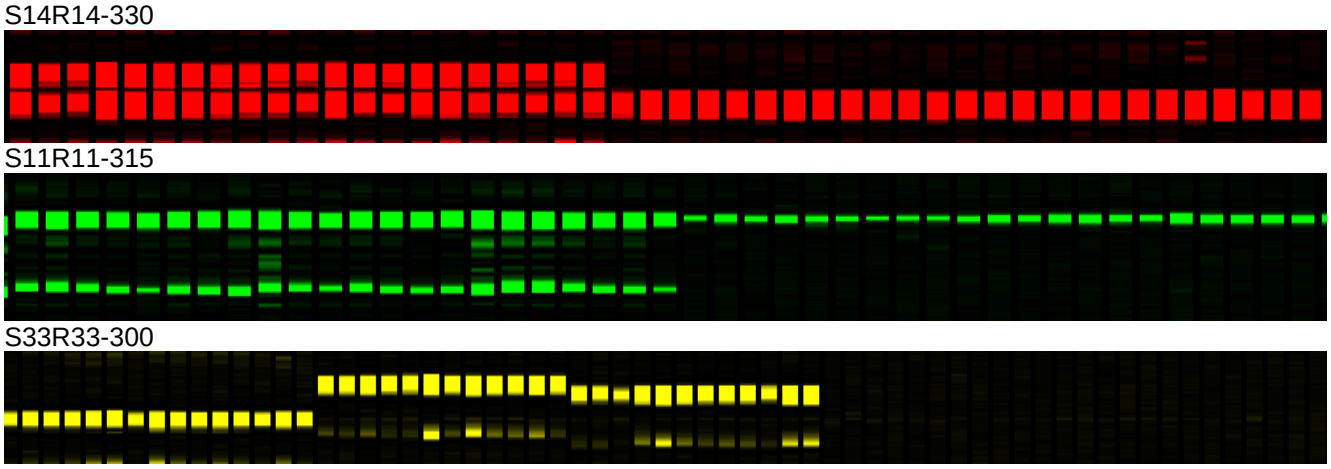


Fig. 4. Development of SSR molecular markers and their linkage with clubroot resistance genes in BC_1 populations of BARxECD1, BARxECD2, BARxECD3 and BARxECD4 of *B. rapa*.

Pyramiding multiple clubroot resistance genes in canola

All four turnip, two cabbage and one kale accessions were used to produce synthetic *B. napus*. Seeds from most interspecific crosses have been harvested and crossed to canola. These synthetic *B. napus* combined the resistance genes in *B. rapa* and *B. oleracea.* After preliminary testing, we found all synthetic *B. napus* were totally resistant to the Canadian clubroot isolates (Table 5). With the known genome location and molecular markers linked to the clubroot resistance loci, we will be able to easily pyramid multiple clubroot genes in canola through marker assisted selection and this research work is underway at the University of Manitoba. Obviously, we are using extensive gene pyramiding to develop canola materials carry different clubroot resistance genes.

SRAP markers are being used to map the third clubroot resistance gene locus in the ECD accession. The high density genetic map will allow us to identify this clubroot resistance gene locus and its location in the *B. rapa* genome will be determined. With all the known clubroot resistance genes, we are able to transfer these new clubroot resistance genes into canola for developing canola hybrids with multiple clubroot resistance genes.

Table 5. Testing clubroot resistance of Synthetic *B. napus* derived from ECD accessions

Crosses	Line code	Resistant plant #	Susceptible plant #
ECD-01/ECD-11	C1	7	0
ECD-01/ECD-15	H1	11	0
ECD-01/ECD-15	H2	6	0
ECD-02/ECD-11	N1	5	0
ECD-02/ECD-11	K1	12	0
ECD-02/ECD-15	A2	7	0
ECD-02/ECD-15	B1	5	0
ECD-03/ECD-11	D2	5	0
ECD-03/ECD-11	D9	11	0
ECD-03/ECD-12	A1	5	0
ECD-03/ECD-12	A8	7	0
ECD-03/ECD-15	A2-2	4	0
ECD-03/ECD-15	B2	7	0
ECD-04/ECD-11	B1	8	0
ECD-04/ECD-12	A2	5	0
ECD-04/ECD-15	A2	9	0
ECD-04/ECD-15	D2	8	0
ECD-04/ECD-15	G2	4	0
ECD-04/ECD-15	I2-1	7	0
ECD-04/ECD-15	I3	7	0

RBPI 1370 - An integrated strategy for sustainable management of clubroot of canola

Official start date of project: 1-April, 2009
Projected end date of project: 31-Mar, 2013
Date of report: 28-June, 2013

Project ID: RBPI 1370
Project Title: Objective I. Surveillance of clubroot and study of *Plasmodiophora brassicae* inoculum dispersal in western Canada
Name of Principal Investigator (PI): Randy Kutcher
List of collaborators:
Debbie McLaren
Gary Peng
T. Kelly Turkington

Project ID: RBPI 1370
Project Title: Objective II. Study interaction of climate, soil and pathogen biology on management of clubroot of canola
Name of Principal Investigator (PI): Bruce Gossen
List of collaborators:
Dennis Pageau

Project ID: RBPI 1370
Project Title: Objective III. Screening Brassica Germplasm for Resistance and Developing Canola Lines against Prairie Races of *Plasmodiophora brassicae*
Name of Principal Investigator (PI): Kevin Falk
List of collaborators:
Gary Peng
Fengqun Yu
Richard Gugel

Project ID: RBPI 1370
Project Title: Objective IV. Developing Microbial Technologies for Biocontrol of Clubroot
Name of Principal Investigator (PI): Gary Peng
List of collaborators:
Dennis Pageau
Russel Hynes
Susan Boyetchko
Bruce Gossen

Project ID: RBPI 1370
Project Title: Objective V. Formulation Development for Biological Control of Clubroot on Canola
Name of Principal Investigator (PI): Russel Hynes
List of collaborators:
Susan Boyetchko

Abstract of the RBPI 1370 Projects

By 2008, clubroot was emerging a serious threat to canola production in western Canada, with increasing number of diseased fields found in Alberta and a general lack of resistance in canola cultivars. There was a lack of expertise or research infrastructure in AAFC to tackle this issue at that time. Under the Growing Forward funding, a team of AAFC scientists with background in disease survey/epidemiology, molecular biology, host resistance, agronomy and disease management, was assembled rapidly. The goal was to work collaboratively with scientists at universities and other levels of government to develop the information and materials that can be utilized to minimize the risk of pathogen spreading on the prairies and disease impact on canola production. Disease survey, pathogen biology studies, resistance development, chemical/biological/agronomic assessment were carried out and the goal was to develop an integrated strategy involving a range of management measures for sustainable control of clubroot on canola. Tremendous progress has been made, with critical information generated and disseminated to producers and industry, and new clubroot-resistant germplasm developed for technology transfer. The milestones set for this study were met fully, with a large number of new resistance genotypes identified and characterized, and clubroot resistant canola germplasm developed. The impact of temperature and pH on infection of root hairs and cortical tissues is better demonstrated using a comparative approach and different hosts or pathogen pathotypes. With the field survey results, disease/pathogen distribution across the prairies is now better understood. The value of using a resistant cultivar in heavily infested areas is clearly demonstrated, especially when used with a 3-year crop rotation for maximized yield potential. A total of **109** scientific materials, including **30** refereed papers, have been published or are in press, and over **82** technology transfer items have been recorded. The study also supported three M.Sc. students and five PDFs, providing valuable training to young professionals. Based on the results of current work, new studies are being developed to characterize resistance genes in the materials identified, understand the mechanisms of resistance genes, introgress complimentary R genes into elite canola breeding lines for durable clubroot resistance, and refine the integrated clubroot-management strategy by determining the effect of crop rotation under varying cropping system/climatic conditions and fumigation to eradicate/substantially reduce the pathogen inoculum load in the soil for isolated infestations. Now the AAFC team is well known around the world for its expertise and productivity in clubroot research.

CLUBROOT RISK MITIGATION INITIATIVE

FINAL REPORT

RBPI 1370 Objective 1

Surveillance of clubroot and study of Plasmodiophora brassicae inoculum dispersal in western Canada (project moved to Strelkov's Pathology Pillar in 2011)

Project Lead: RANDY KUTCHER[1]

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Collaborators:

Debbie McLaren
Gary Peng
T. Kelly Turkington

Work plans and milestones

2009-2010

Field surveys were conducted in Saskatchewan and Manitoba and soil samples collected from 120 canola fields to be analysed for the presence of the clubroot pathogen in Dr. Strelkov's lab. Samples testing positive will be bioassayed with a universal susceptible host as well as a highly susceptible canola cultivar. Prepare annual report and conduct or contribute to technology transfer activities.

Establish contact with industry agronomists, farmers and Canola Council of Canada Agronomists in clubroot affected areas and for at risk locations in eastern Alberta and western Saskatchewan; line up collaborators for spring/summer/fall 2010 field season

Depending on when funding is available, hire Post-Doc and begin preliminary trials to evaluate clubroot assessment protocols to be used on sampled soil, order BSNE and surface creep samplers, assemble sampling supplies for dispersal studies and finalize protocols to prepare for the 2010 field season

Finalize locations and arrangements for fields, areas, and routes for the various dispersal study components, and hire potential students

2010-2011

Continue field surveys in Saskatchewan and Manitoba, collect soil samples from additional 96 canola fields for a total of 126 fields (30 fields financed by CARP grant) from SK and MB. Analyse samples using PCR diagnostic test and bioassay any positive samples. Prepare annual report and conduct/contribute to technology transfer activities

Continue collection of soil and water samples as per preliminary protocols and modify as needed. Store samples under appropriate conditions and carry out further processing and analysis

Process soil and water samples collected during the previous spring-fall and analyze using PCR protocols and bioassays

Modify sampling and assessment protocols as necessary based on experience from the first year of the study

2011-2012

Continue field surveys in Saskatchewan and Manitoba, collect soil samples from 144 fields from SK and MB. Modify the survey plan as required depending on previous year's findings. Analyse samples using PCR diagnostic test and bioassay any positive samples. Prepare annual report and conduct /contribute to technology transfer activities

Continue to collect and process soil and water samples using PCR protocols and bioassays, and modify sampling protocols as necessary based on experience from the first and second years of the study

2012-2013

Continue field surveys in Saskatchewan and Manitoba, collect soil samples from 144 canola fields from SK and MB. Modify and expand survey as required depending on previous year's findings. Analyse samples using PCR diagnostic test and bioassay any positive samples. Prepare final report and conduct and contribute to technology transfer activities

Conduct planning meetings with AAFC, U. of A., AARD, SMA and MAFRI staff to assess the need to continue field surveying, Identify potential new locations, make arrangements for fields, areas, and routes as necessary, and line up new collaborators for subsequent sampling activities as required. Complete collecting and processing soil/water samples and all analyses, conduct comprehensive analyses of data, prepare final report, and contribute to technology transfer activities

Introduction

Extensive field surveying for clubroot on canola has been on-going in Alberta since 2003, and an increasing number of infested fields have been identified each year. So far clubroot has been found in 405 canola fields distributed over 14 counties in Alberta (Strelkov et al. 2009). The worst affected area is around the city of Edmonton, but infested fields in Flagstaff county to the south and east of Edmonton, Newell county to the south and east of Calgary, and Cypress county in the extreme south and east of Alberta and bordering on southern

Saskatchewan have also been identified (Strelkov, personal communication).

Little effort, however, has been placed on clubroot field surveys in Saskatchewan and Manitoba. A survey of 30 fields in Saskatchewan in 2008 has indicated that a field in the west-central part of the province harbours the pathogen based on a DNA test of soil samples, although no disease symptoms were observed in the crop or during a subsequent growth chamber bioassay (Falk et al. 2009). This result may imply that the pathogen that causes clubroot is present in Saskatchewan but the level of infestation is possibly too low to cause visible symptoms on canola plants. Similarly, a single site in Manitoba has tested positive for the pathogen (personal communication, Philip Northover - MAFRI). Therefore, it is imperative to expand the field surveys in Saskatchewan and Manitoba to assess the presence and potential distribution of the clubroot pathogen due to the detrimental impact of this disease to canola crops. In addition, a better understanding of the potential for dispersal of *P. brassicae* inoculum via soil movement from heavily infested areas is urgently needed. Movement of soil infested with resting spores of the clubroot pathogen is believed to be the main mechanism of spread, but little knowledge exists regarding the potential and range for inoculum dispersal via soil movement as a result of wind and water erosion, or the movement of equipment in western Canada, especially over medium to long distances.

This project will be enhanced by intensifying field survey intensity in Saskatchewan and Manitoba and adding the study on *P. brassicae* inoculum dispersal to provide knowledge of the prevalence of P. brassicae in the prairie region as well as potential movement via soil erosion and a variety of field equipment. This knowledge will be used to implement proactive disease management strategies targeted to high-risk areas in Alberta, Saskatchewan and Manitoba, further enhancing the surveillance of clubroot. Knowledge of the presence or absence of pathogen inoculum and the potential for soil-mediated dispersal will help to develop and promote management strategies in order to restrict, or at least delay, the entry and spread of the clubroot pathogen to new areas/fields in AB, but more importantly into and within SK and MB. This objective included to acquiring basic information on clubroot occurrence and distribution in SK & MA based on field surveys and soil sample analysis, and to better understand the primary means of *P. brassicae* inoculum dispersal in western Canada

Research methodologies

Field surveys: Canola producers will be solicited as co-operators for the field surveys. These co-operators will be called after seeding (June and July) to seek permission to collect soil samples from their canola fields. At this time, additional agronomic information such as the crop rotation followed, the canola variety seeded, tillage practices used, etc. will be requested for the field to be surveyed. The survey will take place in August, preferably prior to swathing. In the event that not all fields can be accessed prior to swathing, surveying will continue after swathing.

In each of 80 fields, 10 plants at each of 10 locations along the arms of a W pattern and at least 20 m from each other and 20 m from the edge of the field, will be dug from the soil and examined for clubroot galls and assessed for other disease symptoms such as blackleg and Sclerotinia stem rot. Any plants or a subset of plants with symptoms resembling clubroot will be collected and contained in plastic tubs or bags for laboratory analysis. At the same time, a 1 litre soil sample will be collected for diagnostic analysis using the PCR (polymerase chain reaction) protocol of Cao et al. (2007) in Dr. Strelkov's lab at university of Alberta.

Each soil sample should be comprised of a mixture of small scoops of soil taken at each of the 10 sampling locations of each canola field. Clubroot is most likely to arrive on soil attached to vehicles and field equipment. Therefore if the entrance to the field is evident, five new sites will be selected in the area limited to the vicinity of this approach for the soil collection only. Note: survey data for other diseases will still be collected from 10 sites throughout the W sampling pattern. To collect the soil sample, crop residue should be cleared away from the soil surface, and approximately 1 cup will be scooped from the top 5-10 cm of soil at each site (total 1 litre from all 10 sites combined). The samples will be air-dried in paper envelopes/boxes/bags before analysis. Survey personnel will wear disposable shoe covers, and will also sterilize soil sampling equipment (shovels) in bleach after leaving the field but prior to entering the vehicle. Every precaution will be taken by surveyors to eliminate transport of infested soil outside of the sealed soil samples. Fig. 1 shows locations of soil samples collected in Saskatchewan.

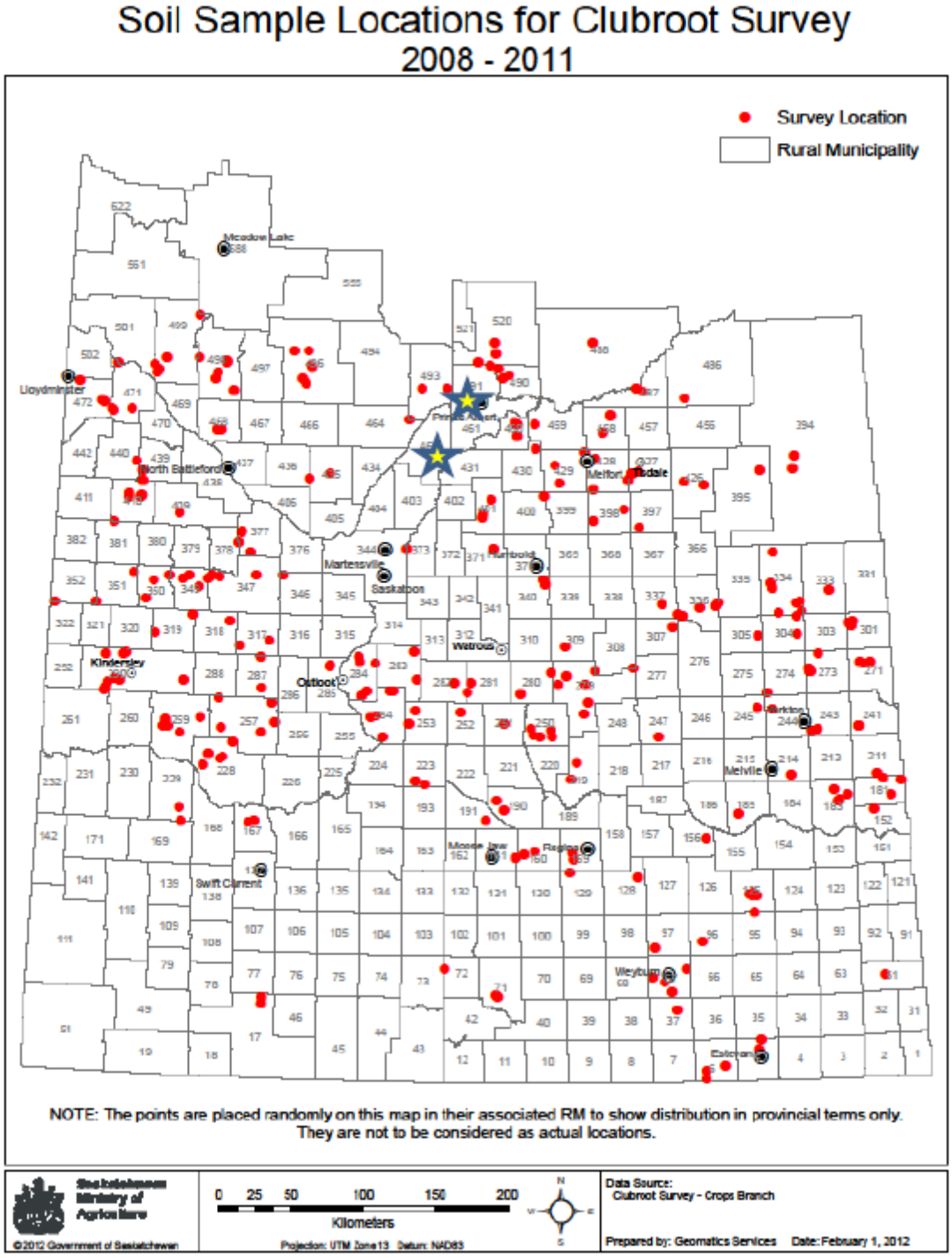

Fig. 1. Locations of soil samples collected in Saskatchewa for the test of Plasmodiophora brassicae infestation. The two star indicate RM's where clubroot disease was identified.

Dispersal of clubroot pathogen inoculum

Wind erosion: Based on prior survey information and communication with industry staff and farmers, 3-5 fields under conservation and conventional tillage will be identified, respectively. Within each field, wind aspirated dust samplers and surface creep dust samplers will be placed as follows:1) at 3-5 sites per field a ½" steel EMT pipe will be anchored in the ground and on each pipe, 3-4 Big Spring Number Eight (BSNE) samplers (Fig. 2 - Custom Products, Big Spring, TX) will be mounted between 10 cm to 5 meters above the soil surface; and 2) at 3-5 sites per field a surface creep sampler (SCS) will be buried in the soil and will sample material from between 3 to 20 mm above the soil surface. The BSNE and SCS samplers will be placed in the fields by mid-May depending on snow melt and seeding date and kept in the fields up to mid-summer. Depending on access and availability of fields the BSNE samples may also be placed in the fields in the fall and maintained over the winter period.

Fig. 2. BSNE soil samplers used to collected soil particles of varying sizes to determine potential dispersal of Plasmodiophora brassicae resting spores with wind erosion.

On a weekly to biweekly basis, material trapped in the sampling pans/canisters will be collected and kept separate for each sampler. Collected material will be air dried at ambient temperatures and then weighed. Soil particle samples will then be stored at 4ºC until further processing can be done. A diagnostic analysis using the PCR protocol of Cao et al. (2007) will be done in Dr. Strelkov's lab to assess for the presence and quantity of *P. brassicae* DNA. Direct evaluation of resting spores will also be attempted using a fluorescent staining technique reported by Donald et al. (2002). Bioassay assessments may also be considered depending on the amount of soil collected.

***Water erosion*:** Based on prior survey information and communication with industry staff and farmers, 3-5 fields suitable to evaluate water erosion will be identified. Between the end of snow melt and early June, fields will be visited and where possible 1-10 litre fluid samples from running water within or adjacent to clubroot affected fields will be collected. Samples will be stored at 4ºC until further processing. Subsamples of the liquid will be taken and put through either a filter or layers of cheesecloth to remove large pieces of debris. The subsamples will then be centrifuged to clean up the suspensions (Donald et al. 2002) before further assessment using PCR or fluorescent staining. Bioassay assessments may also be considered, where subsamples of the collected fluid are used to inoculate highly susceptible Chinese cabbage seedlings under controlled environmental conditions. Sediments

associated with water erosion will also be collected and stored at either 4 -20ºC prior to further processing as above.

***Within field resting spore gradients associated with water runs*:** Similarly, 3-5 fields suitable to evaluate in-field resting spore gradients associated with water erosion will be identified. Between the end of snow melt and early June, these fields will be visited and soil samples collected along 3-5 transects per field. Sample sites along the transect will start at the centre of the water run and then be spaced at 1-5 m intervals up to 50 m from the centre of the water run. Samples will also be taken at distances of up to 250-500 m from the centre of the water run. All sampling equipment will be cleaned between sample sites to prevent cross-contamination. Samples will be stored at 4ºC until the qualitative and quantitative assessment using the PCR assay. Bioassays will also be conducted on the collected sediment using standard procedures.

Results and discussion

Field surveys were conducted in Saskatchewan and Manitoba in 2010. In Saskatchewan 265 fields were inspected for visual symptoms on plants, none were observed. Soil samples were taken from 76 of the 265 fields, which were analysed for the presence of the pathogen using the PCR diagnostic test. The pathogen was not found to be present in any sample. In Manitoba, 145 fields were surveyed for visual symptoms and from 79 of these soil samples were analysed for the presence of *P. brassicae*. No visual symptoms were reported by surveyors and the pathogen was not detected in any of the 79 soil samples.

Soil samples from five locations in each of five commercial fields in the Edmonton area have been collected with sites being referenced via GPS in 2010, 2011 and 2012. In 2010, soil samples were sent to AAFC Saskatoon for assessment of the clubroot pathogen using a bioassay procedure as describe elsewhere, while in 2011 and 2012 samples were sent to the U of A for testing using qPCR as described elsewhere. There was only one field planted to canola in 2010 and it was planted to a clubroot resistant variety. At each of 5 sites, 40 plants were rated for a total of 200 main crop plants per field. Also at each site a total of 10 off-type plants were rated for a total of 50 plants per field.

Several BSNE dust samplers were placed around two commercial fields (one in central Alberta and another in southern Alberta) during the 2010 season. The dust from each sampler was collected at the end of the growing season and analyzed by PCR (Cao et al. 2007). All dust samples from the field in central Alberta tested negative. However, one positive was found in dust from one of the samplers in the field in southern Alberta. This finding suggests the potential for the spread of *P. brassicae* inoculum via wind erosion, although the amount of inoculum present remains to be quantified. Soil and water samples were also collected in 2010 from water runs in infested fields, a river passing through a region of high infestation, as well as highway rest stops and car washes, etc., in a gradient away from areas of infestation.

In 2011, warm summer weather and an extended period of high temperatures at harvest allowed producers to harvest the crop in a timely fashion. A total of 241 canola (*Brassica napus*) fields were surveyed between August 8 and September 25 in the major canola production regions of Saskatchewan. Soil samples were taken from 99 of the fields. The number of fields surveyed per region was approximately proportionate to the amount of canola grown in each of the regions. In Manitoba, A total of 100 fields were surveyed and soil samples taken from 69 fields for pathogen detection. Most of the fields were surveyed before swathing when plants were between growth stages 5.1 and 5.5. Clubroot symptoms were not observed in any of the fields surveyed. There were 11 soil samples from SK and 2 from MB that showed positive in PCR testing for pathogen presence in the soil, but further tests using qPCR and a bioassay suggested the pathogen was below quantifiable levels and unable to cause disease symptoms. In Saskatchewan, however, clubroot disease was reported for the first time in two disease nurseries in the north-central region.

Soil samples were collected in the fall of 2011 from the same five commercial fields in the Edmonton region as per previous years. Three of the fields were seeded to canola so the soil collection was done from soil close to the canola plants and from between plants at each of the five GPS referenced sites for a total of 10 soil samples per field. The other 2 commercial fields were not planted to canola so just one soil sample was taken from each of the

GPS referenced sites. In 2012, the same fields were surveyed again. The soil samples from 2012 are still being tested at the University of Alberta using qPCR. Substantial variability was found either via the bioassay assessments of samples from 2010 or for the qPCR assessments done at the U of A. Sites within the same field varied from no or trace clubroot detection to moderate to high levels of detection. Results indicate the extremely variable nature of the clubroot pathogen within individual fields, which suggests that when surveying for this disease relatively large numbers of sampling sites will be needed to get a true picture of clubroot presence, level and distribution within individual fields.

Soil samples from the central Alberta experimental plots were taken from georeferenced positions and analysed for *P. brassicae* infestation levels. These data points were analysed using a kriging geoprocessing technique to generate a fine scale inoculum level map. This information is being tracked to estimate the rates of inoculum spread and to guide our BSNE sampler placement and positioning. Levels of inoculum were found to be heterogeneous in the field and this is in agreement with popular beliefs regarding inoculum distribution. This work can be improved by reconciling the kriging maps with survey information results and through the use of differential GPS which has a higher degree of resolution.

Several BSNE dust samplers were placed in five fields in central Alberta (2 commercial production fields, 3 experimental plots) and 3 field locations in southern Alberta. The dust from each sampler was collected periodically throughout the growing season and analyzed by PCR (Cao et al. 2007). Eight dust samplers from three of the fields in central Alberta tested conventionally PCR positive and had quantifiable levels of infestation (by qPCR). It was not possible to conduct a bioassay from these samplers due to the small amounts of dust collected. Likewise, samplers were deployed in three locations in southern Alberta. Eight out of the 24 samples were conventionally PCR positive and had quantifiable levels of infestation. One of the samplers was conventionally PCR positive but did not have quantifiable levels of infestation. 42 of the total samples from Alberta had amounts of dust lower than that required for the DNA extraction procedures (<0.15g). Analysis of these samples was not conducted for Southern Alberta due to difficulty in processing such a small amount of sample. All of the positive samples in Central Alberta were >0.15g, with some as high as 16g. Irrigation water samples collected from southern Alberta were found to have very low levels of sediment, and in the two (out of 16 samples) that had sufficient sediment load for analysis, neither had any detectable levels of *P. brassicae* inoculum.

These findings suggest the potential for the spread of *P. brassicae* inoculum via wind erosion although no obvious trends in resting spore amounts are apparent in the data. Higher amounts of dust were collected from the traps on two of the highly managed experimental plots compared to no-till commercial fields. Tangential work has been initiated to investigate the interactions of roots and clubroot galls and the effect of UV light (which dust borne inoculum would be exposed) on resting spore viability. So far, a greenhouse experiment where plants are exposed to differing distributions of clubroot galls has been initiated and preliminary data is expected early summer. Preliminary data suggests that UV-A and UV-B light did not have a significant effect on the viability of resting spores. This experiment is under revision to assess the intensity and integrity of the UV bulbs used and modifications will be made as necessary.

For 2012-2013, field surveys in Saskatchewan and Manitoba was continued. The current BSNE sampler data and fine scale inoculum maps was used to develop a further optimized strategy for BSNE placement and positioning. As high levels of dust were collected at lower heights, two surface creep collectors have been purchased to collect saltating particles and will be deployed in the areas indicated in the data as having the highest infestation levels. Fine resolution inoculum mapping was continued with a higher resolution differential GPS system using the spatial analysis extension of ArcGIS available at the University of Alberta. Once, topographical maps are generated for the experimental fields, Gerlach troughs will be placed in areas of high runoff potential. Irrigation water sampling will continue as before.

Overall, three years of field surveys in SK and MB identified fields with low levels of clubroot infestation and no severely damaged fields have been observed. The information has been conveyed to both provinces and several provincial/commodity group's news releases were announced publically based on this information to increase the awareness. The potential for long range dispersal of resting spores is indicated by this study.

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CLUBROOT RISK MITIGATION INITIATIVE

FINAL REPORT

RBPI 1370 Objective II

Study interaction of climate, soil and pathogen biology on management of clubroot of canola

Project Lead: BRUCE GOSSEN

Collaborators:
Dennis Pageau

Work plans and milestones

2009-2010

1. Initiate studies on techniques to assess infection in seedlings, and trials to compare the reaction of canola and pak choy to clubroot across a range of temperatures, to determine if pak choy could be used as a quick-growing, small-stature model system for canola.
2. Conduct date of seeding trials to assess the impact of temperature on seedling infection and subsequent symptom development.
3. Recruit a post-doctoral fellow and additional graduate student(s) to participate in studies at the University of Guelph. One graduate student has already been hired to participate in this project.
4. Initiate studies on the impact of boron on infection and symptom development.

2010-2011

1. Conduct studies on techniques for assessing pathogen proliferation prior to symptom development in young plants.
2. Complete studies on the impact of temperature on seedling infection, and initiate studies on the interaction of pH and temperature on seedling infection.
3. Repeat studies to identify a replacement for Granaat (Chinese cabbage, universal susceptible), and screen for replacement candidates for other problem cultivars in the ECD differential set.
4. Continue studies on the impact of seeding date on seedling infection and subsequent symptom development.
5. Conduct the first year of field trials to assess the impact of boron on clubroot development at the U of G site.
6. Conduct studies to assess the uniformity and extent of the clubroot infestation at the U of G site. This information will be used to optimize the placement of trials and non-host crops at this site.

2011-2012

1. Complete studies on the interaction of pH and temperature on seedling infection, and initiate studies on the interaction of pH and temperature on pathogen proliferation on young plants.
2. Initiate studies on the impact of biofungicides on clubroot infection, development and symptom expression.
3. Initiate studies on the impact of host resistance on clubroot infection, development and symptom expression.
4. Complete studies to identify replacements for selected cultivars in the ECD differential set.
5. Initiate studies to assess the interaction of inoculum density and host resistance to clubroot on infection and symptom development.
6. Continue studies to identify replacement differentials for the ECD cultivars.
7. Conduct field trials to assess the impact of boron on clubroot development at U of G and Edmonton sites.
8. Initiate studies under controlled conditions to assess the interaction of boron, calcium, and other soil factors on clubroot development and expression.

2012-2013

1. Complete studies on the interaction of pH and temperature on pathogen proliferation in young plants.
2. Complete trials to assess the impact of host resistance and biocontrol agents on infection, pathogen development and symptom development.
3. Complete studies to assess the interaction of inoculum density and host resistance to clubroot on infection and symptom development.
4. Complete trials to assess the impact of boron on clubroot development at the U of G and Edmonton sites.
5. Complete studies under controlled conditions to assess the interaction of boron, calcium, and other soil factors on clubroot development and expression.

1. Effect of temperature and seeding date

Methods

Root hair infection, symptom development and clubroot severity: Root hair infection, symptom development and clubroot severity Ten-day-old seedlings were grown in liquid-sand culture, inoculated with resting spores suspension (300 µL of 108 spores of P. brassicae /mL) and maintained in growth cabinets at 10°, 15°, 20°, 25° and 30°C. Seedlings were harvested at 2-day intervals, starting 2 days after inoculation (DAI) and continuing until swelling of the tap root was observed (maximum 28 days). The root was washed with water, stained with aniline-blue and observed under the microscope. Briefly, 100 root hairs per plant at 0–2 cm below the soil surface were counted and used to calculate the percentage of root hair infection and stage of development of primary infection. Each root was also assessed for symptom development. The day when the swelling of the tap root and hypocotyl were first observed was recorded. Clubroot severity was rated using a 0–3 scale.

Cortical infection, symptom development and clubroot severity : Ten-day-old seedlings were grown, inoculated, and maintained as described above. Seedlings were harvested at 2-day intervals, starting 8 days after inoculation (DAI) and continuing until 42 DAI. A 0.5-cm root segment from the top 0–1 cm of each main root was dehydrated in an ethanol series, embedded in paraffin, and 6-ųm-thick cross-sections were cut and stained using methylene blue. Four stains (periodic acid schiff, fast green, haematoxylin + eosin and methylene blue) were assessed. Methylene blue was selected for the analysis because it provided consistent results that were compatible with image analysis. Five digital images per root at 125x magnification were collected and used to estimate the mean proportion of the area of each field of view that stained for P. brassicae. Development of P. brassicae was classified based on the developmental stages: i) young plasmodia, ii) mature plasmodia, and iii) resting spores. The number of cells that contained plasmodia or resting spores in each microscope field at 125x magnification was counted in five fields of view within one section from each root. In addition, cortical infection (%) was estimated using image analysis for each field of view. he area (%) occupied by plasmodia and resting spores was estimated in digital images of each microscope field at 125x magnification using image analysis software

Results

Root hair infection, symptom development and clubroot severity: Temperature affected every stage of clubroot development. RHI and clubroot severity were highest at 25°C, intermediate at 20°C and 30°C, and lowest at 15°C and 10°C (Fig. 1). Root hair infection was observed at every temperature, but clubroot symptoms developed only above 15°C. A substantial delay in the development of the pathogen was observed at 10°C and 15°C. Secondary zoospores were released at 16 DAI at 15º C, 8 DAI at 20º and 30º C, and 6 DAI at 25ºC. RHI was highest at 25° C, intermediate at 20 and 30°C and lowest at 10–15°C. No symptoms were observed at 28 DAI in plants grown at 10°C. Swelling of the tap root was visible at 28 DAI in plants at 15°C, 14 DAI at 20°C and 30°C, and 10 DAI at 25°C.This result demonstrates that RHI is reduced at temperatures below the optimum for the pathogen.

Cortical infection, symptom development and clubroot severity: Cortical infection (CI) was strongly and positively correlated with clubroot severity. The image analysis software readily separated infected and non-infected areas, and so provided a rapid technique for assessing cortical infection. Cortical infection was highest and symptoms observed earliest at 25° C, intermediate at 20° C and 30° C, and lowest and latest at 15° C. No cortical infection or symptoms were observed at 42 DAI in plants grown at 10° C. A substantial delay in the development of the pathogen was observed at 15° C. Resting spores were first observed at 38 DAI in plants at 15° C, 26 DAI at 20° C and 30° C, and 22 DAI at 25° C (Fig. 2). These results support the observation in companion studies that cool temperatures result in slower development of clubroot symptoms in Brassica crops, and demonstrate that the temperature has a consistent pattern of effect throughout the lifecycle of the pathogen. We conclude that assessment of cortical infection can be a useful supplement to other parameters of pathogen quantification, such as qPCR. Assessment of CI at 30 DAI at 25º C would be optimum for many studies because this ensures a high CI in susceptible lines in a relatively short time.

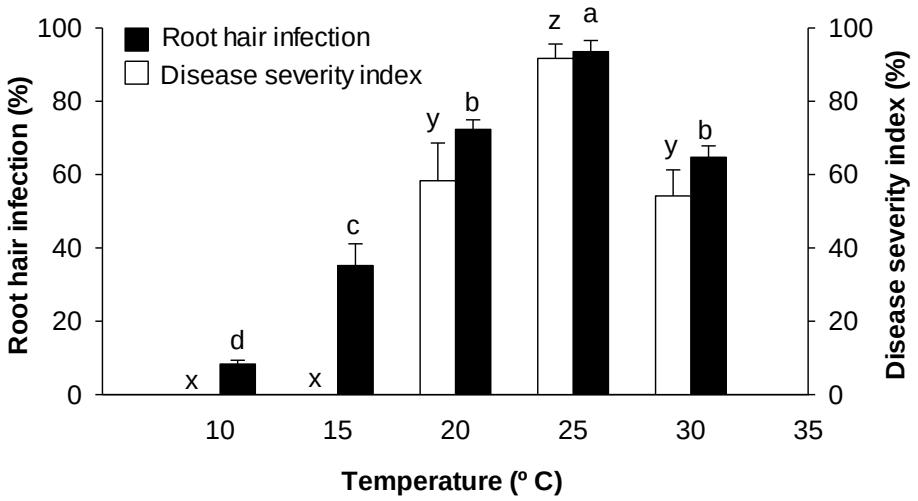

Fig. 1. The impact of temperature on infection of canola root hairs caused by *Plasmodiophora brassicae*.

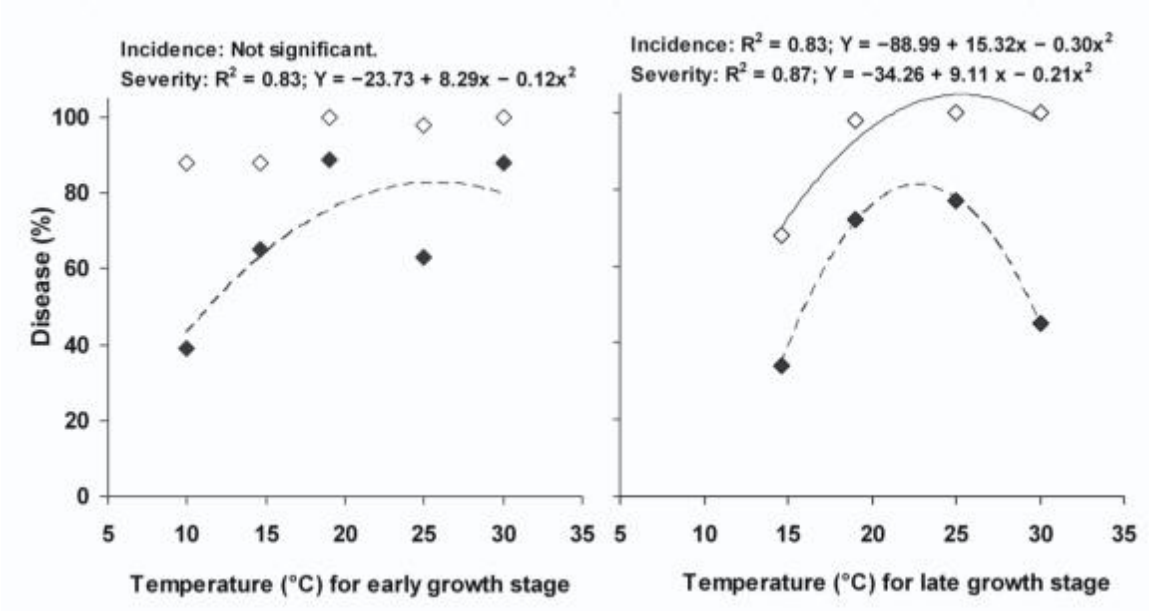

Fig. 2. The impact of temperature on infection and clubroot development on canola caused by *Plasmodiophora brassicae*.

2. Interaction of pH, temperature and soil type

The interaction of temperature (10°, 15°, 20°, 25°, 30°C) and pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) on root hair infection (RHI) and clubroot symptom development in canola roots was studied in trials under controlled conditions in non-calcareous sand at optimum moisture levels. The trials were conducted to assess the influence of host susceptibility, pathotype, and growth medium on the efficacy of two biofungicides against *Plasmodiophora brassicae*. Three experiments were conducted under controlled environmental conditions (25°/20° C day/night, 16-hr photoperiod, 65% RH) and two experiments under field conditions (15°–22° C) with canola and Shanghai pak

choy or napa cabbage. The growth media tested in the growth room trials were: muck soil (pH 6.2), mineral soil (pH 6.8), non-calcareous sand (pH 6.5) and soil-less mix (pH 6.0). The field experimental plots had muck soil (pH 6.2) with a high concentration of resting spores (3 x 10^7 spores g^{-1}).

Results

High RHI (up to 61%) and clubroot incidence (up to 100%) were observed at acidic pH (5.0 to 6.5) when temperatures were optimum for clubroot development (20° and 25°C). Clubroot was suppressed but not totally eliminated at alkaline pH (7.5 and 8.0) and optimum temperature (Fig. 3). Clubroot severity was low at 10°C and 15°C, regardless of pH. There was a negative correlation between clubroot severity and shoot weight at 25°C at each pH. Although clubroot symptom development on canola is reduced at high pH, moderately high clubroot severity can still develop when moisture and temperature are optimum.

In the growth room trials, both biofungicides reduced clubroot levels on canola and Shanghai pak choy in muck soil, mineral soil and sand. Clubroot levels were low in soil-less mix, but increased with increasing soil bulk density. The biofungicides did not reduce clubroot under the high disease pressure of the field trials. Both Shanghai pak choy and napa cabbage were highly susceptible to *P. brassicae*, and the mean incidence and severity of clubroot on both crops from inoculation with pathotype 3 was slightly higher than with pathotype 6. The interaction of biofungicides with growth media in the growth room experiments indicates that biofungicide efficacy needs to be evaluated in field soils under controlled environmental conditions before testing their performance under field conditions.

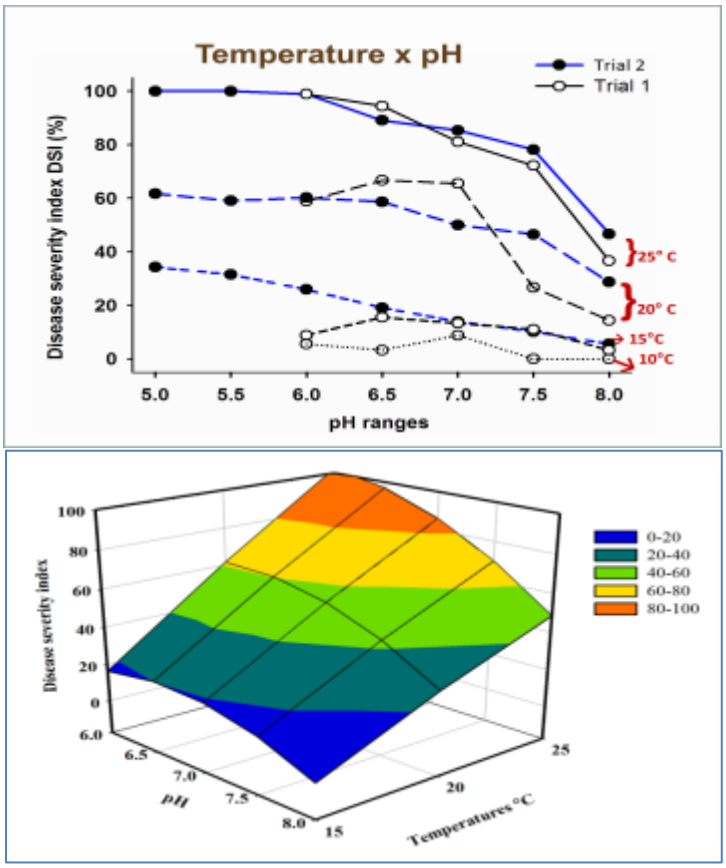

Fig. 3. Interaction of temperature and soil pH on infection of canola by *P. brassicae*.

3. Model Crops and Potential Differentials

The clubroot reaction of 86 lines of *Arabidopsis thaliana* and 5 lines of the Rapid Cycling Brassica Collection (RCBC) (*Brassica carinata, B. juncea, B. napus, B. oleracea, B. rapa*) were evaluated for their reaction to pathotypes 2, 3, 5 and 6 (William's system), which are the predominant pathotypes in Canada. The highly susceptible Shanghai pak choy cv. Mei Qing Choy (*B. rapa* var. *communis*) was included as an susceptible control. Seedlings were grown individually in soil-less mix, inoculated with 3 x 10^6 resting spores of *P. brassicae*, and maintained at 25º/20ºC day/night. Seedlings were assessed for clubroot incidence and severity at 6 wk after inoculation using a 0–3 scale

Most of the lines of *A. thaliana* were susceptible to each of the pathotypes and no line was resistant to all of the pathotypes assessed. Lines with a differential reaction to the pathotypes were generally moderately resistant, rather than immune. The RCBC lines displayed a strong differential response to the pathotypes. These results indicate that lines of *A. thaliana* and RCBC may have potential for use in a new differential set to characterize Canadian pathotypes of *P. brassicae* in Canada.

4. Effect of boron (B)

Trials were conducted under controlled conditions to assess the impact of application rates (0, 0.25, 0.5, 1, 2, 4, 8, 16, 32 kg ha^{-1}), application timing, and commercial formulations of B (Solubor, BoronMax, Boron) on primary and secondary infection in a susceptible canola cultivar (cv. 46A76, Pioneer Hi-Bred).

Increasing rates of B reduced primary infection and symptom development. However, phytotoxicity to canola seedlings occurred at rates higher than 2 kg B ha^{-1}. Application of 2 kg B ha^{-1} reduced slight RHI incidence from 81% to 65%, but delayed the development of each stage of primary infection. There were no substantial differences in response when B was applied before RHI (pre-emergence), before cortical infection (post-emergence), or split into two applications (pre- + post-emergence). All three formulations of B exhibited a similar response under controlled conditions. In the initial field trials on high organic matter soil (70% O.M.) in 2010, 4 kg ha^{-1} was the most effective rate that was not phytotoxic when B was applied as drench prior to seeding. Clubroot severity at 6 weeks after seeding was reduced by 64% compare to a non-treated control. However, in 2011, when B was broadcasted and incorporated in high O.M. and three mineral soils (at locations across Canada), none of tested rates of B, up to16 kg ha^{-1}, effectively suppressed severity in a moderately susceptible cultivar (cv. Invigor 5030 LL, Bayer Crop Science). The results indicate that B might be one component in an integrated management program for clubroot in canola if the issue of rate could be cleared up. Further studies are planned for 2012 to determine whether higher B rates (up to 32 kg ha^{-1}) are phytotoxic to canola and other crops, and can suppress clubroot on canola in under field conditions.

5. Expression of resistance in canola

Knowledge on timing of expression of resistance against pathogen in recently registered clubroot-resistant canola cultivars is lacking. To determine when resistance is developed in a resistant cultivar (cv. 45H29, Pioneer Hi-Bred), time series assessments of RHI and cortical infection by two pathotypes (P3 and P6) were made and compared with that in a moderately susceptible (cv. Invigor 5030 LL, Bayer Crop Science), susceptible (cv. 46A76, Pioneer Hi-Bred), and a pathotype-differential cultivar (cv. 45H21, Pioneer Hi-Bred) (susceptible to P3, resistant to P6). RHI occurred quickly in compatible interactions (susceptible cultivar x virulent pathotype) and more slowly in intermediate and incompatible interactions when assessed at 4, 8 and 12 days after inoculation (DAI). The maximum RHI for both compatible and incompatible interactions was 65–70%; maximum RHI was slightly but significantly lower (59%) in the intermediate interaction type. At 28 DAI, the cortical colonization was high in '46A76' (P3 = 45%, P6 = 35%), intermediate in 'Invigor 5030 LL' (P3 = 23%, P6 = 16%), and no colonization (0%) was observed for either pathotype in '45H29'. In '45H21', cortical colonization by P3 was high (35%), but inoculation

with P6 resulted in no colonization. In the compatible interactions, cortical colonization by P3 was consistently higher than by P6. There were small differences in the pattern of RHI associated with resistance, but resistance was most clearly expressed during cortical infection. There was clearly increased extent of lignification for xylem cells in the stele of resistant canola roots (Fig. 4) relative to that in susceptible root where the lignified cells expanded and became thin-walled (Fig. 5) in after the colonization by the plasmodium of the pathogen.

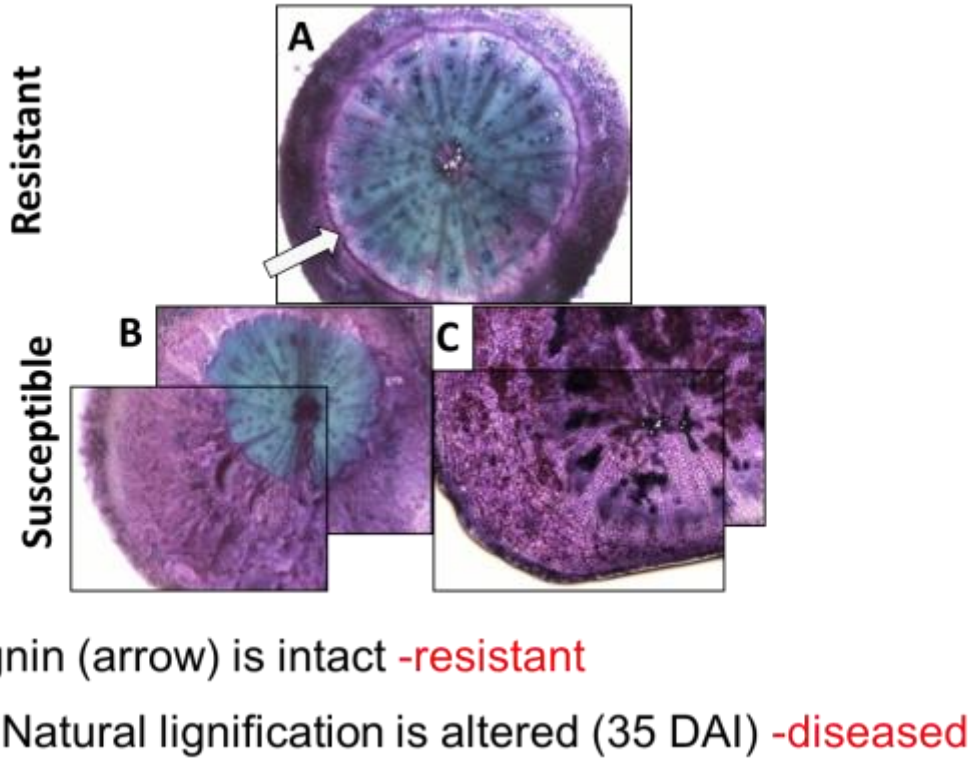

Fig. 4. Lignification of xylem cells in the stele of susceptible canola root (B) is reduced.

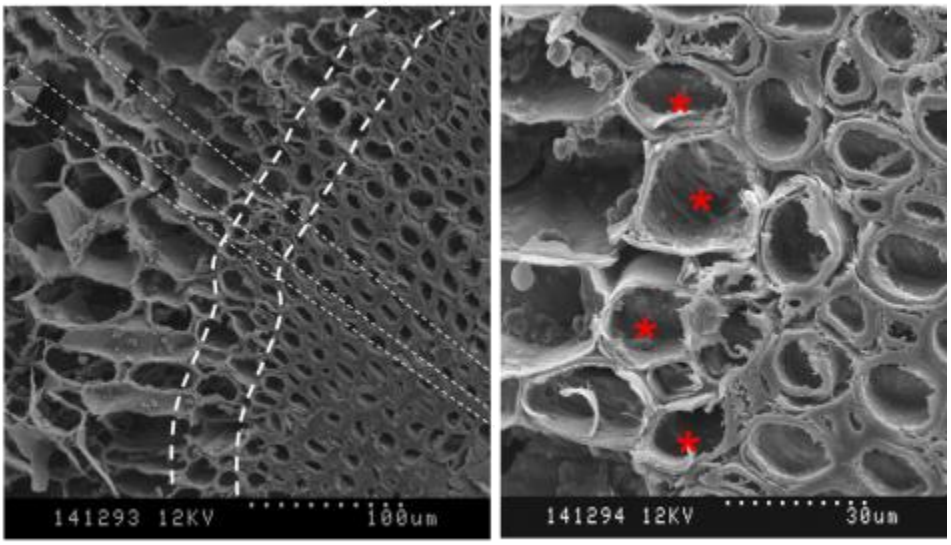

Fig. 5. Lignified cells expand and become thin-walled in susceptible canola roots after colonization by *Plasmodiophora brassicae*

CLUBROOT RISK MITIGATION INITIATIVE

FINAL REPORT

RBPI 1370 Objective III

Screening Brassica Germplasm for Resistance and Developing Canola Lines against Prairie Races of *Plasmodiophora brassicae*

Project Lead: KEVIN FALK

Collaborators:

Gary Peng
Fengqun Yu
Richard Gugel

Work plans and milestones

2009-10
Initiate screening of *B. rapa*, *B. oleracea*, and *B. juncea* germplasm for resistance using the clubroot bioassay in the containment facility at AAFC Saskatoon

2010-11
Complete screening of *B. rapa*, *B. oleracea*, and *B. juncea* lines, and initiate screening of *Camelina sativa* and *B. carinata* germplasm. In addition, initiate introgression of resistant genes into adapted *B. rapa* germplasm in greenhouse

2011-12
Complete screening of brassica germplasm, and initiate introgression of resistance genes into adapted *B. juncea, B. carinata* and *C. sativa* germplasm. Continue introgression of clubroot resistance in to *B. rapa* strains

2012-13
Continue introgression of clubroot resistance in to *B. rapa, B. juncea, B. carinata* and *C. sativa* strains, and initiate evaluation of selected lines in combination with microbial biocontrol formulations in controlled as well as field conditions

1. Screening *Brassica* germplasm for clubroot resistance – Falk, Peng, Yu, Gugel

Introduction

Cultivar resistance is a cornerstone for the management of clubroot (Hirai, 2006; Diederichsen et al., 2009). Resistant cultivars have been developed on Chinese cabbage (B. rapa var. pekinensis) in Japan (Hirai, 2006; Saito et al., 2006; Sakamoto et al., 2008; Kamei et al., 2010) and on rapeseed (B. napus) in Europe (Gowers, 1982; Diederichsen et al., 2006). Almost all clubroot-resistance (CR) Brassica crops are single-gene based and often race specific (Diederichsen et al., 2009), with CR genes that may be traced back to the sources identified originally from European fodder turnips (Crute et al., 1980; Piao et al., 2009). All canola cultivars in western Canada were highly susceptible to clubroot (Strelkov et al., 2006) until 2009 when a resistant cultivar was first introduced. A few more resistant cvs. were commercially available in last couple of years, but all of them carry a single CR gene. Historically, such single-gene-based resistant cultivars tend to be not durable for clubroot control as observed on Chinese cabbage in Japan (Hatakeyama et al., 2006) and on winter oilseed rape in Europe (Oxley, 2007). LeBoldus et al., (2012) exposed one of the CR canola cultivars to a pathotype-3 population of P. brassicae under controlled conditions and observed a substantial decline in the strength of resistance after only two repeated cycles. Therefore, it is prudent to identify a diverse CR background and develop canola germplasms with complementary modes of action for durable resistance. These efforts will help broaden the CR basis and enhance the long-term effectiveness of clubroot management.

CR materials were first documented in the 1930's, but the efforts to CR screening have been more noticeable since the 1960's when highly resistant European turnips were identified (Wit & Van De Weg, 1964; Tjallingii, 1965). So far, CR materials have been reported more frequently in certain crop types, including turnips (B. rapa), rutabagas (B. napus), white cabbage or kale types of B. oleracea (Diederichsen et al., 2009). Some of the most resistant materials have been found in B. rapa (Hirai, 2006) that tended to be more consistent against different P. brassicae pathotypes than other species of brassicas (Toxopeus et al., 1986). Only some of the pathogen isolates from Europe or Japan have been able to overcome the resistance in B. rapa, while more isolates were able to cause clubroot on B. napus and B. oleracea lines of the European Clubroot Differential (ECD) set (Toxopeus et al., 1986). The turnip (B. rapa) cultivars Siloga, Gelria, Milan White and Debra have also been used to breed different CR cultivars (Diederichsen et al., 2009). Brassica oleracea is potentially another important CR source, and has been assessed extensively. Crisp et al., (1989) evaluated close to 1000 B. oleracea accessions, and confirmed the resistance in several north and west European kale and cabbage lines. Carlsson et al., (2004) also found CR

materials during the screening of 52 entries of landraces, old cultivars and wild accessions of B. oleracea and closely related Brassica species. Clubroot resistance has also been introduced into B. oleracea from B. napus (rutabaga) (Chiang et al., 1977) via interspecific hybridization. Based on the test of ECD hosts, Crute et al. (1983) found that resistant B. oleracea lines would more likely be overcome by P. brassicae isolates which might not be virulent on the ECD B. rapa and B. napus lines, due possibly to different CR mechanisms in the B. oleracea hosts. In Canada, clubroot resistance has previously been sought against the pathotypes predominant in the central and eastern provinces. Chiang & Crête (1972) evaluated 334 Brassica lines against pathotype 2 and 6 collected in eastern Canada and reported that several cabbage lines were highly resistant. Similarly, Vigierl et al. (1989) observed several moderately resistant lines among 31 spring canola (B. napus) lines against pathotypes 2 and 6. The Canadian cabbage cv. Acadie was resistant to pathotypes 1 and 6, and cv. Rechelain was resistant to 2, 6 and 7 (Chiang & Crête 1985, 1989). A recent study (Hasan et al., 2012) evaluated 275 Brassica lines against five P. brassica pathotypes identified in Canada, and found resistance materials against all five pathotypes identified in Canada in the diploid species B. rapa, B. nigra, and B. oleracea and the amphidiploid species B. napus. There were relatively small numbers of B. rapa entries (36) included in this study, and this A-genome group of Brassica is considered the most important source for clubroot resistance (Toxopeus et al., 1986; Hirai. 2006).

The objectives of this study were to: 1) screen a large collection of Brassica spp. germplasm, especially the materials from B. rapa, for resistance against pathotype 3 of P. brassicae; 2) evaluate selected CR materials in larger plant populations and under higher pathogen inoculum pressure for the effectiveness and consistency of resistance; and 3) determine the resistance spectrum of these selected CR materials against all P. brassicae pathotypes identified in Canada.

Methodologies

Sources of Brassica germplasm

A total of 955 accessions of *Brassica* spp. were obtained from eight sources in Canada, China, Germany, UK and USA, including 94 *B. napus*, 718 *B. rapa*, 93 *B. juncea*, 12 *B. carinata*, 30 *B. oleracea* and 8 *B. nigra* (Table S1-S6). The majority of *B. rapa* lines were collected by Plant Gene Resource of Canada (PGRC) at the Saskatoon Research Centre (SRC), Agriculture and Agri-Food Canada (AAFC). Most of the *B. juncea* lines were provided by Viterra Inc. and *B. napus* lines by PGRC, Anhui Academy of Agricultural Science and Sichuan Academy of Agriculture Science, China, respectively. Several commercial *B. rapa* (Chinese cabbage) and *B. oleracea* (cabbage) cultivars with reported resistance to clubroot were received from Bajo Seeds (Oceano, CA, USA 9344) and Syngenta Seeds UK Ltd (Fulbourn, Cambridge CB21 5XE, UK). In early screening trials, a clubroot susceptible (CS) canola cv. Fortune RR was used as a control (inoculated). In later trials in which selected candidates were tested for resistance spectrum, the *B. rapa* cv. Granaat (Chinese cabbage) was used as a universally susceptible control (ECD-05). Throughout the study, non-inoculated plants did not produce clubroot symptoms, so they were no regularly included in test trials.

Pathogen inoculum

For screening of the large number of germplasm (Test I) or characterizing the resistance level and pattern of selected CR candidates (Test II), clubroot galls gathered in a severely diseased canola field near Leduc, Alberta was used. The pathogen population in this field consisted predominantly of the *P. brassicae* pathotype 3 (Williams 1966) which is highly virulent on canola (Strelkov et al., 2007, Xue et al., 2008). The pathogen inoculum was increased by soil-drenching inoculation of a susceptible canola cultivar (Fortune® RR) using a *P. brassicae* resting-spore suspension (1×10^7/ml) at seeding under controlled-environment conditions. About 3 g of galls were immersed in 50 ml of distilled water for 2 h to soften the tissue and homogenized in a Warring blender at high speed for 1 min. The resulting suspension was filtered through eight layers of cheese cloth, and the concentration of resting spores was estimated with a haemocytometer. About 5-6 wks after inoculation, galls from diseased plants were harvested, dried at room temperature for 2 wks, buried in the Sunshine #3 potting mix (SunGro Horticulture, Vancouver, BC) for about one month to allow maximum conversion of plasmodia into resting spores. The potting mix was kept moist by weekly watering to near saturation. Processed galls were dried at the room temperature and then stored at -20°C until use.

For testing the resistance spectrum of selected cultivars/lines, single-spore isolates of the pathotypes 2, 3, 5, 6, and

8 of *P. brassicae* (Xue et al., 2008), classified also based on the Williams (1966) differentials, were used. These isolates included SACAN-ss3 (pathotype 2), SACAN-ss1 (pathotype 1), ORCA-ss4 (pathotype 5), AbotJE-ss1 (pathotype 6) and ORCA-ss2 (pathotype 8). Galls caused by the single-spore inoculum of the respective *P. brassicae* pathotypes on the universally susceptible *B. rapa* cv. Granaat (Chinese cabbage) were stored at -20°C until use.

Trial 1. Screening for resistance to clubroot caused by pathotype 3 of P. brassicae:

For the assessment of the 955 *Brassica* germplasm, plastic root trainers, also called conetainers (3.5-cm diam. × 20-cm tall, Stuewe and Sons, Corvalis, OR), were filled with the Sunshine #3 potting mix (pH 5.8-6.3). The growth medium in each conetainer was infested by pipetting 5 ml of a *P. brassicae* pathotype-3 resting spore suspension (1×10^7 spores ml^{-1}) to obtain about 1×10^6 spores cc^{-1}growth medium. Immediately after adding the pathogen inoculum, two seeds were sown into each conetainer at about 1-cm depth, and later thinned to one seedling at the 1-true leaf stage. Seeded conetainers were placed on a holding rack in a growth cabinet at 18-23ºC (night-day) with a 14-h photoperiod at 512 µmol m^2 s^{-1} for 5 wks. The growth medium was saturated during the first week post seeding by daily watering and maintained at high moisture levels by watering every other day after the first week.

Each entry consisted of 7 plants (replicates) and each batch included 13 entries plus a CS control (also 7 plants). Each seeded conetainer was thinned to a single plant at about the 1-true-leaf stage and placed randomly on a holding rack. The experiment was a completely randomized design. Five wks after inoculation, plants were assessed for clubroot severity using a 0–3 scale adopted from Kuginuki et al., (1999); 0 = no galling; 1= small galls on less than 1/3 of roots; 2= small to medium-size galls on 1/3 to 2/3 of roots; and 3 = severe galling, with medium- to large-size galls on more than 2/3 of roots. The average clubroot severity on each entry was compared against that on CS plants included in the same test. A test would be deemed valid only when all CS plants were diseased, with an average clubroot severity exceeding the severity scale 2. This initial testing was done once to select highly resistant candidates for further assessment, and a total of 75 batches of testing were carried out between 2009 and 2011.

Trial 2. Performance of selected CR candidates under higher pathogen inoculum

Ten cultivars/lines, including 2 *B. nigra*, 2 *B. oleracea* and 6 *B. rapa*, with moderately high to very high resistance ratings in Trial 1, were selected for further assessment using larger host populations to determine the variability or consistency of clubroot resistance with different materials. A protocol similar to that described in Trial 1 was used, but the pathogen inoculum level was increased by 10 fold, reaching 1×10^7 sporesccg^{-1} growth medium, to minimize the chance for disease escape. Each entry consisted of 21 to 90 plants, depending on the CR consistency observed in Trial 1; fewer plants would be used for those with a complete resistance reaction (little variation among individuals) and the test was conducted 1-3 times. For each test, the canola cv. Fortune RR was included as a CS control, and it was always 100% diseased with the highest severity rating 3 for each of these plants in each test. Inoculated plants were placed in a growth cabinet with the same conditions as described earlier for 6 weeks and assessed individually for clubroot severity using the 0-3 scale. The data were expressed as the number of plants under each clubroot severity category (resistance distribution).

Trial 3. The resistance spectrum of selected cultivars/lines against the five pathotypes of P. brassicae identified in Canada

Slightly different experimental protocols were used for this study; seeds of each cultivar or line were pre-germinated on a piece of moistened filter paper in Petri dishes, and 1-wk old seedlings were inoculated with *P. brassicae* inoculum by dipping the entire roots in a resting spore suspension for 10 s prior to being transplanted into the Sunshine LA4 potting mix in 6-cm plastic pots individually. Resting spores were extracted separately from frozen galls of each of the five single-spored *P. brassicae* pathotype isolates using a protocol described by Strelkov et al., (2006) and adjusted to a concentration of 1×10^7 spores /ml using a haemocytometer. The inoculated seedlings were placed in a research greenhouse ($20 \pm 2^{\circ}$C) for 6 wks to allow disease symptoms to develop. The growth medium was saturated with tap water during the first wk after inoculation, and then kept highly moist by regular watering. Ten cultivar/lines with confirmed clubroot resistance in both Trial 1 and 2 were assessed against the five *P. brassicae* pathotypes (2, 3, 5, 6, and 8) identified in Canada. A universally susceptible cultivar (ECD-05) of Chinese cabbage (Granaat) was used as a CS control in these trials.

The resistance of the selected materials to each of the pathogen pathotype was assessed individually with in two repetitions in greenhouses at the University of Alberta and the Crop Diversification Centre North, Alberta Agriculture and Rural Revitalization (Edmonton, AB), respectively. The experiment was a randomized complete block design with 3 blocks used in each of the repetitions. Within a block, 12 plants were inoculated for each of the selected cultivars/lines, including the CR control. About 6 wks after inoculation, each plant was uprooted and scored for clubroot symptom severity using the 0-3 scale described above. For statistical analysis, a disease severity index (DSI) was calculated using the following formula modified from Horiuchi and Hori (1980) by Strelkov et al., (2006):

Data analysis:

The screening data (Trial 1) for each entry were averaged over 7 replicated plants for the mean clubroot severity and compared with that of the CS control included in the test for the percentage of clubroot reduction. The clubroot reduction was a relative value against CS control, and was therefore used to rank the resistance of multiple entries within a species (Table S1-S6). The data for selected cultivars/lines evaluated under higher pathogen inoculum pressure and larger populations (Trial 2) were organized as a total number of plants falling into each clubroot severity category (0-3) to characterize the consistency or variation in a cultivar or line. The DSI data from the study of resistance spectrum with selected cultivars/lines (Trial 3) were analyzed using the. A square-root transformation was used to normalize the distribution of percentage data. Data from repeated trials were homogeneous based on the Bartlett`s Test, and were therefore pooled prior to analysis. Analysis of variance was performed initially using PROC ANOVA, and DSI means associated with each cultivar/lines were separated using Fisher's Protected $LSD_{0.05}$ when the ANOVA indicated the significance (at $P \leq 0.05$). Means presented in results are based on non-transformed data.

Results and discussion

Trials 1. Screening for resistance against pathotype 3 of P. brassicae

Of the 955 accessions screened, the majority (627) showed equal or higher susceptibility to pathotype 3 of *P. brassicae* relative to that of CS canola cv. Fortune RR (Table S1 to S6) while 50 accessions showed only slight resistance, with 25-50% less disease severity than on the control. A total of 35 accessions demonstrated >50% clubroot reduction, among which 15 were completely resistant with no visible symptoms 5 wks after inoculation. Highly resistant materials (>75% clubroot reduction) were found in *B. rapa* (17) (Table S2), *B. nigra* (4) (Table S6) and *B. oleracea* (2) (Table S4), but not in any of the amphidiploid species tested. Four of the *B. napus* accessions showed low to moderate resistance against the pathotype 3 (Table S1) while the rest were highly susceptible. Many of the materials in this species are rapeseed cultivars/breeding lines from China and Europe. The rutabaga cv. Askegarde and canola lines SW98323030 and SW02763 showed clear resistance to clubroot caused by pathotype 2, but not to that caused by pathotype 3 of *P. brassicae* (Table S1).

Brassica rapa represented the largest group of germplasm in this screening, with a total of 718 entries (Table S2). Although only <3.6% of the materials showed >50% clubroot reduction, a large number of highly resistant cvs./lines were identified this group, including the var. *chinensis* (Pak choy), var. *pekinensis* (Chinese cabbage), and the var. *rapifera* (fodder and vegetable turnips). Ten of the cvs./lines demonstrated complete resistance (clubroot free) to pathotype 3, including canola (96-6991, 96-6992), Chinese cabbage (Bejo 2833, Bilko, Emiko, Jazz Napa Cabbage), Pak choy (Flower Nabana), and turnip (Toronda, Vedette, Vollenda). There were also a fair number of *B. rapa* lines that showed moderate to high levels of resistance (50-93% clubroot reduction), and most of them are canola or turnip lines. A few of them consistently had smaller galls than those on the CS control, but most seemed to show resistant/susceptible segregation.

Several *B. juncea* lines showed slight resistance, with noticeably smaller galls relative to those on the CS control. However, none of the entries achieved >50% clubroot reduction and most of the lines were highly susceptible (Table S3). Only limited numbers of *B. carinata* lines were assessed in this study, and all of them were susceptible or highly susceptible to pathotype 3 (Table S4). While the majority of *B. oleracea* cvs./lines were highly susceptible, several showed a moderate to high level of resistance against pathotype 3 (Table S5). Most of the CR *B. oleracea*

materials are cabbage while Gruner Angeliter is kale. Two commercial cabbage cvs. Kilaherb and Tekila, showed complete resistance to pathotype 3, but the others CR materials exhibited some resistant/susceptible segregation. Although there were only eight *B. nigra* entries, half of them showed a high level of resistance to pathotype 3, with BRA 192/78, CR2120 and CR2716 exhibiting immunity (Table S6). A couple of PI 219576 plants had only small galls while the others seemed to be completely clubroot free.

Trial 2. Consistency of selected CR candidates under higher pathogen inoculum pressure

Under 10-fold higher pathogen inoculum load than in the previous trial, the ten selected resistance lines, including *B. rapa*, *B. oleracea*, and *B. nigra*, showed variable resistance patterns against pathotype 3 of *P. brassica*. Flower Nabana, Jazz Napa Cabbage, and Tekila were completely resistant with no sign of disease on any of the roots inoculated (Table 1), whereas PI 219576, Purple Top fodder turnip, and Purple Top White Globe vegetable turnip had an almost 1:1 ratio of resistant/susceptible segregation. PI 219576 was highly resistant (although not immune) in Trial 1, but the two turnip cvs. were only moderately resistant. For the rest of selected CR candidates, while most of the plants in the population appeared immune (clubroot free), there were incidental plants with slight to severe (scale 1 to 3) clubroot symptoms.

Trial 3. Resistance spectrum for selected CR candidates: The same CR materials (as in Trial 2) were tested against five *P. brassicae* pathotypes found in Canada. Throughout the study, the universally susceptible control (ECD-05) developed severe clubroot symptoms (DSI >97.0%) after inoculation with each of the pathotypes (Table 1). BRA 192/78, Kilaherb, Tekila, Flower Nabana, and Jazz Napa Cabbage were immune or highly resistant to each of these pathotypes, reducing DSI consistently by >90% relative to the CS control. For each of these cultivars/lines, there was little variation in resistance towards different pathotypes (P >0.05, Protected LSD). Emiko and Bejo appeared slightly less resistant than the above-mentioned cultivars/lines, but their effect towards different pathotypes did not differ (P >0.05, Protected LSD). PI 219576, Purple Top and Purple Top White Globe turnips, however, were only moderately resistant against these pathotypes; reducing DSI by 43-64% (Table 1). There was some variation towards different pathotypes for these materials; the PI 219576 was slightly less resistant to pathotype 3 than to the 2 or 5 and Purple Top White Globe turnip was slightly less resistant to pathotype 3 than to the 2 or 8 (P <0.05, Protected LSD). In contrast, the Purple Top fodder turnip was more resistant to pathotype 2 and 3 than to the 8 (P <0.05, Protected LSD).

Table 1. Resistance spectrum of selected clubroot resistant brassica accessions.

Table II-1. Clubroot severity on selected resistance lines in response to inoculation with five *Plasmodiophora brassicae* biotypes identified in Canada.

Common name	Accession/line	P-type 2	P-type 3	P-type 5	P-type 6	P-type 8
Chinese cabbage	ECD-05 (CK) [a]	97.0	98.6	98.9	97.7	97.5
Black mustard	BRA 192/78	0	0	0	1.4	0
Black mustard	PI 219576	40.7	51.9	38.4	45.8	44.9
Cabbage	Kilaherb	0	0	0	0	2.3
Cabbage	Tekila	0	1.4	0.9	2.4	0
Chinese cabbage	Bejo	3.2	15.7	17.6	16.2	10.2
Chinese cabbage	Emiko	16.7	18.1	25.0	25.5	19.0
Pak choy	Flower Nabana	5.7	6.7	0.7	2.8	2.4
Chinese cabbage	Jazz Napa	0.9	0.9	2.3	1.0	2.3
Turnip (forage)	Purple Top	34.7	43.1	45.8	55.1	55.6
Turnip (vegetable)	PT White Globe	43.1	53.7	49.1	47.7	39.8

[a] **Universally susceptible (control).**

Screening for clubroot resistance has been reported previously, but most has focused on pathotypes predominant in other continents (Tjallingii, 1965; Toxopeus & Janssen, 1975; Dias *et al*., 1993; Voorrips & Visser, 1993; Kopecký *et al*., 2012), or on vegetable crops in North American (Ayers & Lelacheur, 1972; Chiang & Crete, 1985; Miller & Williams, 1986). One recent study did target the pathotypes on canola and western Canada (Hasan *et al*., 2012), and examined materials from A-, B-, and C-genome of *Brassica* species and their amphidiploids. The number of lines in each groups ranged from 24 to 77, with the most of candidates from *B. nigra*. The current study focused particularly on *B. rapa* due to the fact that most of the highly resistant materials found previously were in this species group (Hirai, 2006), to identify a variety of candidates for the development of new CR canola germplasm to be used in western Canada. Overall only a very small number out of the 955 accessions turned out to be highly resistant (>75% disease reduction) against the pathotype 3, but these CR materials represented A-, B-, and C-genome species. In contrast, their amphidiploids were generally much less resistant.

In the current study, most of the highly resistant materials were found in *B. rapa*, with a large number of them being turnip lines. Several oriental crucifer vegetable crops, including pak choy and Chinese cabbage, also showed strong resistance although the origin of CR sources in these vegetable cultivars is not clear. In previous studies, highly resistant *B. rapa* also showed less variability against different *P. brassicae* pathotypes than other CR *Brassica* spp. (Toxopeus *et al*., 1986). European fodder turnip was particularly resistant to clubroot (Diederichsen *et al*., 2009) and some even believed that CR genes in *B. rapa* would exist only in European turnips (Crute *et al*., 1980; Yoshikawa, 1993). The turnip cvs. Siloga, Gelria, Milan White, and Debra have been used for breeding different CR cultivars (Deiderichsen *et al*., 2009), and the cvs. Gelria (ECD-02) and Debra (ECD-01) have been used in the European differential set (Crute *et al*., 1980). One may question if some or all of the CR genes in the oriental vegetable cultivars are from turnip (Yoshikawa, 1993). In our study many turnip lines were only moderately resistant to pathotype 3, while the CR oriental vegetable cvs. were almost immune. Further study of Purple Top (fodder) and Purple Top White Globe (vegetable) turnips showed that the lower resistance observed with these two cvs. was more due to their genetic heterogeneity, with clear resistant/susceptible segregation when tested in larger

populations (Table 1). As a typical cross-pollenated species, most *B. rapa* population should be expected to be genetically heterogenetic, In contrast, the CR oriental vegetable cultivars are all hybrids and would have more homogenetic background. The origin and similarity of CR genes in these *B. rapa* materials are still being sorted out via molecular mapping (Yu *et al*., 2013), but several of these materials do serve as good CR sources against the predominant *P. brassicae* pathotype in western Canada. The turnip cvs. Siloga, Taronda, Vedette and Vollenda were also highly resistant to pathotype 3. Detailed assessment was not done on these materials due to poor seed viability, but Hasan *et al*., (2012) reported five turnip cultivars they tested were highly resistant to all *P. brassicae* pathotypes found in Canada. It appears that many useful CR *B. rapa* materials have been identified through the current study that are effective against the pathogen pathotypes highly virulent on canola.

Most of the *B. napus* lines showed little resistance to the pathotype 3. Even the rutabaga cultivar Wilhelmsburger, which showed resistance against many *P. brassicae* isolates in France (Somé *et al*., 1996), was only slightly resistant. Many of the entries in this group are rapeseed cultivars or breeding lines from China and Europe, including some of cultivars that showed resistance to the pathogen pathotypes in local areas such as Zheping #4 in southern Anhui province, China (personal communications, Prof. B.C. Hu, Anhui Academy of Agriculture Science) and Tosca in Europe (Diederichsen *et al.*, 2009). The origin of CR genes in Zheping #4 is not clear but Tosca received a single resistance gene from CR turnip (Diederichsen *et al*., 2009). It is clear that the CR genes in these two rapeseed cultivars are race specific and not effective against pathotype 3. Similar results were also seen with two of the European rutabaga cvs. Askegarde and York, as well as with two spring canola lines (SW 02763 and SW 98323030) that acquired CR genes from Askegarde and York, respectively. These rutabaga cultivars and canola lines were not resistant or only marginally resistant (York) to pathotype 3, but highly to moderately resistant to pathotype 2 (Table S1). It appears that CR genes in rutabaga, including cvs. Askegarde, York and Wilhelmsburger, are race specific and ineffective against pathotype 3 in western Canada. Strelkov *et al*., (2006) reported pathotype 3 and 5 were more virulent on canola and 48 canola (*B. napus*) cultivars were all highly susceptible to pathotype 3. In a study by Hasan *et al.,* (2012), only 3 *B. napus* lines out of 36 were resistant to the pathotype 3 and 1 was resistant to the pathotype 5. Canola (*B. napus*) cultivars resistant to pathotypes 3 and 5 have been developed and available in western Canada since 2009 (Leboldus *et al.*, 2012). Although CR genes highly resistant to pathotype 3 or 5 are not common the amphidiploid species *B. napus*, canola cultivars may receive strong CR genes from other sources, especially *B. rapa*, as evidenced during the development of the rapeseed cvs “Mendel” (Diederichsen *et al.*, 2006).

Several CR cultivars/lines were identified from *B. nigra* and *B. oleracea*. There was a higher proportion of *B. nigra* materials with a high level of resistance relative to other species tested. The resistance to clubroot had been rarely reported in *B. nigra* until the study by Hasan *et al.* (2012) in which 66 of the 77 lines examined were resistant to all *P. brassica* pathotypes identified in Canada. In our study, three of the eight *B. nigra* lines showed complete resistance to pathotype 3, and the line BRA 192/78 was also resistant to all pathotypes found in Canada. The line PI 219576 was noticeably heterogenetic, with clear resistant/susceptible segregation where plants were either disease free or severely diseased. This segregation pattern was consistent against each of the pathotypes found in Canada, with disease-free plants obtained under each of the cases. These results highlight the additional CR sources identified effective against the all five pathogen pathotypes identified in Canada.

Previously, Crisp *et al*., (1989) assessed about 1000 *B. oleracea* accessions, and confirmed the existence of clubroot resistance in several north and west European kale and cabbage lines, with a new CR source identified from the cabbage cv. Eire. Based on the test of ECD lines, Crute *et al.* (1983) found that CR *B. oleracea* cvs. would more likely be overcome by the *P. brassicae* isolates that were not virulent on the highly resistant *B. rapa* and *B. napus* cvs. in the ECD set. The C-genome group of *Brassica* is considered to carry CR genes less infrequently the other species (Piao *et al.*, 2009), or CR genes in *B. oleracea* is less resistant (Diederichsen *et al.*, 2009). CR genes have also been introduced into *B. oleracea* from *B. napus* (rutabaga) via interspecific hybridization (Chiang *et al*., 1977) and it is difficult to sort out the origin and similarity of CR genes in *B. oleracea* due to generally poor record of the pedigree on most of the original CR sources. Several CR cabbage lines developed in Canada were resistant to the pathotypes 1, 2, 6, and 7 (Chiang *et al*., 1977, Chiang & Crête 1985, 1989), but their resistance against pathotypes 3 or 5, ones that are more relevant to clubroot on canola in western Canada, was not reported. Among the 48 *B. oleracea* lines tested by Hasan et al. (2012), five showed resistance to pathotype 3 and three were resistant to pathotype 5. In our study, five of the 30 *B. oleracea* lines were resistant to pathotype 3, with the cvs.

Kilaherb and Tekila (cabbage) being highly resistant against all five pathotypes found in Canada. These findings highlight potentially highly effective CR sources from C-genome species against pathotypes 3 and 5 on canola.

No highly resistant candidates were identified with the amphidiploid species *B. juncea* or *B. carinata*, despite a relatively large number entries screened for the former. Several lines showed "moderate resistance", but root galls were always present (severity 1-2) on these plants and the plant growth was often negatively affected (data not shown). These results are consistent with those from other studies where CR materials were not reported with *B. juncea* or *B. carinata* as reviewed by Diederichsen *et al*., (2009). Hasan *et al*., (2012) also reported that none of the 48 *B. juncea* or 24 *B. carinata* cvs. carried the resistance to any of the pathotypes found in Canada. They postulated that if CR *B. nigra* lines had been involved in the evolution of *B. juncea* or *B. carinata* in nature, it would be possible that the B-genome resistance in these two amphidiploids is hypostatic. It is difficult to explain the lack of resistance materials in *B. juncea* and *B. carinata* while CR genes exist in the B-genome species so commonly based on the observations by Hasan *et al*. (2012). We phenotyped a small F1 population (30 plants) between a CS double haploid *B. carinata* line and the CR BRA 192/78 (*B. nigra*) cross, and observed an almost 1:1 ratio of resistant/susceptible segregation against pathotype 3 (data not shown). This may indicate that the clubroot resistance with the heterozygous BRA 192/78 is controlled by a single dominant gene towards this pathotype.

This is one of the largest studies attempting to identify diverse CR materials for development of canola germplasm to enhance the effectiveness and durability of clubroot resistance in western Canada. The screening of the *B. rapa* group is extensive, including a variety of crop types with a large number of CR candidates identified. The ten selected CR candidates, representing A-, B-, and C-genome species, appear promising with highly resistant plants obtained from each source that are effective against all five pathotypes of *P. brassicae* found in Canada. Further studies will need to characterize CR genes in these selected materials and incorporated complementary one into canola breeding lines.

2. Molecular mapping of clubroot-resistance genes and introgression of resistance genes into canola germplasm using marker-assisted selection

Introduction

To develop a broad-based pool of resistance germplasm for long-term management of clubroot, diverse resistant materials should be sought with CR genes characterized and differentiated. Genetic mapping has been used to identify clubroot resistance genes in *B. rapa* (Piao *et al*., 2004, Hirai *et al*., 2004), *B. oleracea* (Landry *et al.*, 1992; Voorrips *et al*., 1997), and *B. napus* (Manzanares-Dauleux *et al*., 2000). So far, eight CR loci have been mapped in five *B. rapa* linkage groups (Suwabe *et al*., 2003, 2006; Hirai *et al*., 2004; Hayashida *et al*., 2008; Sakamoto *et al*., 2008; Ueno *et al.,* 2012). Several highly resistant materials were identified in current studies against all five *Pb* pathotypes found in Canada, and a study was carried out further to map the CR genes in selected highly resistant materials and developed molecular markers to facilitate the introgression of the resistance into canola breading lines using marker-assisted selection (MAS).

Methodologies

The commercial pak choy cv. Flower Nabana (FN, *B. rapa* subsp. *chinensis*) was selected due to its outstanding resistance against a broad spectrum of pathotypes. A resistant/susceptible population was developed by crossing a CR FN plant (FN_1) with a self-compatible CS *B. rapa* canola DH line ACDC, and used for mapping of CR genes in FN. A total of 318 microsatellite markers were used to screen the polymorphism between the parents ACDC and FN, and a subset of F_1 population was used to further analyze polymorphic markers.

The *Rpb1* gene was introgressed into elite canola lines using a marker-assisted selection (MAS) approach. To verify the effectiveness of the markers sN8591 and sR6340I for selection of *Rpb1* in *B. rapa* and *B. napus* populations, two BC_1 populations, derived from crosses between F_1 of a CS *B. napus* or *B. rapa* breeding line × CR FN, were tested using these markers in comparison with phenotyping results.

Results and discussion

A resistance gene was mapped in a segregating population of FN consisting of a total of 1299 F_1 plants which were either highly resistant or susceptible against *Pb* pathotype 3, with an approximately 1:1 segregation ratio (X^2 = 2.98, *P* = 0.084) (Fig. 1). Clubroot resistance can be controlled by a major gene or several minor genes in *B. rapa* (Crute *et al*., 1980; Yoshikawa *et al*., 1993; Kuginuki *et al.*, 1997; Mastumoto *et al.*, 1998; Suwabe *et al*., 2003; Hirai *et al*., 2004), but the results indicates that the resistance in FN is controlled by a single dominant CR gene. Six molecular markers flanked this resistance gene, designated as *Rpb1* hereafter, with three markers on each side (Fig. 2). The marker sN8591 and sR6340I are the closet ones from each side, at a distance of 0.54 cM and 0.77 cM to *Rpb1*, respectively. Based on the sequence BLAST search of four CR loci reported in the linkage group A3, the *Rpb1* locus appeared to be independent of the loci *CRb, CRk* and *Crr3* mapped earlier (Hirai *et al.*, 2004; Piao *et al*., 2004; Saito *et al.*, 2006; Sakamoto *et al*., 2008), but very close to that of CRa (Ueno *et al*. 2012) or CRb revealed in a new study (Kato *et al.* 2013).

Fig. 1. Segregation of the F1 population between susceptible *Brassica rapa* DH line ACDC (top) × resistant JNC.

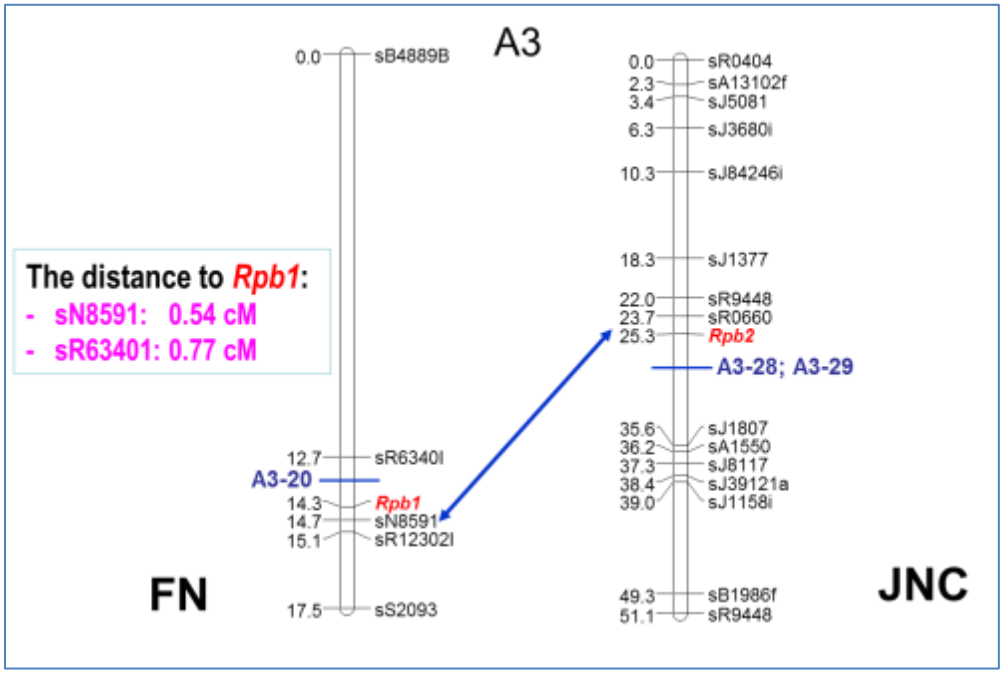

Fig. 2. Mapping of clubroot resistance genes (*Rph1* and *Rpb2*) in FN and JNC (*B. rapa*).

Because clubroot resistance was not found originally in oriental Brassica vegetables, CR genes from European fodder turnip, including cvs. Gelria R, Siloga, Debra, and Milan White (Hirai *et al*., 2006), have been introgressed into Chinese cabbage through breeding efforts. Among those CR genes mapped in the linkage group A3, CRa and CRb were from the turnip lines ECD-02 (Matsumoto *et al*., 2005) and ECD-01 (Piao *et al*., 2004), respectively. Crr3 was from the cv. Milan White (Hirai *et al*., 2004; Saito *et al.*, 2006), and CRk from the cv. Debra (Sakamoto et al., 2008). Previously, these four CR genes were thought to be independent. Markers for *Crr3* were near the QTL region of CRk but additional investigation did not prove these CR alleles are really present at these QTL loci (Sakamoto et al., 2008). However, a genotype of *CRa* closest marker clearly matched those of *CRb* (Kato *et al*. 2013). *Rpb1* described in the current study was derived from the pak choy cv. Flower Nabana (*B. rapa* var. *chinensis*) but the origin of CR gene was not known. Based on the mapping locations, it appears that *CRa, CRb*, and *Rpb1* can be the same gene. It is also noteworthy that *CRb* is also effective against *Pb* pathotype 3 (Kato *et al.* 2013).

In general, MAS was effective in identifying *Rpb1* in resistant BC_1 plants, with sN8591being correct at 100% in both *B. napus* and *B. rapa* populations (Table 2). The marker sR6340I showed some errors, especially on *B. napus* samples, a circumstance possibly attributable to its further distance to *Rpb1* than the marker sN8591. It is unreliable to use sR6340I independently but this marker may be used in conjunction with sN8591 to maximize the accuracy of MAS. With this tool, introgression of *Rpb1* into five *B. napus* and one *B. rapa* canola breeding lines is progressing rapidly.

Table 2. Marker-assisted selection of *Brassica napus* and *B. rapa* plants carrying the *Rpb1* gene in backcross populations for resistance *to P. brassicae* pathotype 3.

Molecular markers	No. plants	Phenotyping		Genotyping R plants		Genotyping S plants	
		R[a]	S[a]	+ marker allele	- marker allele	+ marker allele	- marker allele
BC_1 (*B. napus*)[b]							
sN8591	144	76	68	75	1	0	68
sR6340I	127	65	62	64	1	2	60
BC_1 (*B. rapa*)[b]							
sN8591	144	71	73	69	2	0	73
sR6340I	144	71	73	69	2	0	73

[a] Plant showing resistant (R) and susceptible (S) reactions, respectively.
[b] BC_1 populations for *B. napus* × (*B. napus* × FN) and *B. rapa* × (*B. rapa* × FN), respectively.

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Supplementary tables (S1 to S6)

Resistance of *Brassica* spp. to clubroot (*Plasmodiophora brassicae*, the pathotype 3) in screening trials. The clubroot reduction (%) was based on the comparison of average clubroot severity of each entry (n = 7) with that of a susceptible canola cultivar (Fortune® RR) used in each trial.

Source of germplasm:

1. AAAS: Anhui Academy of Agriculture Science, No. 40 South Road, Hefei, Anhui, China
2. AAFC-PGRC: Agriculture and Agri-Food Canada, Plant Gene Resources of Canada, 107 Science Place, Saskatoon, Saskatchewan S7N 0X2, Canada
3. AAFC-SRC: Agriculture and Agri-Food Canada, Saskatoon Research Centre, 107 Science Place, Saskatoon, Saskatchewan S7N 0X2, Canada
4. Bajo Seeds: Bajo Seeds Inc., 1972 Silver Spur Place, Oceano, California, USA 9344
5. IPK: Leibniz Institute of Plant Genetics and Crop Plant Research, OT Gatersleben, Corrensstrasse 3, D-06466 Stadt Seeland, Germany.
6. SAAS –Sichuan Academy of Agriculture Science, 20 Jing-Ju-Si Road, Chengdu, Sichuan, China
7. Syngenta Seeds: Syngenta Seeds UK Ltd. CPC4, Capital Park, Fulbourn, Cambridge CB21 5XE, UK (provided by Dr. M.R. McDonald, Department of Plant Agriculture, University of Guelph, Guelph, Ontario, Canada)
8. Viterra: Viterra Inc., Research and Development, 201- 407 Downey Road, Saskatoon, Saskatchewan S7N 4L8, Canada

Table S1. Resistance of *Brassica napus*.

Germplasm	Source of collection	Clubroot reduction (%)	Germplasm	Source of collection	Clubroot reduction (%)
Askegarde	Viterra	100 [a]	CN44110	AAFC-PGRC	0
SW 02763	Viterra	78 [a]	CN44113	AAFC-PGRC	0
SW 98323030	Viterra	50 [a]	CN44119	AAFC-PGRC	0
Wilhelmsberg	HRI, UK	44	CN44122	AAFC-PGRC	0
CN44116	AAFC-PGRC	26	CN44123	AAFC-PGRC	0
York	Viterra	26	CN44125	AAFC-PGRC	0
CN4411	AAFC-PGRC	25	CN44126	AAFC-PGRC	0
Deyou 8	AAAS	20	CN44128	AAFC-PGRC	0
Krashodarskij	AAFC-PGRC	20	CN44131	AAFC-PGRC	0
CN44096	AAFC-PGRC	17	Deyou 5	AAAS	0
Dichotoma	AAFC-PGRC	16	Dwarf Essex Rape	Viterra	0
Tosca	Viterra	16	Echo	AAFC-PGRC	0
CN44127	AAFC-PGRC	14	Green Oil #9	AAFC-PGRC	0
CN44133	AAFC-PGRC	11	Heyou 202	AAAS	0
CN44105	AAFC-PGRC	10	Heyou 46	AAAS	0
CN44097	AAFC-PGRC	7	Heyou 56	AAAS	0
CN44112	AAFC-PGRC	7	Hu You 17	AAAS	0
CN44132	AAFC-PGRC	7	Huaxie 102	AAAS	0
Qinyou 10	AAAS	6	Ke Yuan You 1	SAAS	0
Qinyou 11	AAAS	6	Ke Yuan You 2	SAAS	0
Toria TL15	AAFC-PGRC	6	Latvijskij 6	AAFC-PGRC	0
TS-72	AAFC-PGRC	6	Laurentian Golden	Viterra	0
Wanyou 23	AAAS	6	Nemercanskij 2268	AAFC-PGRC	0
Wanyou 29	AAAS	6	Polar	AAFC-PGRC	0
Candle	AAFC-PGRC	5	Red Russian	Viterra	0
CN44124	AAFC-PGRC	4	Rongyou 11	SAAS	0
Saron	AAFC-PGRC	3	Span	AAFC-PGRC	0
09F8030	AAAS	0	SW 02763	Viterra	0
09F8031	AAAS	0	SW 98323030	Viterra	0
09F9089	AAAS	0	Tianhe 98	AAAS	0
09FR05	AAAS	0	Topaz	AAFC-SRC	0
American Purple Top	Viterra	0	Torch	AAFC-PGRC	0
Arlo	AAFC-PGRC	0	Toria Itsa	AAFC-PGRC	0
Askegarde	Viterra	0	Toria TLC1	AAFC-PGRC	0
Canaspan	AAFC-PGRC	0	Tsao Yutunri	AAFC-PGRC	0
Chuan You 21	SAAS	0	Vinnickij 15/59	AAFC-PGRC	0
Chuan You 22	SAAS	0	Vinnickij 21	AAFC-PGRC	0
Chuheza1	SAAS	0	Wanyou 13	AAAS	0
Chuheza2	SAAS	0	Wanyou 14	AAAS	0
CN44098	AAFC-PGRC	0	Wanyou 15	AAAS	0
CN44099	AAFC-PGRC	0	Wanyou 16	AAAS	0
CN44100	AAFC-PGRC	0	Wanyou 18	AAAS	0
CN44101	AAFC-PGRC	0	Wanyou 20	AAAS	0
CN44102	AAFC-PGRC	0	Wanyou 25	AAAS	0
CN44103	AAFC-PGRC	0	WIR 4170	AAFC-PGRC	0
CN44104	AAFC-PGRC	0	Yellowish #1	AAFC-PGRC	0
CN44106	AAFC-PGRC	0	Yuchoy	Viterra	0
CN44107	AAFC-PGRC	0	Zhe Ping 4	AAAS	0
CN44109	AAFC-PGRC	0			

[a] Inoculated with the pathotype 2 of *P. brassicae.*

Table S2. Resistance of *B. rapa*.

Germplasm	Source of collection	Clubroot reduction (%)	Germplasm	Source of collection	Clubroot reduction (%)
96-6991	AAFC-SRC	100	SRS 3432	AAFC-PGRC	25
96-6992	AAFC-SRC	100	SRS 3505	AAFC-PGRC	25
Bejo 2833	Bejo Seeds	100	SRS 3533	AAFC-PGRC	25
Bilko	Bejo Seeds	100	K-110	AAFC-PGRC	24
Emiko	Bejo Seeds	100	PAK 85547	AAFC-PGRC	24
Flower Nabana	Viterra	100	SRS 1030	AAFC-PGRC	24
Jazz Napa Cabbage	Viterra	100	Waido (mizuna)	Viterra	24
Taronda	AAFC-PGRC	100	SRS 3447	AAFC-PGRC	23
Vedette	AAFC-PGRC	100	Muzuna Greenstreak	Viterra	22
Vollenda	AAFC-PGRC	100	PAK 85649A	AAFC-PGRC	22
96-6990	AAFC-SRC	93	Pakchoi -Joi choi	Viterra	22
Siloga	AAFC-PGRC	92	Royal crown	Viterra	22
SRS 3464	AAFC-PGRC	82	Tokyo Bekana	Viterra	22
SRS 3426	AAFC-PGRC	81	BS113 (ARL)	Viterra	21
Nozawana	Viterra	81	Mibuna Green Spray	Viterra	20
96-6993	AAFC-SRC	77	SRS 3497	AAFC-PGRC	20
T38	AAFC-SRC	77	97-7225	AAFC-SRC	19
T35	AAFC-SRC	70	K-949	AAFC-PGRC	19
SRS 2102	AAFC-PGRC	61	Pingba Tian Youcai	AAFC-PGRC	19
T49	AAFC-SRC	57	Siao Baje Tacaj	AAFC-PGRC	19
PYS-6	AAFC-PGRC	56	SRS 1034	AAFC-PGRC	19
96-6994	AAFC-SRC	56	SRS 1063	AAFC-PGRC	19
Y.S.M.	Viterra	54	SRS 3327	AAFC-PGRC	19
L-141P2	Viterra	54	SRS 3425	AAFC-PGRC	19
Purple Top (forage turnip)	Viterra	52	SRS 3460	AAFC-PGRC	18
SRS 1048	AAFC-PGRC	50	T63	AAFC-SRC	18
22398-6 Sylvestors	AAFC-SRC	48	K-947	AAFC-PGRC	17
96-6989	AAFC-SRC	48	Opava	AAFC-PGRC	17
Feng Quing Choi	Viterra	48	Purple Mizuna	Viterra	17
White Globe (vege turnip)	Viterra	47	Spectrum	AAFC-PGRC	17
SRS 3454	AAFC-PGRC	46	SRS 1768	AAFC-PGRC	17
Shuka -Autumn Torch	Viterra	44	SRS 3341	AAFC-PGRC	17
SRS 3339	AAFC-PGRC	44	SRS 961	AAFC-PGRC	17
PAK 85530	AAFC-PGRC	40	Michihli	AAFC-PGRC	16
SRS 3452	AAFC-PGRC	39	SRS 1021	AAFC-PGRC	16
K-946	AAFC-PGRC	38	SRS 3476	AAFC-PGRC	15
Y-S-L-5	AAFC-PGRC	38	SRS 3485	AAFC-PGRC	15
PAK 85538	AAFC-PGRC	37	SRS 3526	AAFC-PGRC	15
SRS 3461	AAFC-PGRC	36	SRS 3528	AAFC-PGRC	15
SRS 3467	AAFC-PGRC	36	Tsoi-Sim	Viterra	15
SRS 3469	AAFC-PGRC	36	C.B. -1	Viterra	14
SRS 3480	AAFC-PGRC	35	K-927	AAFC-PGRC	14
PAK 85531	AAFC-PGRC	25	K-932	AAFC-PGRC	14
SRS 2372	AAFC-PGRC	25	K-934	AAFC-PGRC	14
SRS 3424	AAFC-PGRC	25	SRS 1028	AAFC-PGRC	14

Table S2. Resistance of *B. rapa* cont.

Germplasm	Source of collection	Clubroot reduction (%)	Germplasm	Source of collection	Clubroot reduction (%)
SRS 3431	AAFC-PGRC	14	SRS 1047	AAFC-PGRC	10
Luodian Xiao Youcai	AAFC-PGRC	13	SRS 3329	AAFC-PGRC	10
96-6982	AAFC-SRC	13	SRS 3331	AAFC-PGRC	10
96-6988	AAFC-SRC	13	SRS 3488	AAFC-PGRC	10
CB 9948	AAFC-PGRC	13	SRS 3499	AAFC-PGRC	10
K-41	AAFC-PGRC	13	SRS 3501	AAFC-PGRC	10
Nepalganj CL-3	Viterra	13	SRS 3502	AAFC-PGRC	10
PAK 85522	AAFC-PGRC	13	SRS 3503	AAFC-PGRC	10
PAK 85530	AAFC-PGRC	13	SRS 3509	AAFC-PGRC	10
PAK 85651	AAFC-PGRC	13	SRS 3527	AAFC-PGRC	10
PI 340193	AAFC-PGRC	13	Tendergreen	Viterra	9
PI 340196	AAFC-PGRC	13	SRS 3473	AAFC-PGRC	9
PI 340201	AAFC-PGRC	13	SRS 3474	AAFC-PGRC	9
Saturn	AAFC-PGRC	13	SRS 3530	AAFC-PGRC	9
SRS 3378	AAFC-PGRC	13	SRS 3444	AAFC-PGRC	8
SRS 3430	AAFC-PGRC	13	SRS 3448	AAFC-PGRC	8
SRS 3525	AAFC-PGRC	13	YC-20	Viterra	8
TC-40	Viterra	13	PAK 85549	AAFC-PGRC	7
TC-50	Viterra	13	96-6990	AAFC-SRC	7
IB 1515	AAFC-PGRC	12	Dushan Youbaicai	AAFC-PGRC	7
SRS 415	AAFC-PGRC	12	PAK 85649B	AAFC-PGRC	7
CN19061	AAFC-PGRC	11	Palle	AAFC-PGRC	7
PAK 85501	AAFC-PGRC	11	PI 340200	AAFC-PGRC	7
PAK 85539	AAFC-PGRC	11	SRS 3433	AAFC-PGRC	7
PAK 85540	AAFC-PGRC	11	SRS 3438	AAFC-PGRC	7
SevenTop	Viterra	11	SRS 3442	AAFC-PGRC	7
SRS 3338	AAFC-PGRC	11	SRS 390	AAFC-PGRC	7
SRS 3344	AAFC-PGRC	11	SRS 944	AAFC-PGRC	7
SRS 3349	AAFC-PGRC	11	AC Sunshine	AAFC-PGRC	6
SRS 3354	AAFC-PGRC	11	CN19046	AAFC-PGRC	6
SRS 3362	AAFC-PGRC	11	PAK 85513	AAFC-PGRC	6
SRS 3459	AAFC-PGRC	11	PI 340190	AAFC-PGRC	6
Hee Ju Ta Cai	AAFC-PGRC	10	Qing 13	AAFC-PGRC	6
97-7163	AAFC-SRC	10	Quinyou #15	AAFC-PGRC	6
AC Sunbeam	AAFC-PGRC	10	SRS 1046	AAFC-PGRC	6
Huahong Caizi 1	AAFC-PGRC	10	SRS 1049	AAFC-PGRC	6
K-1033	AAFC-PGRC	10	SRS 3337	AAFC-PGRC	6
K-1081	AAFC-PGRC	10	SRS 3340	AAFC-PGRC	6
K-458	AAFC-PGRC	10	SRS 3342	AAFC-PGRC	6
K-511	AAFC-PGRC	10	SRS 3365	AAFC-PGRC	6
K-676	AAFC-PGRC	10	SRS 3395	AAFC-PGRC	6
K-749	AAFC-PGRC	10	SRS 3411	AAFC-PGRC	6
K-773	AAFC-PGRC	10	SRS 948	AAFC-PGRC	6
K-829	AAFC-PGRC	10	SRS 953	AAFC-PGRC	6
PAK 85550	AAFC-PGRC	10	SRS 954	AAFC-PGRC	6
PI 264139	AAFC-PGRC	10	SRS 963	AAFC-PGRC	6
PI 340222	AAFC-PGRC	10	Taixian Youcai	AAFC-PGRC	6
SRS 1039	AAFC-PGRC	10	Xionghua	AAFC-PGRC	6

Table S2. Resistance of *B. rapa* cont.

Germplasm	Source of collection	Clubroot reduction (%)	Germplasm	Source of collection	Clubroot reduction (%)
Zunyi Zao Hei Youcai	AAFC-PGRC	6	93-6777	AAFC-SRC	0
B-12-68	AAFC-PGRC	5	93-6778	AAFC-SRC	0
CB 9946	AAFC-PGRC	5	96-6998	AAFC-SRC	0
Dafang Fugi Youcai	AAFC-PGRC	5	97-7223	AAFC-SRC	0
Hezhang Xiaobai Youcai	AAFC-PGRC	5	97-7224	AAFC-SRC	0
JinYou 6	AAFC-SRC	5	97-7226	AAFC-SRC	0
K-1032	AAFC-PGRC	5	AC Parkland	AAFC-PGRC	0
K-16	AAFC-PGRC	5	AC Sungold	AAFC-PGRC	0
K-384	AAFC-PGRC	5	ACDC10	AAFC-SRC	0
K-673	AAFC-PGRC	5	Agena	AAFC-PGRC	0
K-698	AAFC-PGRC	5	Agrani	AAFC-PGRC	0
K-788	AAFC-PGRC	5	Anlong Aijiao Youcai	AAFC-PGRC	0
K-833	AAFC-PGRC	5	Anshun Tian Youcai 1	AAFC-PGRC	0
K-890	AAFC-PGRC	5	B.A.S	AAFC-PGRC	0
K-900	AAFC-PGRC	5	B.C. -64	AAFC-PGRC	0
K-909	AAFC-PGRC	5	Baiyou #1	AAFC-PGRC	0
K-933	AAFC-PGRC	5	Bal Toria	AAFC-PGRC	0
K-940	AAFC-PGRC	5	Bhind	AAFC-PGRC	0
K-955	AAFC-PGRC	5	Bijie Xiao Youcai 1	AAFC-PGRC	0
K-957	AAFC-PGRC	5	Brown Sarsn sel. A	AAFC-PGRC	0
Klondike	AAFC-PGRC	5	C7P4-1-1	AAFC-PGRC	0
PAK 85535	AAFC-PGRC	5	CB 9606	AAFC-PGRC	0
PAK 85652	AAFC-PGRC	5	CB 9625	AAFC-PGRC	0
PI 179182	AAFC-PGRC	5	CB 9947	AAFC-PGRC	0
PI 340209	AAFC-PGRC	5	Ceska Krajova	AAFC-PGRC	0
SRS 1016	AAFC-PGRC	5	Ching Choy Sum	AAFC-PGRC	0
SRS 1037	AAFC-PGRC	5	Civastro R	AAFC-PGRC	0
SRS 1040	AAFC-PGRC	5	CN 107761	AAFC-PGRC	0
SRS 3478	AAFC-PGRC	5	CN 107762	AAFC-PGRC	0
SRS 3495	AAFC-PGRC	5	CN 107770	AAFC-PGRC	0
SRS 3529	AAFC-PGRC	5	CN 107771	AAFC-PGRC	0
SRS 3531	AAFC-PGRC	5	CN 107772	AAFC-PGRC	0
SRS 3535	AAFC-PGRC	5	CN101949	AAFC-PGRC	0
PAK 85486	AAFC-PGRC	4	CN101951	AAFC-PGRC	0
SRS 1014	AAFC-PGRC	4	CN105400	AAFC-PGRC	0
Wangmo Ai Youcai	AAFC-PGRC	4	CN19045	AAFC-PGRC	0
Horizon	AAFC-PGRC	3	CN19060	AAFC-PGRC	0
Guiyang Bai Youcai	AAFC-PGRC	3	CN35408	AAFC-PGRC	0
Huangxinwu	AAFC-SRC	3	CN36092	AAFC-PGRC	0
PAK 85552	AAFC-PGRC	3	CN44108	AAFC-PGRC	0
SRS 3541	AAFC-PGRC	3	CN44108	AAFC-PGRC	0
SRS 3455	AAFC-PGRC	1	CPI 156418	Viterra	0
96-6993	AAFC-SRC	1	Dafang Heibaicai	AAFC-PGRC	0
214-3-8	Viterra	0	Dejiang Bai Youcai 1	AAFC-PGRC	0
4-7-7	Viterra	0	Dhali	AAFC-PGRC	0
(Toria A)	AAFC-PGRC	0	Dijie Chuan Baicai	AAFC-PGRC	0
1007	AAFC-PGRC	0	Dong Du	AAFC-PGRC	0
93-6750	AAFC-SRC	0	Doudi Youcai	AAFC-PGRC	0

Table S2. Resistance of *B. rapa* cont.

Germplasm	Source of collection	Clubroot reduction (%)	Germplasm	Source of collection	Clubroot reduction (%)
DS 42/179	Viterra	0	K-648	AAFC-PGRC	0
DS-17M	AAFC-PGRC	0	K-652	AAFC-PGRC	0
Dunyun Youcai	AAFC-PGRC	0	K-660	AAFC-PGRC	0
Esko	AAFC-PGRC	0	K-662	AAFC-PGRC	0
Greenwich Michihli	Viterra	0	K-674	AAFC-PGRC	0
Guanling Tian Youcai	AAFC-PGRC	0	K-693	AAFC-PGRC	0
Hankkija's simeoni	AAFC-PGRC	0	K-694	AAFC-PGRC	0
Heixinwu	AAFC-SRC	0	K-697	AAFC-PGRC	0
Hezhang Bendi Youcai	AAFC-PGRC	0	K-752	AAFC-PGRC	0
Hezhang Ziaohei Youcai	AAFC-PGRC	0	K-774	AAFC-PGRC	0
Hongbo Youcai	AAFC-PGRC	0	K-794	AAFC-PGRC	0
Hontsai Tai	Viterra	0	K-803	AAFC-PGRC	0
Huangping Bai Youcai	AAFC-PGRC	0	K-804	AAFC-PGRC	0
Hubei Youcai	AAFC-PGRC	0	K-815	AAFC-PGRC	0
IB 126	AAFC-PGRC	0	K-843	AAFC-PGRC	0
IB 129	AAFC-PGRC	0	K-868	AAFC-PGRC	0
IB 132	AAFC-PGRC	0	K-879	AAFC-PGRC	0
IB 133	AAFC-PGRC	0	K-880	AAFC-PGRC	0
IB 134	AAFC-PGRC	0	K-884	AAFC-PGRC	0
IB 1454	AAFC-PGRC	0	K-888	AAFC-PGRC	0
IB 1456	AAFC-PGRC	0	K-889	AAFC-PGRC	0
IB 1509	AAFC-PGRC	0	K-894	AAFC-PGRC	0
IB 1516	AAFC-PGRC	0	K-896	AAFC-PGRC	0
IB 1750	Viterra	0	K-903	AAFC-PGRC	0
IB 55	Viterra	0	K-905	AAFC-PGRC	0
Jancaj	AAFC-PGRC	0	K-910	AAFC-PGRC	0
Jiangkou Dahua Youcai 2	AAFC-PGRC	0	K-915	AAFC-PGRC	0
Jinsha Xiao Caizi	AAFC-PGRC	0	K-919	AAFC-PGRC	0
Jinsha Xiao Youcai	AAFC-PGRC	0	K-94	AAFC-PGRC	0
Jinsha Xiaobaicai	AAFC-PGRC	0	K-942	AAFC-PGRC	0
K-100	AAFC-PGRC	0	K-953	AAFC-PGRC	0
K-1016	AAFC-PGRC	0	K-956	AAFC-PGRC	0
K-1071	AAFC-PGRC	0	K-963	AAFC-PGRC	0
K-1075	AAFC-PGRC	0	K-968	AAFC-PGRC	0
K-17	AAFC-PGRC	0	Kaiyang Ai Youcai	AAFC-PGRC	0
K-366	AAFC-PGRC	0	Kal Toria	AAFC-PGRC	0
K-374	AAFC-PGRC	0	Kelta	AAFC-PGRC	0
K-408	AAFC-PGRC	0	Komatsuna	Viterra	0
K-459	AAFC-PGRC	0	Kosaitai	Viterra	0
K-460	AAFC-PGRC	0	Kurikara	AAFC-PGRC	0
K-529	AAFC-PGRC	0	Kyona (Mizuna)	Viterra	0
K-549	AAFC-PGRC	0	L-145P5	Viterra	0
K-569	AAFC-PGRC	0	Leega	AAFC-PGRC	0
K-571	AAFC-PGRC	0	Leielander	AAFC-PGRC	0
K-589	AAFC-PGRC	0	Loa Tsai	AAFC-PGRC	0
K-600	AAFC-PGRC	0	Longevity hybrid	Viterra	0
K-608	AAFC-PGRC	0	Maleksberger	AAFC-PGRC	0
K-645	AAFC-PGRC	0	Manping	AAFC-PGRC	0

Table S2. Resistance of *B. rapa* cont.

Germplasm	Source of collection	Clubroot reduction (%)	Germplasm	Source of collection	Clubroot reduction (%)
Marco	AAFC-PGRC	0	PAK 85551	AAFC-PGRC	0
Matjeslander	AAFC-PGRC	0	PAK 85554	AAFC-PGRC	0
Mengou #3	AAFC-PGRC	0	PAK 85650B	AAFC-PGRC	0
Mengyou #7	AAFC-PGRC	0	PI 251240	AAFC-PGRC	0
Mibuna	AAFC-PGRC	0	PI 312846	AAFC-PGRC	0
Michihli	Viterra	0	PI 340194	AAFC-PGRC	0
Milanskaja Belaja	AAFC-PGRC	0	PI 340195	AAFC-PGRC	0
Mizuna	AAFC-PGRC	0	PI 432364	AAFC-PGRC	0
Mosa	AAFC-PGRC	0	PI 432365	AAFC-PGRC	0
Muskovskij	AAFC-PGRC	0	PI 432366	AAFC-PGRC	0
Mutjeslander	AAFC-PGRC	0	PI 432369	AAFC-PGRC	0
Namanganskaja	AAFC-PGRC	0	PI 432370	AAFC-PGRC	0
Nikko	Bejo Seeds	0	PI 432374	AAFC-PGRC	0
Nobitter R	AAFC-PGRC	0	PI 432375	AAFC-PGRC	0
Noko	AAFC-PGRC	0	PI 432375	AAFC-PGRC	0
Nozaki Early	AAFC-PGRC	0	PI 432376	AAFC-PGRC	0
ORE 74-191	AAFC-PGRC	0	Pingba Bai Youcai	AAFC-PGRC	0
ORE 75-207	AAFC-PGRC	0	Pingba Youcai	AAFC-PGRC	0
ORE 76-225	AAFC-PGRC	0	Ponda	AAFC-PGRC	0
Oregon Sel II	AAFC-PGRC	0	Primax	AAFC-PGRC	0
Oregon Sel. 1	AAFC-PGRC	0	PT-303	AAFC-PGRC	0
Pachuca	AAFC-PGRC	0	PT-303	AAFC-PGRC	0
PAK 85379	AAFC-PGRC	0	PT-507	AAFC-PGRC	0
PAK 85384	AAFC-PGRC	0	Puebla #2021	AAFC-PGRC	0
PAK 85397	AAFC-PGRC	0	Puebla #2025	AAFC-PGRC	0
PAK 85485	AAFC-PGRC	0	Qianxi Hei Caizi	AAFC-PGRC	0
PAK 85494	AAFC-PGRC	0	Qing You #5	AAFC-PGRC	0
PAK 85496	AAFC-PGRC	0	Quinyou N.5	AAFC-PGRC	0
PAK 85497	AAFC-PGRC	0	Quinyou N.7	AAFC-PGRC	0
PAK 85498	AAFC-PGRC	0	Quinyou No.11	AAFC-PGRC	0
PAK 85499	AAFC-PGRC	0	Quinyou No.9	AAFC-PGRC	0
PAK 85502	AAFC-PGRC	0	R-500	Unkown	0
PAK 85503	AAFC-PGRC	0	Reward	AAFC-PGRC	0
PAK 85504	AAFC-PGRC	0	Rongjiang Ai Youcai	AAFC-PGRC	0
PAK 85505	AAFC-PGRC	0	Rongjiang Ai Youcai	AAFC-PGRC	0
PAK 85508	AAFC-PGRC	0	Salusia	AAFC-PGRC	0
PAK 85510	AAFC-PGRC	0	San' Jusmancincaj	AAFC-PGRC	0
PAK 85512	AAFC-PGRC	0	Sansui Youcai	AAFC-PGRC	0
PAK 85514	AAFC-PGRC	0	Shigian Bai Youcai	AAFC-PGRC	0
PAK 85532	AAFC-PGRC	0	Siloza Kweekbedrijk	AAFC-PGRC	0
PAK 85533	AAFC-PGRC	0	Sinan bai youcai	AAFC-PGRC	0
PAK 85536	AAFC-PGRC	0	Soup-spoon	AAFC-PGRC	0
PAK 85541	AAFC-PGRC	0	SRS 1013	AAFC-PGRC	0
PAK 85542	AAFC-PGRC	0	SRS 1015	AAFC-PGRC	0
PAK 85543	AAFC-PGRC	0	SRS 1017	AAFC-PGRC	0
PAK 85544	AAFC-PGRC	0	SRS 1018	AAFC-PGRC	0
PAK 85546	AAFC-PGRC	0	SRS 1019	AAFC-PGRC	0
PAK 85548	AAFC-PGRC	0	SRS 1020	AAFC-PGRC	0

Table S2. Resistance of *B. rapa* cont.

Germplasm	Source of collection	Clubroot reduction (%)	Germplasm	Source of collection	Clubroot reduction (%)
SRS 1022	AAFC-PGRC	0	SRS 3385	AAFC-PGRC	0
SRS 1023	AAFC-PGRC	0	SRS 3386	AAFC-PGRC	0
SRS 1026	AAFC-PGRC	0	SRS 3387	AAFC-PGRC	0
SRS 1027	AAFC-PGRC	0	SRS 3388	AAFC-PGRC	0
SRS 1029	AAFC-PGRC	0	SRS 3389	AAFC-PGRC	0
SRS 1031	AAFC-PGRC	0	SRS 3390	AAFC-PGRC	0
SRS 1033	AAFC-PGRC	0	SRS 3393	AAFC-PGRC	0
SRS 2103	AAFC-PGRC	0	SRS 3394	AAFC-PGRC	0
SRS 2307	AAFC-PGRC	0	SRS 3396	AAFC-PGRC	0
SRS 328	AAFC-PGRC	0	SRS 3397	AAFC-PGRC	0
SRS 3326	AAFC-PGRC	0	SRS 3398	AAFC-PGRC	0
SRS 3328	AAFC-PGRC	0	SRS 3399	AAFC-PGRC	0
SRS 3333	AAFC-PGRC	0	SRS 3400	AAFC-PGRC	0
SRS 3334	AAFC-PGRC	0	SRS 3401	AAFC-PGRC	0
SRS 3335	AAFC-PGRC	0	SRS 3402	AAFC-PGRC	0
SRS 3336	AAFC-PGRC	0	SRS 3404	AAFC-PGRC	0
SRS 3343	AAFC-PGRC	0	SRS 3405	AAFC-PGRC	0
SRS 3345	AAFC-PGRC	0	SRS 3406	AAFC-PGRC	0
SRS 3346	AAFC-PGRC	0	SRS 3407	AAFC-PGRC	0
SRS 3347	AAFC-PGRC	0	SRS 3408	AAFC-PGRC	0
SRS 3348	AAFC-PGRC	0	SRS 3409	AAFC-PGRC	0
SRS 3350	AAFC-PGRC	0	SRS 3410	AAFC-PGRC	0
SRS 3351	AAFC-PGRC	0	SRS 3412	AAFC-PGRC	0
SRS 3352	AAFC-PGRC	0	SRS 3413	AAFC-PGRC	0
SRS 3353	AAFC-PGRC	0	SRS 3414	AAFC-PGRC	0
SRS 3355	AAFC-PGRC	0	SRS 3415	AAFC-PGRC	0
SRS 3356	AAFC-PGRC	0	SRS 3416	AAFC-PGRC	0
SRS 3357	AAFC-PGRC	0	SRS 3427	AAFC-PGRC	0
SRS 3358	AAFC-PGRC	0	SRS 3428	AAFC-PGRC	0
SRS 3359	AAFC-PGRC	0	SRS 3429	AAFC-PGRC	0
SRS 3360	AAFC-PGRC	0	SRS 3434	AAFC-PGRC	0
SRS 3361	AAFC-PGRC	0	SRS 3435	AAFC-PGRC	0
SRS 3364	AAFC-PGRC	0	SRS 3436	AAFC-PGRC	0
SRS 3366	AAFC-PGRC	0	SRS 3439	AAFC-PGRC	0
SRS 3367	AAFC-PGRC	0	SRS 3440	AAFC-PGRC	0
SRS 3369	AAFC-PGRC	0	SRS 3441	AAFC-PGRC	0
SRS 3370	AAFC-PGRC	0	SRS 3443	AAFC-PGRC	0
SRS 3371	AAFC-PGRC	0	SRS 3445	AAFC-PGRC	0
SRS 3372	AAFC-PGRC	0	SRS 3449	AAFC-PGRC	0
SRS 3373	AAFC-PGRC	0	SRS 3450	AAFC-PGRC	0
SRS 3375	AAFC-PGRC	0	SRS 3453	AAFC-PGRC	0
SRS 3376	AAFC-PGRC	0	SRS 3456	AAFC-PGRC	0
SRS 3377	AAFC-PGRC	0	SRS 3457	AAFC-PGRC	0
SRS 3379	AAFC-PGRC	0	SRS 3462	AAFC-PGRC	0
SRS 3380	AAFC-PGRC	0	SRS 3465	AAFC-PGRC	0
SRS 3381	AAFC-PGRC	0	SRS 3466	AAFC-PGRC	0
SRS 3382	AAFC-PGRC	0	SRS 3468	AAFC-PGRC	0
SRS 3383	AAFC-PGRC	0	SRS 3471	AAFC-PGRC	0
SRS 3384	AAFC-PGRC	0	SRS 3472	AAFC-PGRC	0

Table S2. Resistance of *B. rapa* cont.

Germplasm	Source of collection	Clubroot reduction (%)	Germplasm	Source of collection	Clubroot reduction (%)
SRS 3475	AAFC-PGRC	0	SRS 958	AAFC-PGRC	0
SRS 3477	AAFC-PGRC	0	SRS 959	AAFC-PGRC	0
SRS 3479	AAFC-PGRC	0	SRS 960	AAFC-PGRC	0
SRS 3481	AAFC-PGRC	0	SRS 964	AAFC-PGRC	0
SRS 3482	AAFC-PGRC	0	SRS 965	AAFC-PGRC	0
SRS 3483	AAFC-PGRC	0	SRS 966	AAFC-PGRC	0
SRS 3484	AAFC-PGRC	0	SRS 967	AAFC-PGRC	0
SRS 3486	AAFC-PGRC	0	SRS 968	AAFC-PGRC	0
SRS 3487	AAFC-PGRC	0	SRS 969	AAFC-PGRC	0
SRS 3489	AAFC-PGRC	0	SRS 971	AAFC-PGRC	0
SRS 3490	AAFC-PGRC	0	SRS 973	AAFC-PGRC	0
SRS 3498	AAFC-PGRC	0	SRS 974	AAFC-PGRC	0
SRS 3504	AAFC-PGRC	0	SRS 979	AAFC-PGRC	0
SRS 3506	AAFC-PGRC	0	SRS 980	AAFC-PGRC	0
SRS 3507	AAFC-PGRC	0	SRS 982	AAFC-PGRC	0
SRS 3508	AAFC-PGRC	0	SRS 984	AAFC-PGRC	0
SRS 3510	AAFC-PGRC	0	Suehlihung #2	Viterra	0
SRS 3511	AAFC-PGRC	0	Summer Jean	Viterra	0
SRS 3512	AAFC-PGRC	0	Sylvestris	AAFC-SRC	0
SRS 3513	AAFC-PGRC	0	Szczecinski	AAFC-PGRC	0
SRS 3514	AAFC-PGRC	0	T-4	AAFC-PGRC	0
SRS 3515	AAFC-PGRC	0	T-8	AAFC-PGRC	0
SRS 3516	AAFC-PGRC	0	T-9	AAFC-PGRC	0
SRS 3534	AAFC-PGRC	0	Taisai	AAFC-PGRC	0
SRS 3536	AAFC-PGRC	0	Tatsoi	Viterra	0
SRS 3537	AAFC-PGRC	0	TC-100	Viterra	0
SRS 3538	AAFC-PGRC	0	TC-36	Viterra	0
SRS 3539	AAFC-PGRC	0	TC-74	Viterra	0
SRS 3540	AAFC-PGRC	0	Texi	AAFC-PGRC	0
SRS 3542	AAFC-PGRC	0	Tian Youcai	AAFC-PGRC	0
SRS 3543	AAFC-PGRC	0	Tlaxcala #7	AAFC-PGRC	0
SRS 3545	AAFC-PGRC	0	Tobin	AAFC-PGRC	0
SRS 3546	AAFC-PGRC	0	Tongzi Hei Caizi 2	AAFC-PGRC	0
SRS 3547	AAFC-PGRC	0	Toria (P2-13-74)	AAFC-PGRC	0
SRS 3548	AAFC-PGRC	0	Toria (SR2)	AAFC-PGRC	0
SRS 3549	AAFC-PGRC	0	Toria SOS	AAFC-PGRC	0
SRS 3550	AAFC-PGRC	0	Trofee	AAFC-PGRC	0
SRS 3551	AAFC-PGRC	0	TS-29	AAFC-PGRC	0
SRS 388	AAFC-PGRC	0	Tsoi-sim Early Green	AAFC-PGRC	0
SRS 472	AAFC-PGRC	0	Tylor	AAFC-PGRC	0
SRS 589	AAFC-PGRC	0	Valtti	AAFC-PGRC	0
SRS 726	AAFC-PGRC	0	Vitamin Green	Viterra	0
SRS 937	AAFC-PGRC	0	Wasslander	AAFC-PGRC	0
SRS 939	AAFC-PGRC	0	White Lady hibrid	Viterra	0
SRS 945	AAFC-PGRC	0	Wong Bok	AAFC-PGRC	0
SRS 952	AAFC-PGRC	0	Wongun Youcai 3	AAFC-PGRC	0
SRS 955	AAFC-PGRC	0	Xiao Ri Qi	AAFC-PGRC	0
SRS 956	AAFC-PGRC	0	Xiaobaicai	AAFC-SRC	0
SRS 957	AAFC-PGRC	0	Xiuwen Ai Youcai	AAFC-PGRC	0

Table S2. Resistance of *B. rapa* cont.

Germplasm	Source of collection	Clubroot reduction (%)
Y.S. (AND) -189	Viterra	0
YC-213	Viterra	0
YC-232	Viterra	0
YC-26	Viterra	0
YC-3	Viterra	0
YC-49	Viterra	0
YC-79	Viterra	0
Y-S-L-1	AAFC-PGRC	0
YST-151	AAFC-PGRC	0
YST-151	Viterra	0
Yu Tsai Sum	Viterra	0
Yuche Tac Man	AAFC-PGRC	0
Yuquig Dabai Youcai	AAFC-PGRC	0
Yuquing Bendi Hei Youcai	AAFC-PGRC	0
Zhenning Tian Youcai 1	AAFC-PGRC	0
Zhijin Hei Youcai	AAFC-PGRC	0
Zijing Bai Youcai	AAFC-PGRC	0
Zsc 4042	AAFC-PGRC	0
Zunyi Ai Youcai	AAFC-PGRC	0
Zunyi Bai Youcai	AAFC-PGRC	0
Zunyi Hei Youcai	AAFC-PGRC	0
Zunyi Xiao Youcai	AAFC-PGRC	0

Table S3. Resistance of *B.juncea.*

Germplasm	Origin	Source of collection	Clubroot reduction (%)	Germplasm	Origin	Source of collection	Clubroot reduction (%)
Shi-Li-Hon	China	Viterra	48	Ekla	France	Viterra	0
Big Stem	USA	Viterra	47	Gao Chan Huang You Cai	China	AAFC-SRC	0
T.M. 12	Egypt	Viterra	44	Golden Frill	Unknown	Viterra	0
Red-Giant	USA	Viterra	40	Green Wave	Unknown	Viterra	0
Bau-sin	China	Viterra	38	Horned Mustard	Unknown	Viterra	0
Garnet Giant	USA	Viterra	36	Huang Jing You	China	AAFC-SRC	0
San-Ho Gianty Chirimen	China	Viterra	33	Huang you cai-2	China	AAFC-SRC	0
Hakarashi	Unknown	Viterra	32	J072-01904	Canada	Viterra	0
Nan-fong	China	Viterra	28	J072-07013	Canada	Viterra	0
Red Mustard	Unknown	Viterra	28	Kekkyu Tekana	Unknown	Viterra	0
Gai-choi	China	Viterra	24	Ma wei you cai	China	AAFC-SRC	0
Southern Giant	USA	Viterra	24	Miike Giant	Unknown	Viterra	0
Unknown	Ethiopia	Viterra	24	MRS88 01	Turkey	Viterra	0
Hatakena	USA	Viterra	22	MRS88 02	Turkey	Viterra	0
Miike Giant Purple	Unknown	Viterra	22	MRS88 03	Turkey	Viterra	0
Peacock-tail	Unknown	Viterra	19	MRS88 20	Turkey	Viterra	0
Tsunga 4270	Zimbabwe	Viterra	19	MRS88 21	Turkey	Viterra	0
Ho-mi Z (Dragon Tongue)	Unknown	Viterra	17	MRS88 61	Ethiopia	Viterra	0
Yukina Savoy	Unknown	Viterra	17	MRS88 22	Turkey	Viterra	0
Golden Streak	Unknown	Viterra	16	MRS88 335	Morocco	Viterra	0
Wasabina	Unknown	Viterra	16	MRS88 359	Ethiopia	Viterra	0
MRS 90-017	Zimbabwe	Viterra	14	MRS88 377	Ethiopia	Viterra	0
Tsunga 4283	Zimbabwe	Viterra	14	Ndakapulca	Zimbabwe	Viterra	0
Sawtooth mustard	Unknown	Viterra	13	Ndakapulca 4273	Zimbabwe	Viterra	0
Huang You Cai-1	China	AAFC-SRC	11	Ndakaupuka 4285	Zimbabwe	Viterra	0
Pizzo	Unknown	Viterra	11	Osaka Purple	Unknown	Viterra	0
MRS88 04	Turkey	Viterra	10	Pacific Gold Mustard	Unknown	Viterra	0
MRS 90-016	Zimbabwe	Viterra	10	PI 169077	Turkey	Viterra	0
MRS 90-019	Zimbabwe	Viterra	10	PI 169085	Turkey	Viterra	0
MRS 90-021	Zimbabwe	Viterra	10	PI 173861	India	Viterra	0
Mustard -Old Fashioned	Unknown	Viterra	10	PI 179183	Turkey	Viterra	0
Saryan	CN101942	AAFC-PGRC	7	PI 193761	Ethiopia	Viterra	0
Juncea aus Aeth	Ethiopia	Viterra	6	R2458	Egypt	Viterra	0
Ndakaupuka 4282	Zimbabwe	Viterra	6	Savanna - Hybrid Mustard	Unknown	Viterra	0
Bau-sin	Unknown	Viterra	5	Serifon	Unknown	Viterra	0
CN101945	Unknown	AAFC-PGRC	5	Tendergreen	Unknown	Viterra	0

Germplasm	Origin	Source of collection	Clubroot reduction (%)	Germplasm	Origin	Source of collection	Clubroot reduction (%)
Tsunga 4269	Zimbabwe	Viterra	5	Tsunga	Zimbabwe	Viterra	0
Tsunga 4284	Zimbabwe	Viterra	5	Tsunga 4266	Zimbabwe	Viterra	0
Broadleaf	Unknown	Viterra	2	Tsunga 4271	Zimbabwe	Viterra	0
Bamboo leaf	China	Viterra	0	Tsunga 4275	Zimbabwe	Viterra	0
Bingu	Zimbabwe	Viterra	0	Tsunga 4313	Zimbabwe	Viterra	0
BRA 434/79	Yugoslavia	Viterra	0	Xian Huang You 9	China	AAFC-SRC	0
Chembere				Yanagawa			
Dzagumhana	Zimbabwe	Viterra	0	Takama	Unknown	Viterra	0
CN101939	Unknown	AAFC-PGRC	0	Zi Ye Jie	China	AAFC-SRC	0
CN101940	Unknown	AAFC-PGRC	0				0
CN101941	Unknown	AAFC-PGRC	0				0
Covo	Zimbabwe	Viterra	0				0

Table S4. Resistance of *B. oleracea.*

Germplasm	Source of collection	Clubroot reduction (%)
Tekila	Syngenta Seeds	100
Kilaherb	Syngenta Seeds	100
Pee Wee	AAFC-PGRC	65
Gruner Angeliter	AAFC-PGRC	59
CN 35413	AAFC-PGRC	56
Primevert	AAFC-PGRC	46
Ghobi	AAFC-PGRC	15
Kirghizkaja 1	AAFC-PGRC	5
A028147	AAFC-PGRC	0
Boreal	AAFC-PGRC	0
Canadian Ace	AAFC-PGRC	0
Chou cavalier	AAFC-PGRC	0
Colt	AAFC-PGRC	0
Green marrowstem kale	AAFC-PGRC	0
Hous. Evergreen	AAFC-PGRC	0
Junior	AAFC-PGRC	0
Kailann -Big Boy	Viterra	0
Little leaguer	AAFC-PGRC	0
Mihnevskaja	AAFC-PGRC	0
Morden Midget	AAFC-PGRC	0
Mosbahskaja	AAFC-PGRC	0
Movir-74	AAFC-PGRC	0
Mozgoraja Zelenaja	AAFC-PGRC	0
Nadezda	AAFC-PGRC	0
Polycaul	AAFC-PGRC	0
Primevert	AAFC-PGRC	0
Skorospelaja	AAFC-PGRC	0
Tajininskaja	AAFC-PGRC	0
Vrozajnaja	AAFC-PGRC	0
Yuanbaicai	AAFC-SRC	0

Table S5. Resistance of *B. carinata.*

Germplasm	Source of collection	Clubroot reduction (%)
04-201EM	AAFC-SRC	19
05-333EM	AAFC-SRC	14
080798EM-148	AAFC-SRC	7
05-332	AAFC-SRC	0
04-202EM	AAFC-SRC	0
01-0088EM	AAFC-SRC	0
04-199EM	AAFC-SRC	0
05-456EM	AAFC-SRC	0
05-447EM	AAFC-SRC	0
05-451EM	AAFC-SRC	0
99-0011EM	AAFC-SRC	0
080798EM-086	AAFC-SRC	0

Table S6. Resistance of *B. nigra*.

Germplasm	Source of collection	Clubroot reduction (%)
BRA 192/78	Viterra	100
CR2120	IPK	100
CR2716	IPK	100
PI 219576	Viterra	88
34197	Viterra	0
PI 195554	Viterra	0
PI 173861	Viterra	0
MRS90-008	Viterra	0

FINAL REPORT

RBPI 1370 Objective IV

Developing Microbial Technologies for Biocontrol of Clubroot

Project Lead: GARY PENG

Collaborators:

Dennis Pageau
Russel Hynes
Susan Boyetchko
Bruce Gossen

Work Plans and Milestones

2009-10

Hire a PDF to conduct the project and finalize detailed research plans with the PDF and all collaborators

Isolate endophytic and rhizosphere microorganisms from canola roots across the prairie provinces, with a particular attention on "suppressive soils" during later season in Alberta; and develop/validate an evaluation process capable of screening out unsuccessful candidates rapidly

Conduct efficacy trials on selected microbial candidates using the established clubroot bioassay in a containment facility to identify promising organisms/isolates

Evaluate solid-substrate fermentation systems for production of a Canadian strain of *Heteroconium chaetospira* (Hc), a highly efficacious fungal endophyte against clubroot on canola

Evaluate preliminary biological formulations for seed treatment and "in-furrow" delivery to determine minimal rates required (in conjuncture with the project V)

Assess interaction of Hc with R and MR canola cultivars under high inoculum pressure of single and mixed *P. brassica* races

Initiate assessment of biocontrol on retarding clubroot pathogen race population shift under selection pressure of R or MR cultivars

Investigate modes of action for selected biofungicides (or agents) as well as interactions with R and MR canola cultivars in controlled conditions (in conjuncture with the project II)

Initiate study on submerged fermentation for mass production of selected bacterial agents.

In collaboration with scientists in Alberta, identify commercial or research field sites suitable for setting short- and medium-term rotation plots. Background clubroot pathogen inoculum levels and race composition will be evaluated using the PCR and bioassay procedures (linked to other studies)

2010-11

Continue efficacy trials on selected microbial candidates from the *in vitro* bioassay and identify most promising biocontrol agents for further biological assessments including efficacy determined from the clubroot bioassay and safety to important field crops

Continue trials to evaluate the effectiveness of the biofungicides Serenade and Prestop in naturally infested fields

Continue studying modes of actions for clubroot biocontrol and complete assessment of selected biofungicide interactions with canola cultivars for control of clubroot under varying pathogen-race and inoculum pressure conditions and identify most synergistic combinations

Continue assessment of biocontrol on retarding clubroot pathogen race population shift under selection pressure of R or MR cultivars, and modify study protocols as required

Continue studies on microbial interactions with R and MR canola cultivars

Continue evaluation of refined biofungicide formulations for seed treatment and soil applications in controlled environment, and determine minimum rates required for sufficient colonization of canola roots and effective control of clubroot disease (linked to project V)

Investigate biofungicide interaction with canola cultivars for under varying soil moisture, temperature, and pH conditions (to be revealed in the project II)

Continue refinement of submerged fermentation process through nutritional and environmental manipulation for optimal yield, shelf life, and product potency

Maintain the crop rotation plots based on a short (1-2 yr) and medium-term (3-4 yr) crop ration plan. The pathogen inoculum level and race composition will be monitored (linked to other studies)

2011-12

Complete study on biocontrol modes of action and critical elements for efficacy against clubroot were identified

Complete assessment of biofungicide interactions with R/MR canola cultivars against clubroot in controlled conditions and design a synergistic combination of Hc and canola cultivar plus for field testing

Complete assessment of biocontrol on retarding clubroot pathogen race population shift under selection pressure of R or MR cultivars, and determine the applicability of the protocol in field trials

Complete refinement of selected biofungicide formulations and optimal formulation composition and process are determined (linked to the project V)

Establish clubroot infested field plots in Alberta and Quebec, and initiate field trials to evaluate efficacy of using biocontrol in combination with a resistant canola cultivar under varying *P. brassica* inoculum pressure and canola seeding dates (linked to the project II)
Initiate monitoring pathogen race population shift under selection pressure of the R canola cultivar, with and without use of biocontrol or other management tools
Continue maintaining crop rotation plots for potential uses in following years and monitoring clubroot pathogen inoculum level and race structure

2012-13
Continue collaborative trials in filed mini plots to assess the value of combining biocontrol with R canola cultivars under varying *P. brassica* inoculum pressure and canola seeding dates
Complete evaluation of biofungicide formulation based on validation of efficacy in relation to application rates, placement, and root colonization
Initiate a long-term investigation of using multiple management tools to control clubroot on canola in established crop-rotation plots. These tools may include (not limited to) a biocontrol treatment, a selected R or MR canola cultivar, two crop-rotation and two seeding-date scenarios. This trial may need to be continued several years to fully assess the impact of an integrated approach
Continue monitoring pathogen race population shift under selection pressure of R canola cultivar with and without use of biocontrol or other management tools
Develop technology transfer plans in collaboration with the industry partner for biocontrol technologies and demonstrate field trials to growers and other stakeholders.

1. Assessment of indigenous microorganisms and commercial biofungicides/fungicides for control of clubroot on canola

Introduction

Until recently, there was a lack of effective options for control of clubroot on canola, in large part because all of the commercial cultivars were highly susceptible (Strelkov *et al*., 2006). In addition, the impact of agronomic approaches to clubroot management such as early seeding and long cropping rotations out of canola were inadequate when used alone, especially in a longer and warmer growing season (Gossen *et al.*, 2010) or for highly susceptible canola cultivars (Wallenhammar *et al*., 2000). A resistant canola cultivar became available in 2009. While important to clubroot management, genetic resistance has generally been race specific (Diederichsen *et al*., 2006) and can break down when virulent races increase in the pathogen population. Four pathotypes of *P. brassicae* have been identified in the population in Alberta (*Strelkov et al.,* 2007; Xue *et al.*, 2008), and there are potentially more that are present at a low frequency. Therefore, it is prudent to develop an integrated strategy for sustainable management of clubroot that includes but is not entirely dependent on cultivar resistance (Dixon, 2003; Donald & Porter, 2009). Additional measures including soil nutrient management (Webster & Dixon, 1991a, b; Dixon & Page, 1998), cultural practices based on better understanding of pathogen biology (McDonald & Westerveld, 2008; Gossen *et al*. 2009), crop rotation (Wallenhammar, 1996), and fungicides (Cheah *et al*., 1998; Suzuki *et al*., 1995; Takeshi *et al*., 2004) or biofungicides (Cheah *et al*., 2000; Peng *et al*., 2009) will probably help the performance and longevity of genetic resistance.

Several recent studies have illustrated the potential for reducing clubroot using naturally occurring microorganisms (Narisawa *et al*., 1998; Arie *et a*l., 1999; Joo *et al*., 2004). Some of these organisms produce anti-microbial metabolites against *P. brassicae* (Arie *et al*., 1998; Kim *et al*., 2004), while others colonize roots (Hashiba *et al*., 2003; Usuki *et al*., 2007) and induce resistance to the disease (Morita *et al*., 2003). Microbial control of clubroot is attractive because certain soil microbes can colonize root and/or the rhizosphere and so potentially provide durable protection. So far, no biofungicide was available for clubroot control, but several microbial biofungicides, including *Bacillus subtilis* (Serenade), *Gliocladium catenulatum* Gilman & Abbott. [syn. *Clonostachys rosea* f. *catenulate* (Gilman & Abbott) Schroers] (Prestop), *Streptomyces griseoviridis* (Mycostop), *S. lydicus* (Actinovate), and *Trichoderma harzianum* Rifai (Root Shield), have been registered in Canada for control of other soil-borne diseases. If effective, these biocontrol agents may be integrated for clubroot control in Canada.

The mechanism for biocontrol of clubroot disease is not clear. The life cycle of *P. brassicae* consists of a primary infection phase generally in root hairs and a secondary phase in the root cortex (Buczacki, 1983). It starts with the germination of resting spores and production of primary zoospores which infect root hairs, followed by the formation of primary plasmodia in root hairs and release of secondary zoospores that are capable of infecting roots. The characteristic clubbing symptom is a consequence of hyperplasia and hypertrophy of the infected root tissues (Channon & Maude, 1971; Ludwing-Muller *et al.,* 1997). Under optimal conditions, the production of primary and secondary zoospore generally peaks around 7 and 14 days after inoculation (Sharma *et al.,* 2011). Resting spores can tolerate a wide range of adversarial conditions but zoospores are the weakest links in the pathogen life cycle (Dixon, 2006). It was not clear whether Prestop®, when applied as a soil drench at seeding, was sufficiently effective for suppressing both primary and secondary infection. It was also of interest to understand whether this biofungicide would have additional mechanisms of biocontrol, especially relating to its ability to colonize canola roots and induce resistance response of host.
Some biocontrol agents (BCAs) have been reported to colonize rhizosphere and induce plant resistance (Kloepper *et al.,* 2004) via the activation of certain host metabolic pathways, including the synthesis of pathogenicity-related (PR) proteins (Viswanathan & Samiyappan 1999) and/or phenolics (Chen *et al.,* 2000). Phenylalanine ammonia lyase (PAL) is a key enzyme for phenylpropanoid metabolism that regulates the synthesis of several plant-defense-related metabolites, including phenols, phenylpropanoids, monomers of lignin and salicylic acid. Accumulation of these metabolites has been linked to induced plant resistance (Harish *et al.,* 2008). For effective biocontrol, it is useful to understand whether this BCA strain is capable of colonizing canola roots and inducing host resistance and whether it is necessary to optimize product formulation for delivery of the biofungicide in canola fields to target both primary and secondary infection effectively.

The objectives of this study were to: 1) isolate and evaluate rhizosphere and endophytic inhabitants of canola roots indigenous to the Canadian prairies for potential clubroot control, 2) assess commercial biofungicide products for clubroot control; 3) investigate potential delivery options for field application; and 4) evaluate the efficacy of selected biocontrol agents against clubroot under field conditions; 5) characterize the primary modes of action by which the leading biofungicides Serenade or Prestop suppressed the clubroot disease on canola, including the effect on germination and viability *P. brassicae* resting spores, root-hair and cortical infection, and disease development; 6) assess the ability of biofungicides to colonize canola roots and the potential to induce host resistance; and 7) assess the ability of this fungus to affect the expression of genes involved in biosynthesis of certain defense-related metabolites.

Methodologies

Field collection of microorganism samples
Canola root samples were pulled from fields of varying locations in the Black Soil Zone of Alberta and Saskatchewan, and stored at 4ºC until use. Additional root samples from Alberta were air dried, and stored at 4ºC before processing for more robust endophytes or rhizosphere inhabitants. A protocol was put in place for handling canola roots from infested field in Alberta. These roots are placed in a separate container with a warning sign "Clubroot materials, please don't open!" These samples were fetched and processed in a tray and all residuals were autoclaved before disposal. Loose soil was dislodged from roots and one gram of roots was added into 10 mL of 0.05% Tween 80 solution. The sample was agitated, serially diluted, and plated on 0.1x acidified (0.13% lactic acid) potato dextrose agar (PDA) with antibiotics (1.5% penicillin G w/w, 0.65% streptomycin w/w) to isolate fungi, and on 0.1x nutrient agar (NA) to isolate bacteria. The plates were incubated at room temperature for seven days.

Isolation of endophytic microorganisms
Roots were washed free of soil and bundled according to the diameter of the root pieces for surface sterilizing. Fine roots (less than 0.5mm in diameter) were soaked in 70% EtOH for 30 sec, 0.12% hypochlorite for 5 min, 70% EtOH again for 30 sec, rinsed twice in sterile water and once in acidified sterile water (0.1% lactic acid, pH 4) for one min. Thin root pieces 0.5-1 mm in diameter were treated with rapid agitation in 95% EtOH for 60 sec, 0.12% hypochlorite for 2 min, 95% EtOH for 60 sec, rinsed twice in sterile water, and in acidified sterile water for 5 min. Root pieces 1 mm in diameter or greater followed the same procedure as the thin roots, but with modifications only to the time in 0.12% hypochlorite (5 min for roots ~1 mm in diameter (medium roots); 10 min for roots 1-2 mm in diameter (thick

roots)). Very thick root pieces were split into thinner pieces before surface sterilization. Excessive water on the root pieces was removed using sterile paper towels and dried for three hours before plating on 1x PDA + Antibiotics or 1x NA. In addition, root imprints were made on 1x PDA + Antibiotics or 1x NA plates to measure the effectiveness of the surface sterilization based on the recovery of surface microbial contaminants. Plates were incubated at room temperature for 2-6 weeks.

Tiered screening of indigenous microorganisms for biocontrol of clubroot

Due to a large number of soil microorganisms obtained from canola root samples, a tiered system was designed to facilitate screening of these candidates efficiently.

Tier I - Agar-plate assay for detection of antibiosis and competition: Purified bacterial and fungal cultures were transferred onto PDA in a 9-cm Petri plate at four locations 1 cm away from the edge, which could be linked across with two perpendicular lines. Bacterial cultures were smeared and fungal mycelial plugs (5 mm) were placed at the four locations. A mycelial plug of *P. ultimum* (5 mm) was placed at the center of each plate as an indicator target for suppression. Inoculated plates were incubated at 20°C for one wk, and *P. ultimum* colony size was measured along the perpendicular lines. Inhibition was determined by relating the average *P. ultimum* colony (two measurements per plate) to that of the control (blank). The degree of inhibition was expressed using the following scale: "–": no visible inhibition, the indicator colony size was similar to control; "+": slight inhibition, the *P. ultimum* colony was <25%; "++": moderate inhibition, the colony was between 25% and 50% of the control; and "+++": high inhibition, *P. ultimum* colony was reduced by at least 50%. This colony inhibition could be caused by antibiosis or competition.

Tier II. Pythium-canola seedling bioassay: All endophytic isolates were also screened for additional modes of action (induced disease resistance) using a canola seedling assay to identify candidates that reduce the indicator pathogen *P. ultimum* causing seedling damping off. Endophytic isolates were grown on PDA (fungi, 2 wks) or NA (bacteria, 3 days) for inoculum. The *P. ultimum* isolate was initially grown on PDA in Petri dishes for 1 wk, then homogenized in a Waring blender for 15 s, and the culture suspension used to inoculate autoclaved barley grain kernels in microbial incubation bags (with aeration). Inoculated grain cultures are incubated at room temperature for 2 wks, shaken periodically to enhance uniform growth and colonization, air dried and ground in a Wiley mill using a 25-mesh screen to produce a uniform particle size. The *P. ultimum* grain formulation was stored at 4°C until use.Individual plastic seeding trays (6 x12 x17 cm) were filled half way to the top (3-cm depth) with the soil-less mix with 10 g of *P. ultimum* grain formulation sprinkled evenly on the top. At this inoculum dose, the pathogen caused a moderate level of damping off, reducing canola emergence by about 50%. A total of 50 seeds of Fortune RR canola cultivar were sown to each tray, and then 50 ml of endophyte suspensions (fungal spores/mycelial fragments, or bacterial cells) were applied as a soil drench to a tray. Each tray formed an experimental unit. The concentrations of the endophyte suspensions were about 2 $x10^6$ propagules/ml for the fungi and 2 x 10^8 propagules/ml for bacteria. After treatment, seeds were covered with 2 cm of soil-less mix, placed in seeding flats (6 trays/flat), watered to saturation, and kept in a growth room at 15/5ºC (day/night) with 14 h daily lighting to encourage seedling damping off. Non-infested mix and infested mix treated with water were used as control and pathogen check, respectively. After 10-d incubation, total stand counts were taken. Each treatment consisted of 2 replicates (trays), and candidates with greater than 50% efficacy were retested later with 4 replicates. Candidates consistently provided >50% efficacy were advanced to a Tier-III test involving the clubroot pathogen.

Tier III. Canola clubroot screening bioassay: It is more efficient to test only the most promising candidates in the clubroot bioassay due to longer duration of trial, limited space, and higher cost to operate the containment. The testing protocol was similar to the one used in the efficacy trial for biofungicides with the following modification; Pb inoculum (10^7 resting spores/ml) was applied to soil-less mix at 5 ml per container 1 d prior to seeding. A highly susceptible canola cultivar, Fortune RR, was used for screening of biocontrol agents under controlled conditions. Seed was sown in Sunshine #3 potting mix (pH 5.8-6.2) in 10-cm diameter plastic pots. Clubroot galls were collected from infested canola fields in central Alberta. The pathogen population from these collections may represent a mixture of pathotypes, but pathotype 3 would certainly predominate (Strelkov *et al*., 2006; 2007; Xue *et al*., 2008). Galls were air dried and stored at -15ºC until required. To extract resting spores, about 3 g of dried galls were soaked in 150 mL distilled water for 2 h to soften the tissue and then macerated in a blender at high speed for 2 min. The resulting slurry was filtered through four layers of 0.3-mm nylon cloth and the spore concentration estimated using a haemocytometer. In the greenhouse trials, naturally infested field soils were also used as a less

artificial means of infestation. Biocontrol treatments applied as a soil drench at 25 ml per conetainer. Inoculated plants were kept in a growth cabinet at 23/18ºC for 4 wks and clubroot severity rated using the 0-3 scale: 0= no galling; 1= small galls only, on less than 1/3 of roots; 2= small or medium-sized galls on 1/3 to 2/3 of roots; and 3= severe galling, medium to large-size galls on more than 2/3 of roots. Disease severity index (DSI) was calculated based on the weight of each rating class observed. Inoculated plants that received only the same volume of water would be used a control for each trial. The experimental design was CRD with 7 replicates. Candidates consistently provided >50% efficacy were tested further.

Assessment of biofungicides

The commercial formulation of the biocontrol agents *B. subtilis*, *G. catenulatum*., *S. griseoviridis*, *S. lydicus*, *T. harzianum*, *G. virens* Miller, Gilddens, & Foster, and *B. subtilis* var. *amyloliquefaciens* were tested under growth-cabinet conditions. The latter two agents were not included in greenhouse trials due to their poor efficacy in initial tests and non-registration status in Canada. The fungicide fluazinam [3-chloro-N-[3-chloro-2,6-dinitro-4-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2-pyridinamine] was effective against clubroot in crucifer vegetable crops (Cheah *et a*l. 1998; Donald *et al*. 2002) and has been registered recently in Canada for clubroot control on vegetable crops. Cyazofamid [4-chloro-2-cyano-N,N-dimethyl-5-p-tolylimidazole-1-sulfonamid] is a relatively new fungicide with specific activity against Oomycetes (Takeshi *et al*., 2004) and high efficacy against clubroot (Mitani *et al*., 2003).

Efficacy trials under growth-cabinet conditions

In these initial trials, all of the biofungicide and fungicide products were assessed against the pathogen control. Because clubroot had not been reported on canola in the province of Saskatchewan, these trials were carried out in a level-2 containment facility at the Saskatoon Research Center to ensure that the pathogen did not escape. Space in the containment facility was very limited, so each experimental unit consisted of seven plants. The study was laid out in randomized complete block design, with the blocks being three separate time periods. Each product was applied as a soil drench at 3 days after seeding, and suspensions of *P. brassicae* resting spores were added at 7 days after seeding. The plants were inoculated after the treatments were applied to provide an opportunity for the biofungicide agents to become established before being challenged. The plants were maintained in a growth cabinet for 4-5 weeks and then rated for clubroot incidence and severity.

Efficacy trials under greenhouse conditions

Separate experiments were carried out to assess the efficacy of selected products when delivered as a soil-drench or seed treatment. In each experiment, two sources of pathogen inoculum were used: 1) naturally infested field soils were mixed with non-infested soil-less mix at 1:1 and 1:2 (v/v) for trials 1 and 2, respectively, and 2) applying resting spores to non-infested soil-less mix at 7 days after seeding (1 h prior to treatment), at 2×10^7 spores per plant in trial 1 and 1×10^7 spores per plant in trial 2. The field soil was diluted with soil-less mix because of high clubroot DSI (almost 100%) observed in canola plots at the field location. All treatments were applied 7 days after seeding for both naturally infested soil and artificially inoculated soil-less mix (1 h after inoculation). For the soil-drench application, products were applied at 50 mL per pot. For the seed-treatment application, canola seeds were immersed in a product preparation at 10 × the soil-drench concentrations for 5 minutes and then air dried for 1 hour prior to seeding. For each of the greenhouse trials, the study was laid out in a randomized complete block design with four replicates and 10 plants per replicate. The plants were maintained in a greenhouse for 8 weeks and then assessed for clubroot incidence and severity. Each greenhouse trial was repeated.

Effect of water volume on efficacy

The soil-drench treatment at 50 mL per plant represents a large volume of water when translated to a field scale (equivalent to 42,000 L ha^{-1} in an “in-furrow” field application), and would be impractical for product delivery in canola crops. To assess the possibility of using a reduced carrier volume, a commercial formulation of *B. subtilis* (13 L ha^{-1}), *G. catenulatum* (1.4 Kg ha^{-1}), fluazinum (2.9 L ha^{-1}), and cyazofamid (0.54 L ha^{-1}) were applied as an “in-furrow” drench at about 500, 2,500 and 12,500 L ha^{-1} of water volume shortly after seeds were dropped. Soil-less mix in each conetainer was infested with 5×10^7 *P. brassicae* resting spores 2 days prior to seeding and watered immediately after seeding with 15 to 30 mL of acidified water (pH = 6.3), depending on the water volume

applied to provide a similar level of soil moisture to all treatments. Thereafter plants of each treatment were watered as described earlier. The trial was laid out in a randomized complete block design with two blocks (replicates in time) and seven plants per experimental unit. Each plant was assessed for clubroot severity 4-5 weeks after seeding.

Field trials

In 2009, two trials of canola and one of Chinese cabbage [*B. rapa* L. subsp. *Chinensis* (Rupr.) var. *utilis* Tsen and Lee] were conducted to assess the efficacy of four products, based on the efficacy observed in previous trials, against clubroot under field conditions. These treatments were applied as a liquid into the seed furrow at 5.2×10^{13} colony forming unit (cfu) ha^{-1} of *B. subtilis*, 1.4×10^{11} cfu ha^{-1} of *G. catenulatum*, 1.45 kg active ingredient (a.i.) $ha^{-1}$of fluazinum, or 0.22 kg a.i. ha^{-1} of cyazofamid formulation. The liquid volume delivered was about 500 L ha^{-1}. The canola trials were conducted in two commercial fields with heavy clubroot infestation near Edmonton, Alberta. Pathotype 3 of *P. brassicae* predominates at these sites (Strelkov *et al.*, 2007). The Chinese cabbage trial was conducted at the Muck Crop Research Station, University of Guelph near Bradford, Ont., where the soil is infested with pathotype 6 of clubroot pathogen (Cao *et al.*, 2009).

The trials were arranged in a randomized complete block design with four replicates. Each plot consisted of four 6-m-long rows with 21-cm row spacing for canola and 44-cm for Chinese cabbage, seeded at 6.5 Kg of seed ha^{-1} at about 2.5-cm depth. Susceptible (S) and resistant (R) lines were used for both canola and Chinese cabbage trials; cvs. 45H26 (S) and 45H29 (R) for canola (source: Pioneer Hi-Bred Canada), and Mirako (S) and Yuki (R) for Chinese cabbage (source: Mirako – Bejo Seeds Inc., Oceano, CA, USA; Yuki - Stokes Seeds Ltd., Thorold, ON, Canada). All of the products were applied in-furrow at 500 L ha^{-1} using a calibrated backpack sprayer. Water was applied to the control. Clubroot severity was assessed at full bloom in canola and at 8 weeks after seeding in Chinese cabbage, by digging about 25 plants from the central 4-m area of each plot and rating each plant as described previously.

Effect of soil-dryness duration on efficacy

In a growth-cabinet trial to assess the impact of early season drought on treatment efficacy, *B. subtilis*, *G. catenulatum*, fluazinum, and cyazofamid formulations at the rates described above were applied “in furrow” in 500 L ha^{-1} water to canola at seeding. Resting spores of *P. brassicae* had been applied to the growth medium 2 days prior to seeding. The duration of dryness treatments were applied by delaying additional watering after seeding for 0, 1, 2, 3, and 4 weeks to simulate the effect of delayed rain events under field conditions. The average water content of the growth medium was 11.4% (water activity: 0.678 ± 0.011 at 20°C) prior to regular watering. Inoculated but nontreated plants served as a control at each interval of delayed watering. Plants were grown in conetainers arranged in a randomized complete block design with two blocks (replicates in time) and seven plants per experimental unit. Clubroot severity was assessed 4 weeks after watering was initiated for each treatment. Watering provided the high soil moisture conditions for seed germination and root infection.

Mechanisms the biofungicides Serenade and Prestop in control of clubroot on canola

Effect of the biofungicide and its components on infection and clubroot development: The growth medium was infested with *P. brassicae* suspension (1×106 resting spores cc^{-1}) 2 d before seeding. Biocontrol treatments, i.e. 5% Serenade or Prestop, a product filtrate (from a 5% product suspension), or a bacterial cell/fungal conidial suspension (10^6 spores mL^{-1}), were applied as a soil drench (25 mL $plant^{-1}$) immediately after seeding. Controls received only 25 mL of water. Root-hair infection (RHI) was assessed using microscopy. Roots of three treated plants (randomly sampled) removed at 7 or 14 DAS were washed using the procedure described above, cut into 1-cm pieces, and fixed in 70% alcohol until use. Roots of control plants (pathogen-inoculated only) were processed similarly. Root pieces were stained overnight with aceto-carmine (1%) and examined using a light microscope for the presence of primary plasmodium or zoosporangia in root hairs. Up to 40 root hairs were checked for each plant (replicate) and the incidence of infection calculated.

To determine the development of *P. brassicae* in root tissues, pathogen gDNA was extracted from 0.2 g (fresh weight) of bulked roots of three plants removed at 7 and 14 DAS from each treatment block, and quantified using a StepOne™ Real-Time PCR detection System (Applied Biosystems Inc., Montreal, QC). This qPCR reaction was performed in a, approximately 20-µL reaction volume containing 2-µL template DNA, 0.1-µL each primer (50 nM),

10-µL SYBR Green-I PCR Master Mix (Applied Biosystems), and 7.8-µL SDW. The thermocycling profile consisted of an initial denaturation step of 10 min at 95°C, then 50 cycles of 15 s at 95°C, and finally 1 min at 60°C. The cycle threshold (C_t) levels were set automatically by the system and quantification carried out using a standard curve developed by serial dilutions (10^0 to 10^{-4} ng) of gDNA extracted from purified *P. brassicae* resting spores. All qPCR reactions were conducted using the specific primers Pb4-1 (TACCATACCCAGGGCGATT) and PbITS6 (CAACGAGTCAGCTTGAATGC) described by Sundelin *et al.* (2010). These primers were designed to generate a *P. brassicae* DNA product of approximately 139 bp. Three repeated reactions were conducted to establish standard linear regression curves between C_t values and the logarithm of template concentrations. For each amplification, a melting curve as well as electrophoresis (2% agarose gel) was performed to ensure that only the target DNA was amplified.

***Effect of the biofungicide filtrate on* P. brassicae *resting spores*:** Canola root exudate solutions were produced to induce the germination of resting spores in a suspension for this assessment. Seed of cv. Fortune RR canola were disinfected, rinsed with SDW, and placed at 25ºC in darkness for 12 h. Fifty germinating seeds were placed on a nylon mesh fixed to a Styrofoam ring set on a glass jar with 150-mL Hoagland's solution. Seedlings were kept in a growth chamber at 25ºC with a 16-h photoperiod (512 µmoL $m^{-2}s^{-1}$) for 14 d. The solution was filtrated (0.22 µm) with pH adjusted to 6.0, and referred as a root-exudate solution (RES).

Purified *P. brassicae* resting spores were added to RES (1×10^7 mL^{-1}) amended with the biofungiciede filtrate equivalent to 5% product concentration (w/v) or with the fungicide fluazinam (ISK Biosciences Co., Concord, OH) at 0.5%. Prepared spore suspensions were kept in 15-mL Falcon tubes and incubated at 25ºC in darkness for up to 10 days. The germination of resting spores in each treatment was assessed by taking a 20-µL sample from each of 5 replicates daily and stained with acetic-orcein (1%) or acetic-carmine (1%) for 10 min on a glass slide. Up to 100 spores were examined with each sample under a light microscope (400×) using the protocol described by Naiki & Dixon (1987). The empty or shrivelled spores were considered as having germinated (Lahlali *et al.,* 2011). The germination of spores in non-amended RES was used as a control.

The effect of biofungicide filtrate on the viability of *P. brassicae* resting spores was assessed simultaneously with the germination test without using RES to verify the results of the germination test. Resting spores were suspended in SDW amended with the filtrate equivalent to 5% product concentration or in SDW only (control) for 2-10 days of exposure. Unlike in RES, resting spores would not germinate during this process and the spore viability was assessed based on reactions to Evan's blue which would only stain dead spores (Tanaka *et al.,* 1999). At 2-d intervals, a 20-µL sample was taken from each of three replicates in Falcon tubes, stained for 10 min on a glass slide with 1% Evan's blue, and examined under a light microscope (400×). The dye would only stain dead spores which showed a blue cell wall and reddish cytoplasm. Those that did not take up the stain were counted as being live (Lahlali *et al.,* 2011).

Effect of biofungicide-treatment timing on clubroot control: The effect of biofungicide treatment timing was examined using a treatment scheme following the protocols previously described by Naiki & Dixon (1987) and Tanaka *et al.* (1999) for fungicide timing. The Prestop®, a product filtrate, a fungal conidial suspension or water (inoculated control) was applied as a soil drench to a pathogen infested growth medium after seeding. Seedlings were removed at 7 or 14 days after seeding (DAS) with roots washed using procedures described above prior to being transplanted into a non-infested growth medium. This rigorous washing was intended to remove most of the pathogen propagules and biofungicide components on the rhizoplane. After transplanting, the treatments or water was applied again according to a pre arrangement. Treatments applied at seeding was designed to target the root-hair infection (RHI) by primary zoospores, those applied after transplanting would more likely affect secondary zoospores, and two treatments would target both primary and secondary zoospores by maintaining the biofungicide or its components at high doses during most of the infection period. Plants not inoculated with *P. brassicae* resting spores were not used in this trial because many prior trials without inoculation had yielded no clubroot symptoms. This design was based on the timing for peak RHI and the release of secondary zoospores under optimum conditions (Sharma *et al.,* 2011).

Trial procedure: Large plastic pots (15-cm-diam.× 15-cm tall) were filled with Sunshine #3 planting mix drenched with 60 mL of a *P. brassicae* suspension (1×10^7 resting spores mL^{-1}) to obtain about 2×10^6 spores g^{-1} growth

medium. Two days later, fifty seeds of Fortune RR canola cv. were planted in each large pot at 1-cm depth, and the treatments or water (control) was applied at 180 mL pot^{-1}. Seeded pots were placed in the growth cabinet and seedlings were removed at 7 and 14 DAS, respectively. After root washing, seedlings were transplanted individually into non-infested Sunshine #3 potting mix in conetainers. Transplants were arranged randomly on a holding rack, some were treated again with Prestop®, the filtrate, fungal conidial suspension or just water at 15 mL $plant^{-1}$, and all maintained in the growth cabinet. At about 5 wks after seeding, clubroot severity was assessed on all plants. The experiment was laid out as a RCBD and replicated at three different times (blocks) with seven plants for each treatment in a block.

***Host defense response(s) Induced by biofungicides Serenade or Prestop*:** At 14 DAS, roots from three Prestop®-treated canola plants (a replicate) were washed as described above and RNA was extracted from these root samples using an RNeasy Plant Mini Kit. RNA from the first true leaves of each plant was also extracted, and root/leaf samples from non-treated plants were used as controls. Three replicates were used with each treatment. PCR amplification was carried out for each target gene using conditions reported previously (Potlakayala *et al.,* 2007; Zhao *et al.,* 2009). The Actin gene was used as the housekeeping gene control for qPCR (Zhao *et al.,* 2009). The relative expression ratio of a target gene was measured based on the differences in Ct of a sample versus the control. Differences between a treatment and control in relative expression of each gene were determined for significance at $P < 0.05$.

Results and discussion

Microorganisms from canola roots – potential for clubroot control

A total of 5,152 isolates were isolated from canola roots collected in Saskatchewan and Alberta. The majority of them were bacteria (74%) and the rest was fungi. This bacterial-fungal ratio was equally applicable to isolates from the rhizosphere and interior of the roots. The total number of isolates from the rhizosphere and interior of canola roots was fairly close. The majority of bacterial isolates showed no inhibition to the fungal pathogen while fewer than 5% of rhizosphere and endophytic bacteria reduced the colony of indicator pathogen by greater than 50%. A larger percentage of fungi showed higher levels of inhibition, ranging from 30% to 15% for rhizosphere and endophytic fungi, respectively. Overall, more than 250 of soil microbial isolates showing >50% inhibiting to P. untimum in the dual-cultural assay. A total of 650 endophytic isolates that failed to show significant inhibition in the dual-culture bioassay were tested in this plant bioassay to select for induced plant response or direct microbial competition. Isolates with greater than 30% enhancement on canola emergence (about 140 isolates in total) were tested further against clubroot. A total of 390 isolates showing significant antibiosis/competition in the dual-culture bioassay or reduction on the pythium impact on canola emergence were tested in the containment facility against clubroot on canola. Overall, isolates with high efficacy of clubroot control were limited and only 3 fungal isolates resulted in greater than 75% of reduction in clubroot severity. The efficacious microbial treatment also alleviated the negative impact of clubroot on the vigor of canola plants. All highly effective candidates are endophytes capable of colonizing canola roots. Several fungal and bacterial isolates reduced the disease by more than 50%.

Performance of biofungicides/fungicides under field conditions

In a total of ten field trials, the biofungicides Serenade and Prestop, and fungicides fluazinam and cyazofamid, applied as a liquid in furrow (500 L/ha) or granular formulations (50Kg/ha) did not reduce clubroot severity on susceptible or resistant canola cultivars central Alberta and northern Quebec. One trial on Chinese cabbage in Ontario with close to 2000L/ha soil drench volume achieved substantial efficacy for all the products mentioned above and lowered disease severity by 54–84%. It was clear that a long period of dry soil conditions post canola seeding will reduce the efficacy of most of these products.

Modes of action for biocontrol of clubroot

There were some similarities between the biofungicides Serenade and Prestop for the mechanisms of clubroot control: The whole product is consistently more effective than the individual components in reducing infection by *P. brassicae* and two each applications were more effective than one. These biofungicides reduced genomic DNA of *P. brassicae* in canola roots by 26–99% at 7 and 14 days after inoculation the qPCR results were strongly correlated with root hair infection (%) assessed with microscopy at the same time ($r = 0.84–0.95$). The transcript activity of nine host-defense-related genes in biofungicides-inoculated plants was increased relative to that of water

controls at 14 day after treatment. Genes encoding the jasmonic acid (BnOPR2), ethylene (BnACO), and phenylpropanoid (BnOPCL and BnCCR) pathways were up-regulated in plants treated with the biofungicides. This induced defense response can be translocated to the foliage. It is possible that antibiosis and induced resistance are involved in clubroot suppression by the biofungicides Serenade and Prestop. Biocontrol activities against the infection from both primary and secondary zoospores of *P. brassicae* may be required for maximum biocontrol efficacy against clubroot.

The endophytic fungus *Heteroconium chaetospira* (Grove) M.B. Ellis reduced the severity of clubroot on canola by >85% when applied to the soil under controlled conditions (Fig. 1). It is a very slow-growing fungus and there is little change for it to act rapidly after the germination canola seed under field conditions. The treatment was tried on transplants of Chinese cabbage by incorporating 3 g of a granular formulation of *H. chaetospira* in growth media for a longer duration in greenhouse to allow the fungus to establish in transplants. The treated plants were examined for gene expression related to jasmonic acid (JA), ethylene (Et), auxin (IAA), PR protein, and phenylpropanoid (Pp) pathways using qRT-PCR to determine whether induced host-defence responses were involved in biocontrol. In treated plants, higher transcript levels for Et, JA, IAA, and PR-2 were observed in both roots and leaves relative to non-treated controls, but the expression of Pp genes was significant only in roots based on a microarray study. These results indicate that induced host resistance is possibly involved in clubroot suppression by *H. chaetospira* via the modulation of JA, Et and Pp pathways in root tissues. The Pp pathways influence the production of several host-defense secondary metabolites including phenolics, salicylates and flavonoid phytoalexins. Despite the successful establishment of the endophyte in roots of Chinese cabbage transplants, field trials did not prove substantial benefit in either clubroot reduction or yield increase. The endophyte is no longer being pursued as a promising biocontrol agent against clubroot.

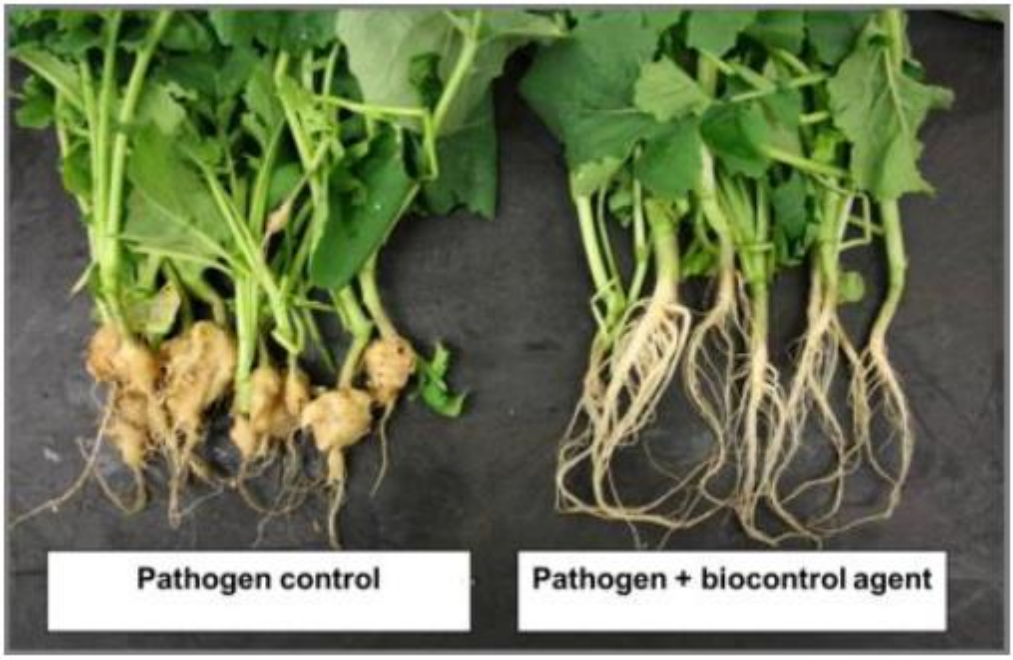


Fig. 1. Efficacy of granular formulation of the endophytic biocontrol agent *Heteroconium chaetospira* against clubroot on canola inder controlled environment.

Additional bacterial candidates

In 2012, twenty *Bacillus subtilis* strains and 4 bacteria belonging to the Bacillus and Pseudomonas spp. were evaluated for clubroot control in canola. Previous studies showed that these bacteria were plant growth promoting and had potential as biopesticides. The efficacy trials did not yield any usable results because of experimental

error, variable disease severity incidence and results therefore did not consistently reach the desired disease levels required to attain significant clubroot symptoms. These inconsistent problems are related to the nature of the pathogen and a better understanding of handling the pathogen and achieving consistent pathogenic needs to be investigated. Some of the leading bacterial candidates underwent detailed taxonomic identification using the chaperonin-60 universal target (cpn60 UT) which is accessible in nearly all bacterial genomes with a single set of universal primers. This was conducted in collaboration with Dr. Tim Dumonceaux at Saskatoon. Some of the leading strains belonged to Pseudomonas fluorescens, Bacillus subtilis, and B. globigii strains. However, due to inconsistent generation of disease pressure (incidence and severity), we could not conclude which bacterial strains should be forwarded as promising biopesticide candidates for clubroot disease of canola (**Boyetchko**).

2. Integrated management of clubroot on canola using crop cultivar resistance in combination with biofungicide seed-treatment or crop rotation

Introduction

Resistant canola cultivars are generally single-gene based and have shown a low level of disease under field conditions (Peng et al., 2011a). The resistance is also race specific and historically this type of resistance is not durable because it can be eroded when pathogen race structure changes. In a study by LeBoldus et al. (2012), a resistance canola cultivar showed substantially increased clubroot severity after being exposed repeatedly to the same *P. brassicae* population. Cultivar resistance is the key to effective clubroot control on canola. It was not clear if additional measures can help the performance and longevity of resistant cultivars. The biofungicide Serenade® ASO (AgraQuest, Davis, CA), a liquid formulation of *B. subtilis*, was highly suppressive to clubroot under controlled-environment conditions when applied as a soil drench, especially when the pathogen inoculum was at lower levels. It boosted efficacy of a moderately resistant (MR) canola line against a heavy load of pathogen inoculum (Peng et al. 2011b). However, a liquid biofungicide is impractical for canola in western Canada due to a large-scale production and general lack of proper equipment. Development of granular or seed-dressing formulations may facilitate the delivery of biofungicide and improve its stability in the soil.

There was generally a lack of specific information on the impact of crop rotation on survival of *P. brassicae* in soils, although a long-term rotation was considered beneficial in reducing pathogen inoculum and alleviating clubroot pressure (Wallenhammar 1996). In recent years, however, many growers in western Canada produce canola in a tight rotation due to differential commodity prices in marketplaces, and it is not known how much this practice may exacerbate the clubroot problem vs. a 4-year rotation (a 3-year break from previous canola) often recommended in the region. Resting spores of *P. brassicae* can survive many years in soil (Dixon 2009) and it would be unrealistic to expect a practical crop rotation removes clubroot pressure entirely. If a 4-year rotation can lower the disease pressure adequately, then an additional treatment such as biofungicide may act more effectively in reducing the disease impact. This study was carried out to assess a biofungicide formulation and crop rotation in combination with cultivar resistance to maximize clubroot control and canola yield potential, while reducing inoculum build up in the soil. This may help extend the longevity of CR cultivars. The objectives of this study were to: 1) Quantify potential effect of crop rotation on reducing *P. brassicae* inoculum in the soil; 2) Explore interaction of cultivar resistance with a biofungicide or crop rotation for enhanced clubroot control; 3) Assess the value of a long crop rotation (3- to 5- year) when used jointly with a biofungicide or CR cultivar for clubroot control in heavily infested fields.

Methodologies

Bacillus subtilis formulations

Two granular formulations were produced for a *B. subtilis* strain and tested with resistant and susceptible canola cultivars. Seed dressing was used with a commercial *B. subtilis* seed treatment formulation and evaluated in crop-rotation plots. The granular formulation A was based on prior experiences on soil-applied biopesticides (Hynes and Boyetchko, 2011); it showed a fast disintegration rate in water, which would facilitate rapid release of active

ingredients in the soil. The formulation B used corn-cob grits as a low-cost carrier. The formulation composition is as follows:

Formulation A	
Corn starch (Tate and Lyle Ingredients Americas Decatur, IL)	193 g
Peat	32 g
Liquid fermentation product	171 mL
Titre range:	9.26E+08 cfu/g (S.E.±0.54E+08)
Formulation B	
Corn-cub grits (Co-op, Quebec City, QC)	100 g
Liquid fermentation product	60 mL
Titre range:	7.80E+08cfu/g (S.E.±0.36E+08)

Field plots. Plots were established on two commercial farms near Leduc and Edmonton, AB, and also on an AAFC Research Farm in Normandin, QC. All these fields were heavily infested by clubroot, with previous DSI generally greater than 80% on susceptible canola cultivars. The *P. brassicae* population at the AB and QC sites are predominantly pathotype 3 and 2, respectively. At all locations, each plot consisted of eight 6-m rows with 18cm row spacing (6.5 kg/ha seeding rate). Clubroot severity in each plot was assessed by pulling 25 plants at about the Harper and Berkenkamp (1975) growth stage 4.3 (late flowering) and each plant was assessed for clubroot severity using a 0-3 scale (Strelkov et al. 2006) as described before. At crop maturity, each plot was harvested separately, seeds dried to approximately10% moisture, and yields taken.

Granular biofungicide formulations (2011). The cultivars 45H-28 (susceptible) and 45H-29 (resistant) were seeded on May 28 (Leduc), June 2 (Edmonton), and June 6 (Normandin), 2011. The two granular formulations of *B. subtilis* strain QST713 were applied in furrow at 50 Kg/ha in mixtures with canola seed. Corn-cub grits coated with the fungicides Allegro® 500F (fluazinam, 725 g/ha) and Ranman® 400SC (cyazofamid, 600 g/ha) were applied similarly for comparisons. The experiment was a split-plot design with cultivar in main plots (4 replicates), and biofungicide/fungicide formulations in sub plots arranged randomly in 4 blocks within each main plot.

Effect of *B. subtilis* seed dressing under different crop rotations (2011). The field in Normandin was about 4 ha and had been found with severe clubroot throughout the field since early 1990s. The distribution of pathogen inoculum was quite even across the whole field. Starting from 1999, this field had been used for a crop rotation study with a total of 112 large plots (8×32 m) maintained as canola-barley-field pea-barley-canola, canola-field pea-barley-barley-canola, or canola-barley-barley-field pea-canola rotation systems, or continuous field pea or barley. The field was plowed in fall annually, and these rotation plots provided choices of varying break duration from a canola crop. Plots with 1- and 3-year breaks (barley and/or field peas) were selected randomly in 2011 trials to represent 2-year and 4-year crop rotations in canola production, and plots that had been 11 years out canola (in continuous barley or field pea only) were used as an extreme scenario for comparisons only. Prior to the trial, 3L soil (top 15 cm) were taken from 5 random sites of each plot and 4 plots from each crop-rotation category were used to estimate pathogen inoculum levels using a bioassay under controlled-environment conditions.

Four equal-increment rates (1×10^5 to 5×10^6 cfu/seed) of the biofungicide Kodiak (*B. subtilis* strain GB03) was used with the seed-treatment adjuvant L1782 and seeds treated with L1782 alone were used for non-treated controls. Two trials, seeded on June 5 and June 15, 2011 respectively, were set up on different sections of the field in a split-plot design with crop rotation as main plots (4 replicates) and biofungicide seed dressing in sub plots in 4 blocks within each main plot. To assess potential impact of varying prior crop residuals on canola emergence, plants in two random 1-m rows of a plot were counted at 7, 14, and 21 d after emergence (the same 1-m row section each time) and plant density calculated based on total counts and row spacing.

To obtain an additional estimate of disease pressure in different plots, five plants were pulled randomly from each subplot of control within each main plot in 2011 trials, roots washed repeatedly with running tap water to remove debris, soil particles, and pathogen inoculum on the root surface. To determine the amount of *P. brassicae* in roots, pathogen genomic DNA from 0.2g root tissue was extracted with the DNeasy and quantified using quantitative PCR

(qPCR) using the procedure described by Sundelin et al. (2010). In 2012 trials, similar soil samples were taken from each rotation replicates and tested directly with qPCR for quantification of *P. brassicae* resting spores in the soil. In addition to the clubroot severity assessment, the above-ground impact by clubroot was also assessed for each plot visually relative to non-disease plots using a 0-4 scale where: 0 = plants are not affected, having developed normally; 1 = plants are slightly affected, with up to 25% of the plants showing the symptom of stunting and yellowing; 2 = about 50% plants showing the symptom; 3 = >75 % plant showing symptoms and the plot is noticeably thinned; and 4 = 100% plants are affected; they are dying or dead, and the plot looks sparse.

Effect of crop rotation in combination with cultivar resistance (2012). Experiment parameters (plot size, seeding rate, row spacing, and agronomic management) similar to those in 2011 field trials were used. Five crop-rotation conditions were selected with continuous canola (0-year break), canola-barley-canola (1-year break), canola-barley-field pea-canola (2-year break), canola-barley-field pea-barely-canola (3-year break), and canola-barley-field pea-barely-fallow (4-year break), respectively. The 11-year break (with barley or field pea only) was not used in this trial. Three canola cultivars, rated as susceptible, MR, and resistant under controlled conditions were seeded to each of the rotation conditions. The experiment used a split-plot design with the rotation in main plots and cultivars in sub-plots. The trial was repeated by a second seeding one week after the first one at a different location of the same field, and assessed using the same methods as described above for disease impact and canola yield.

Data analysis. DSI data were transformed with arcsine square root prior to analysis of variances (ANOVA) using SAS. The data for above-ground systems used a pre-transformed 0-4 scale (Little and Hills 1978) and were subjected to ANOVA directly. Yield data were analyzed on the per plot basis. PROC UNIVARIATE was used to examine the data normality. Data from repeated trials were pooled when the homogeneity of variance was confirmed using the Bartlett's test or analyzed separately if non-homogeneous. Because no significant interaction was detected in any of the field trials, means were separated using least significant difference (LSD) at $P \leq 0.05$. Untransformed data were reported in results.

Results and Discussion

Effect of biofungicide formulations on CR and CS cultivars (2011). The weather condition was generally conducive to *P. brassicae* infection and clubroot development at all sites. The disease pressure was high at each field site with DSI ranging from 69% to 98% on the CS cultivar. The CR cultivar 45H29 showed strong resistance, lowering DSI to <15%. None of the biofungicide/fungicide formulations reduced the disease relative to non-treated controls on either CS or CR cultivar. Overall, the yield for the CS cultivar was much lower than that of CR cultivar, with 73% to 81% reduction at the same field site.

Efficacy of *B. subtilis* GB03 seed dressing under varying crop rotations (2011). Plots of varying rotation history showed a pattern of clubroot potential; the plots with only 1-year break from canola had the highest DSI in the bioassay and greatest amount of early pathogen development in canola roots as revealed by qPCR. There was no substantial difference in plant emergence among all treatments: ranging from 37-40 to 39-42 per meter row in trial 1 and 2, respectively. This indicates little impact from varying crop rotation systems or seed treatments used, and therefore any variations observed between different rotational plots will more likely be caused by other factors (clubroot in this case). The DSI was very high in both trials, generally exceeding 90% and reaching 100% in plots of short rotation. None of the biofungicide seed treatment rates reduced DSI regardless of the crop-rotation scenario. Among the rotational treatments, plots with a 3-year break from canola or longer often showed noticeably smaller galls, despite the fact that most of these plants still fell into the highest (3) disease-severity category based on the rating scale used. Therefore, no substantial difference was found in root symptoms between the crop rotation practices.

Further assessment of disease impact on crop condition based on above-ground symptoms showed a quite different picture; while there was no effect found with any of the seed-dressing treatments, the crop condition was much better in plots with a longer break from canola. The crop in plots with only 1-year break appeared worst, with most plants being either dead or dying. Because the canola cultivar used in this study was a susceptible one, the yield was therefore much lower than that of a resistant cultivar (45H29) used in the other study at the same

location. There was no effect of seed treatment on the yield, but longer crop rotations consistently gave higher yields. Even a 3-year break increased the yield substantially over 1-year break. This yield benefit was possibly due to the impact of longer crop rotation on pathogen inoculum load and disease influence reflected by the crop conditions.

Interaction of crop rotation with cultivar resistance (2012). As in 2011 study, longer crop rotations generally reduced *P. brassicae* inoculum in the soil (Table 1). The effect of crop rotation on CS or MR (MS) cultivar was similar to those on the CS cultivar in 2011; DSI was not reduced while the crop impact and seed-yield reduction by clubroot was alleviated. A 3- or 4-year break from a canola crop increased the yield substantially compared to even a 2-year break, although the yield was very low relative to the CR cultivar in the same trial (Fig. 2 and Fig. 3). The longer rotation did not reduce the clubroot impact sufficiently to allow the CS or MS cultivar to reach maximum yield potential in the heavily infested field. Interestingly, a 2-year break from canola increased the yield of CR cultivar by 25% over the back-to-back canola, despite the indifference in DSI or crop impact observed early on. It is not clear how the yield of CR cultivar is affected in the shorter rotations. The rotation is generally tight on many parts of the prairies in favor of canola production, but producers need to be aware of the shortfalls with clubroot control, especially in areas with heavy infestation of the pathogen inoculum. The benefit of longer crop rotation to canola yield was clearly demonstrated in this study, even for a resistant cultivar. For maximum effect, at a 3-year crop rotation (2-year break) should be considered in combination with a CR cultivar in areas of high clubroot risks.

Table 1. The effect of crop rotation on the amount of *Plasmodiophora brassicae* inoculum load in the soil.

A break from canola (years)	Resting spores /g soil (qPCR)
0	2.7×10^6 bc
1	2.9×10^6 c
2	5.7×10^4 a
3	2.1×10^5 ab
4	1.1×10^5 ab

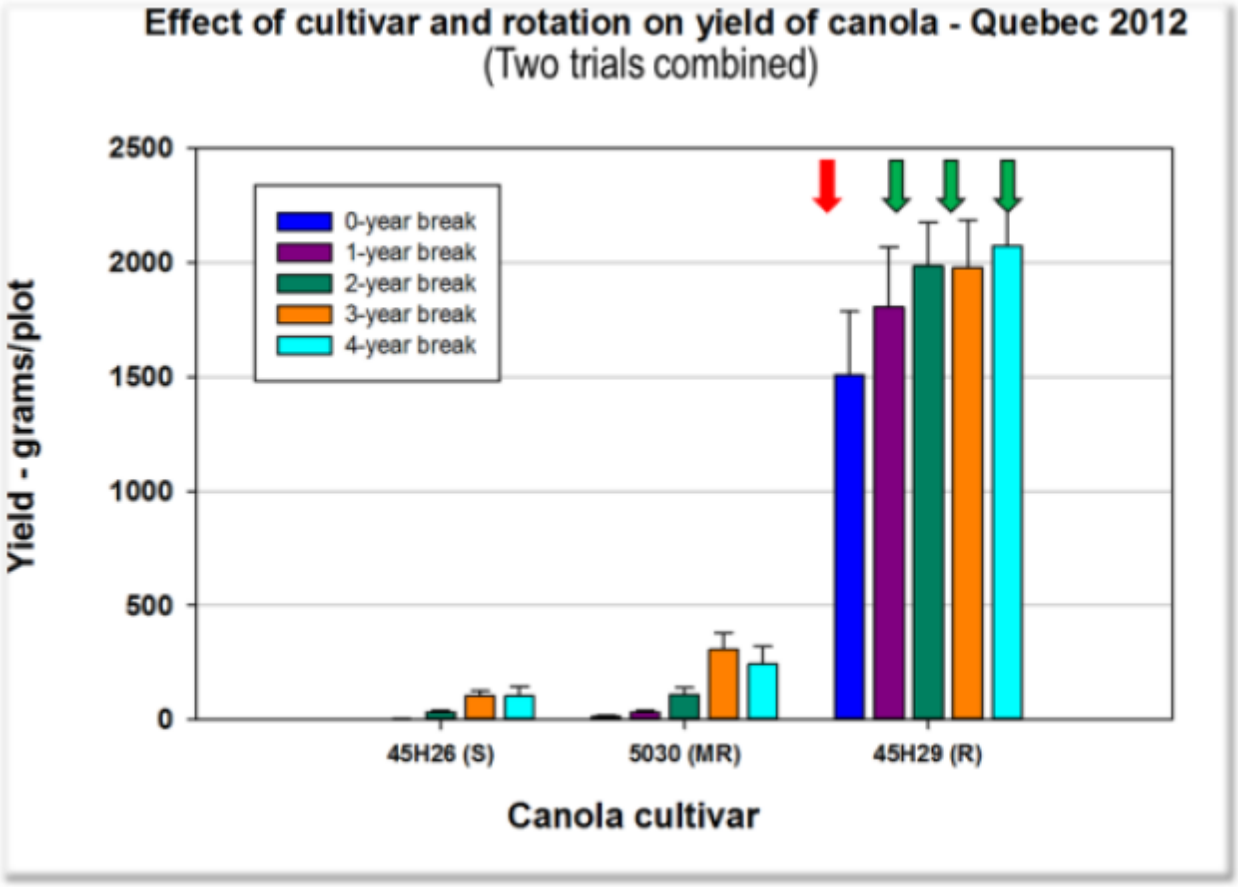
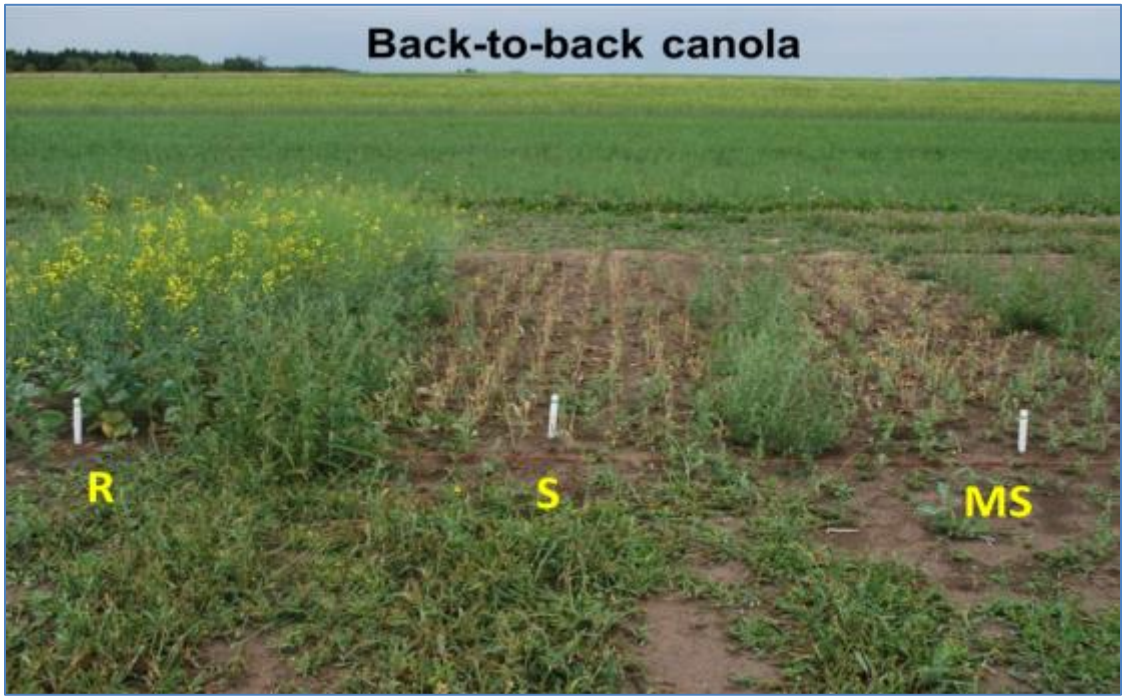
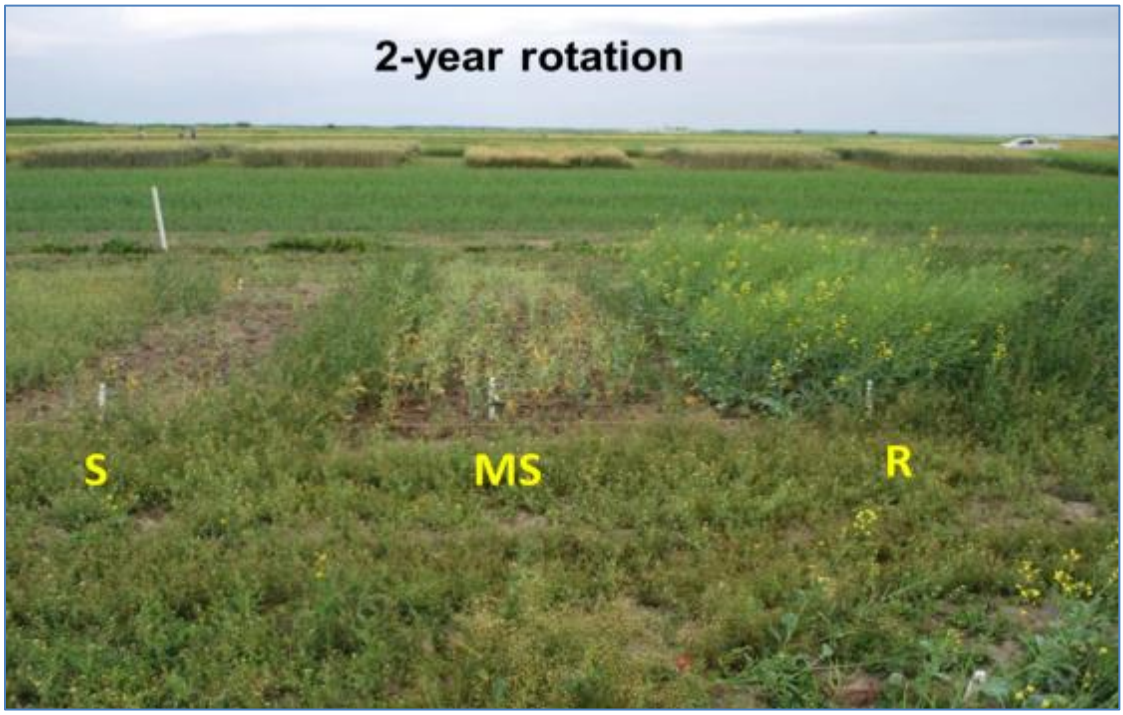
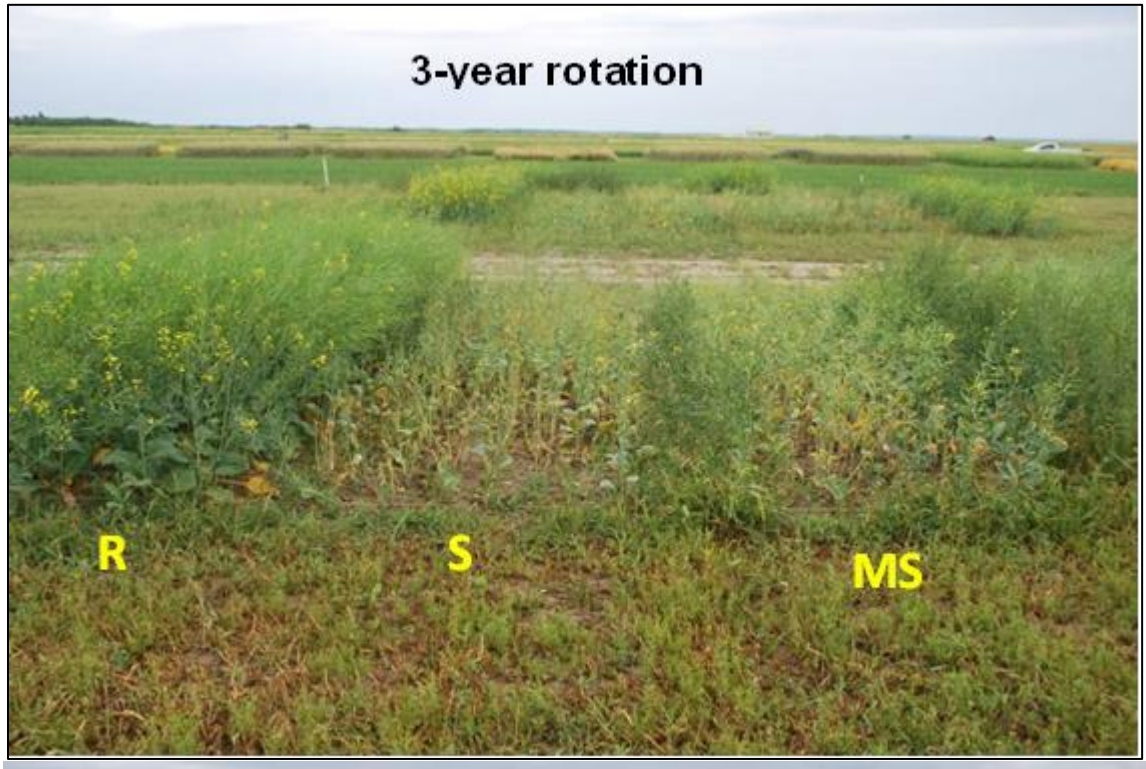
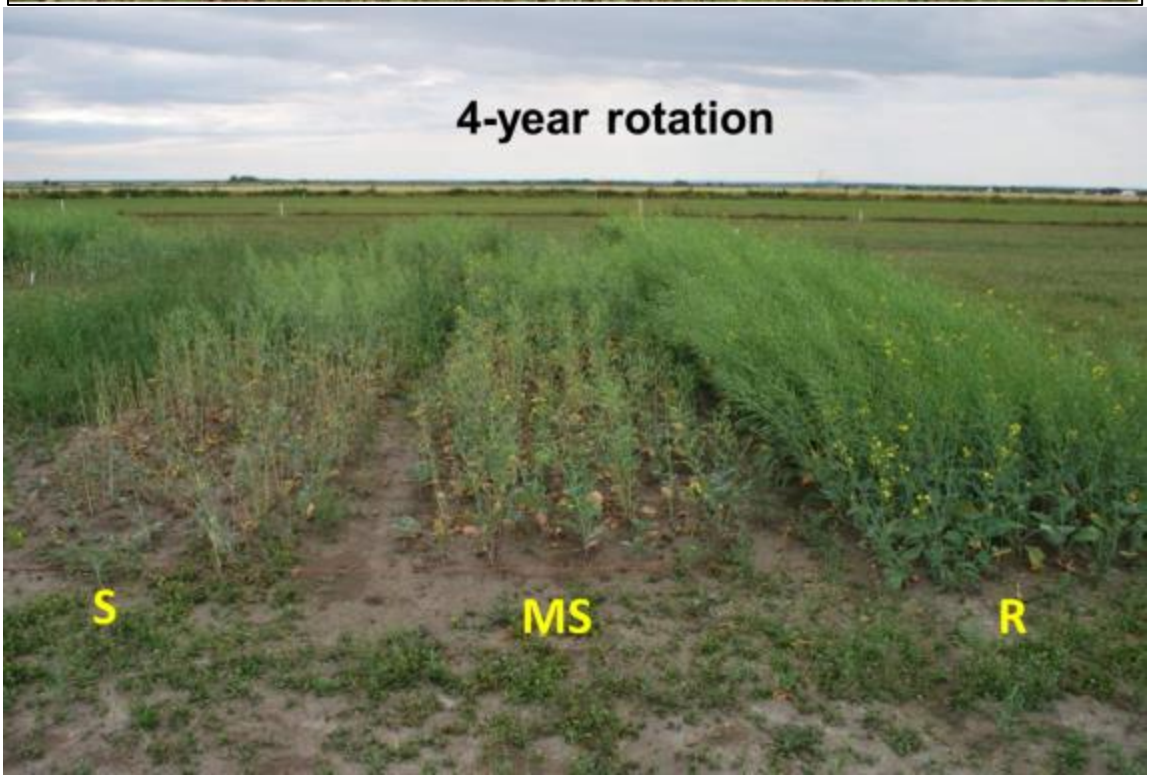


Fig. 2. Effect of crop rotation on the yield of susceptible, moderately susceptible, and resistant canola cultivars in field plots heavily infested by *Plasmodiophora brassicae*.


Back-to-back canola
R
S
MS


2-year rotation
S
MS
R


3-year rotation
R
S
MS


4-year rotation
S
MS
R

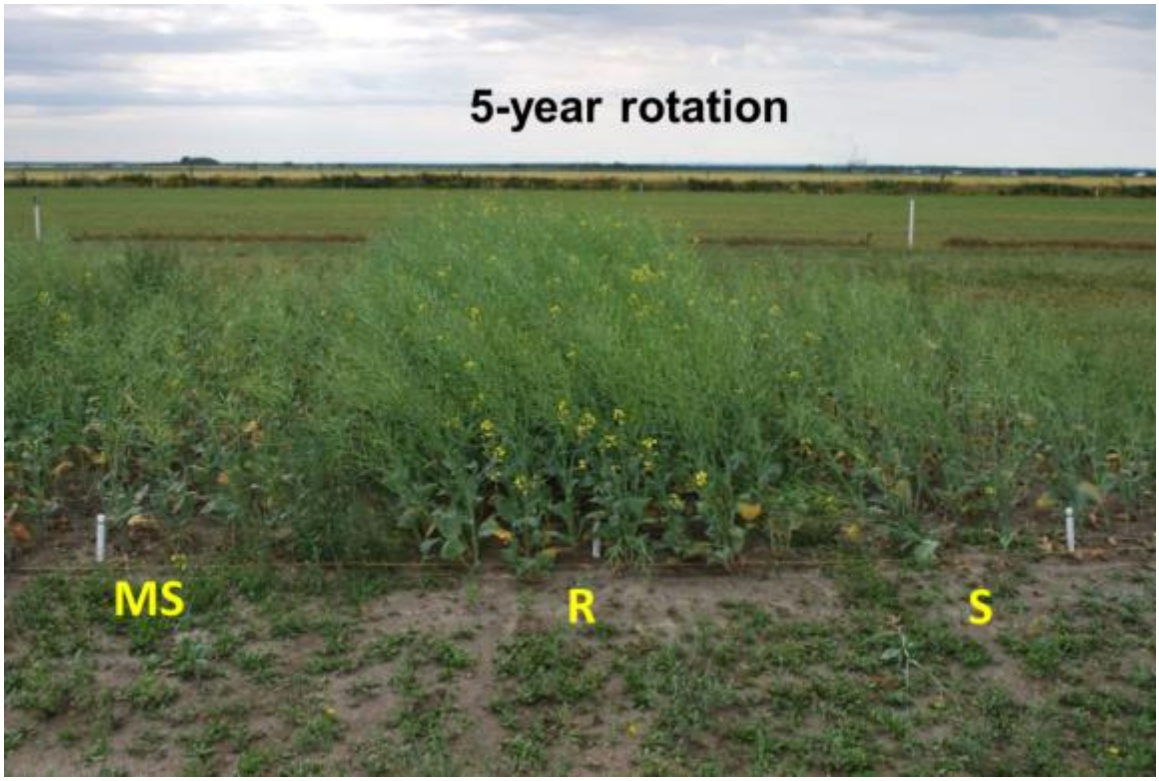


Fig. 3. Crop conditions for a susceptible (S), moderately susceptible (MS), and resistant (R) canola cultivar in a field heavily infested by *Plasmodiophora brassica* under varying crop rotation practices.

Conclusions

The biofungicides or fungicides, with substantial efficacy against clubroot under controlled conditions, showed little effect on disease or canola yield under field conditions, regardless of cultivar resistance. Although the resistance was showed to be the key to successful management of clubroot on canola, a 2-year break from the previous canola also reduced *P. brassicae* inoculum load in soils by 10 fold, alleviating the disease impact. A 2- or 3-year break, however, will not reduce the pathogen inoculum sufficiently in a heavily infested field to allow a susceptible or MS cultivar to reach its yield potential. When a resistant cultivar is used with a >3-year crop rotation (a 2-year break), the pathogen inoculum load in the soil may be reduced and canola yield increased substantially, as opposed to a back to back canola crop.

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FINAL REPORT

RBPI 1370 Objective V

Formulation Development for Biological Control of Clubroot on Canola

Project Lead: RUSSEL HYNES

Collaborators:
Susan Boyetchko

Work Plans and Milestones

2009-10

Evaluate a selected group of formulation ingredients based on prior knowledge and experience for compatibility with target biocontrol agents

Develop seed coating and seed row placed granule formulations for delivering the biocontrol agents to canola rhizosphere and effective clubroot control

2010-11

Continue improving seed coating and seed row placed granule formulations based on canola root colonization, dose effect, and efficacy of clubroot control in controlled environment

2011-12

Optimize and finalize formulation ingredients and process, as well as product delivery strategy in relation to effective and efficient control of clubroot

Initiate shelf-life study for the final formulation of biocontrol agent

2012-13

Complete the shelf-life study for the final formulation of biocontrol agent

Participate in field trials for efficacy of the formulated biocontrol agent in combination with resistant canola cultivar, varying crop seeding dates and/or different crop rotation practices

Materials and methods

Fermentation of *Bacillus subtilis* SER

Mass production of *Bacillus subtilis* SER was achieved using a New Brunswick Scientific (NBS) BioFlo 3000 fermenter. The *B. subtilis* isolate was cultured for 48 hours on Luria-Bertani media before inoculating sterile modified compound media (MCM) in the fermenter and running for 72 hours. The compound media was slightly modified by replacing domestic soybean flour with commercial Sigma soybean flour. Fermenter conditions were set at a minimum dissolved oxygen of 40%, pH = 7 and a temperature of 30°C. Enumerations were completed by diluting with 10 mM phosphate buffer and plated, in triplicates, on nutrient agar with a Spiral Biotech Autoplate 4000. Enumerations of vegetative cells and spores were completed at 22, 24, 28, 36, 47, 48, 53, 59, 70 and 72 hours. To obtain the spore counts, samples were placed in an 80°C water bath for 10-20 minutes after being diluted to 10-1. Vegetative cells were diluted and plated without heating. Cells were incubated for 24 hours at 30°C and counted with a Q Count Model 510.

Coacervate Preparation and Enumeration

The biopesticide *Bacillus subtilis* SER was microencapsulated by complex coacervation followed by lyophilisation to be applied to canola seed coat. The complex coacervate formulation included corn, pea, rice or tapioca starch as filler and protectant for *Bacillus subtilis*. Each starch coacervate was prepared in duplicates. 500 mL of a 2% gum arabic (10g/500 mL) solution with an adjusted pH of ~7 was prepared at room temperature. 50 mL of B. subtilis was transferred to the gum arabic solution. 500 mL of a 2% gelatine solution with an adjusted pH of ~7 was prepared at 35°C. The gum arabic/B. subtilis solution was pumped into the gelatine solution at ~15 mL/min using a Masterflex C/L peristaltic pump. 25 g of Corn, Pea, Rice or Tapioca starch was added and allowed to mix for 15-60 minutes until completely dissolved. The final pH was measured, recorded and then adjusted to 3.75-4.00 with 1 N HCl, added dropwise to facilitate the coacervate reaction between the gum arabic and gelatine. The final solution was transferred to four freeze drying jars, ~260 mL of solution per jar. This solution was frozen with a FTS, Just-A-Tilt shell freezer in -35°C methanol, then freeze dried with a Flexi-Dry MP Freeze Dryer for a minimum of 68 hours. Control coacervates were also made for corn, pea, rice and tapioca starch in a similar fashion excluding the addition of B. subtilis. 10-5 solutions were plated in replicates of 3 with a Spiral Biotech Autoplate 4000 on nutrient agar. A Q Count Model 510 counted the plates following a 24-30 hour incubation period at 30°C. Enumerations for all 4 coacervate starch types were completed every two weeks for eight weeks.

Granular Preparation

A granular formulation was made, in duplicate, with 200 g of corn starch, 41 g peat and 180 mL of *B. subtilis* solution. The solution was mixed with a Viking Professional 1000 water mixer for 1 minute and then extruded

through a domed MG55 Granulator extruder. The extruded substance was then spheronized at 1000 rpm for 10 seconds with a Fuji Paudal Co., Ltd. Benchtop Marumerizer. The resulting granular was then dried in a Microzone Corporation flow cabinet overnight.

Seed Coat and Enumeration

8.8 g of seed were coated in a sterile plastic bag with 200 µL of 1% glycerol, followed by 200 mg of coacervate. Coated seeds were dried overnight in a Microzone Corporation transfer cabinet and enumerated. 1 g of coated canola seed was diluted with 9 mL of 10 mM potassium phosphate buffer (pH 7.0 at 22°C) in a 50 mL sterile conical tube. The solution was vortexed with a Heidolph Multi Reax for five minutes at setting 6. The solution was then serial diluted to 10-4, and 50 µL of the 10-3 and 10-4 solutions were plated in replicates of 3 with a Spiral Biotech Autoplate 4000 on nutrient agar. A Q count Model 510 counted the plates following a 24-30 hour incubation period at 30°C. Enumerations for both trials were completed every two weeks for eight weeks. Cfu/seed was calculated by determining the weight of 100 seeds, in reps of 3. This was extrapolated to determine number of seeds per gram, which was used to convert cfu/1 gram to cfu/seed.

Assessment of Formulations as Bio-fungicidal Agents for *P. brassicae*

Conetainers were filled to ~1 cm from the top with Sunshine #3 soil mix with osmocote. Conetainers were watered with reverse osmosis water and two canola seeds were placed in a small furrow, ~ 1 cm deep. The 1 g of *B. subtilis* granule amended con-tainers did not contain a furrow; 2 untreated canola seeds were placed on top of the soil, followed by 1 g of the granular and a ~ 1 cm covering of soil. 10 mg granular was evenly distributed in the furrow. 4 coacervate starch types containing *B. subtilis* and 4 control coacervates were coated on the seed and placed in the furrow. 1 mL of *B. subtilis* in broth formation was added for the drench formulation. Untreated canola seeds were grown without a treatment as a pathogen control. Pathogen free control plants were grown in a greenhouse and watered every 1-2 days with reverse osmosis water. All 12 treatments were also grown in conetainers with soil inoculated with 5 mL of 105 and 106 P. brassicae spores/mL. *P. brassicae* spores were isolated from dried *B. napus* L galls from infected plants. The galls were homogenized and strained in reverse osmosis water and spore counts were determined with a haemocytometer. The shoot height of control plants were measured once a week for four weeks. The shoot weight was measured for trial two controls and inoculated plants at week four. Weight was measure immediately after removal of shoot from the root, as well as after drying in a heater at 60°C for up to 96 hours. These weights are denoted as wet weight and dry weight, with the dry weight representing the biomass. A disease severity index (DSI) was determined by assessing gall formation on the root of the canola with a rating of 0 to 3.Unpaired t-tests were used to determine significant differences from the control ($P < 0.05$).

Results and Discussion

The population of *Bacillus subtilis* SER spores generated in the Bioflow fermenter after 72 hours of growth was approximately 8 $\log_{10}$ colony forming units (cfu) per mL. Bacillus spores were collected from the fermenter and stored in sterile containers at 4 °C until needed. *Bacillus subtilis* spores were prepared in a granular and complex coacervation (microcapsules) formulations as described in the above. The effect of starch type in the complex coacervation reaction, *Bacillus subtilis* survival in the formulation and on the seed was examined.

Populations of *B. subtilis* detected in the complex coacervate formulation freeze dried powder are reported in Table 1.Starch type, corn, pea, rice or tapioca, did not affect the complex coacervation reaction or the microencapsulation of *B. subtilis*. The granular formulation prepared with spores of *B. subtilis* had 8.3 $\log_{10}$ cfu/g at time 0. Viability, cfu/g, of *B. subtilis* over 16 weeks for the 4 complex coacervate formulations and granular formulation are reported in Fig. 1. There was no significant change in the population of *B. subtilis* over 16 weeks. This is consistent with other formulations (unpublished results, trade secrets and proprietary information) that have been used to carry the highly resistant spores of *Bacillus subtilis*. However, to the best of my knowledge, the complex coacervate formulation developed in this study is the first seed coat delivery system for Bacillus. Viability of *B. subtilis*, expressed as $\log_{10}$ cfu/g coated canola seed, over 8 weeks for the 4 complex coacervate formulations are reported in Fig. 2. There was no significant change in the seed coat population of *B. subtilis* over 8 weeks. These results demonstrate that the complex coacervate formulation when applied to canola seed provide satisfactory protection from naturally occurring seed coat compounds, dry environmental conditions and with the aid of the sticker, glycerol (1%), handling.

Table 1. Effect of starch type on the initial population of *Bacillus subtilis* SER in complex coacervate formulations (reported as colony forming units per g formulation, cfu).

Starch Type	Log_{10} CFU/g ± Standard Error
Corn	8.62 ± 0.02
Pea	8.64 ± 0.03
Rice	8.74 ± 0.06
Tapioca	8.48 ± 0.14

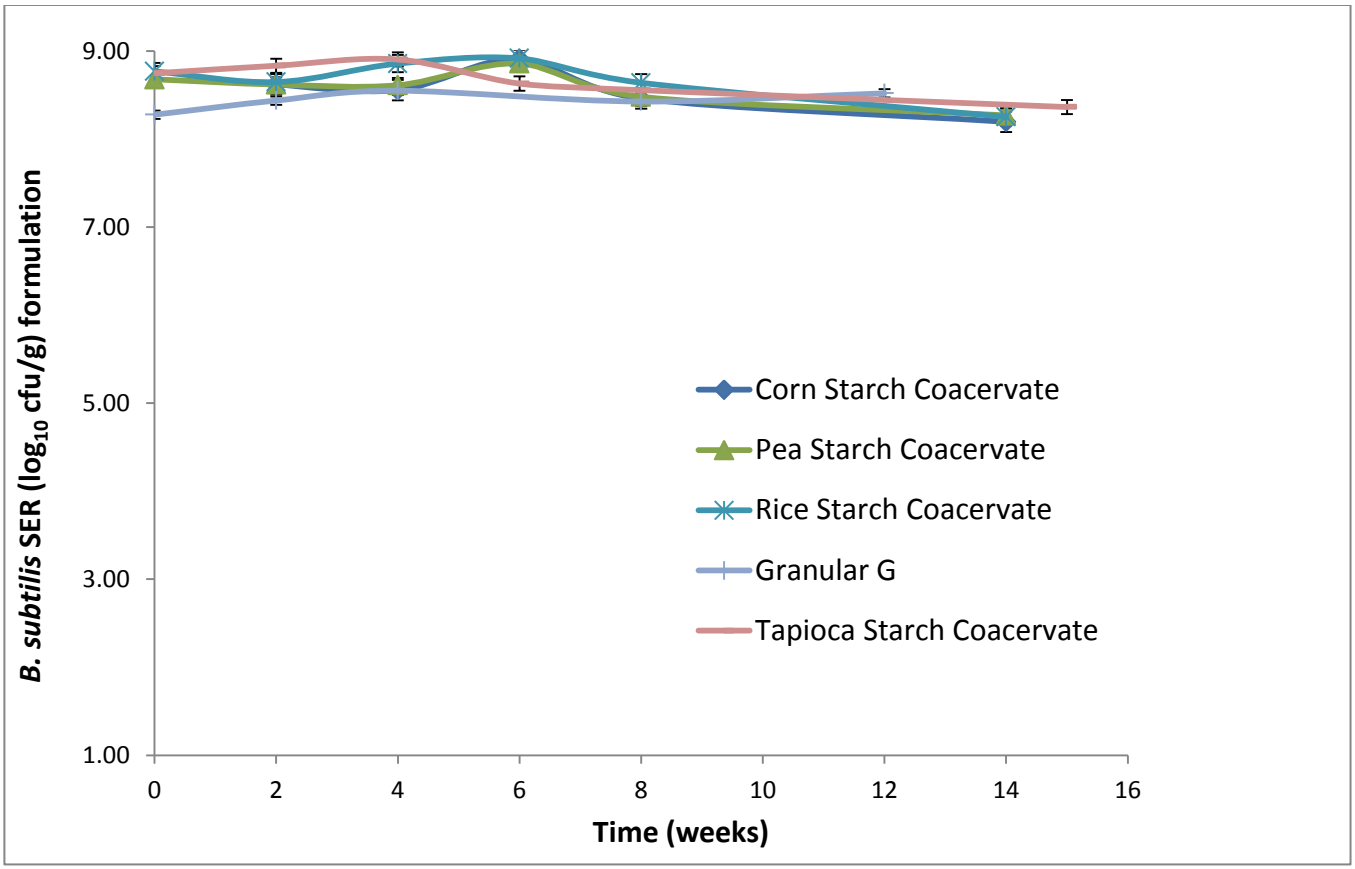

Fig. 1. Shelf-life study of *Bacillus subtilis* SER in complex coacervate formulation prepared with 4 starches, corn, pea, rice, tapioca and the granular (G) formulation. Error bars indicate stardard errors of the means of two experiments.

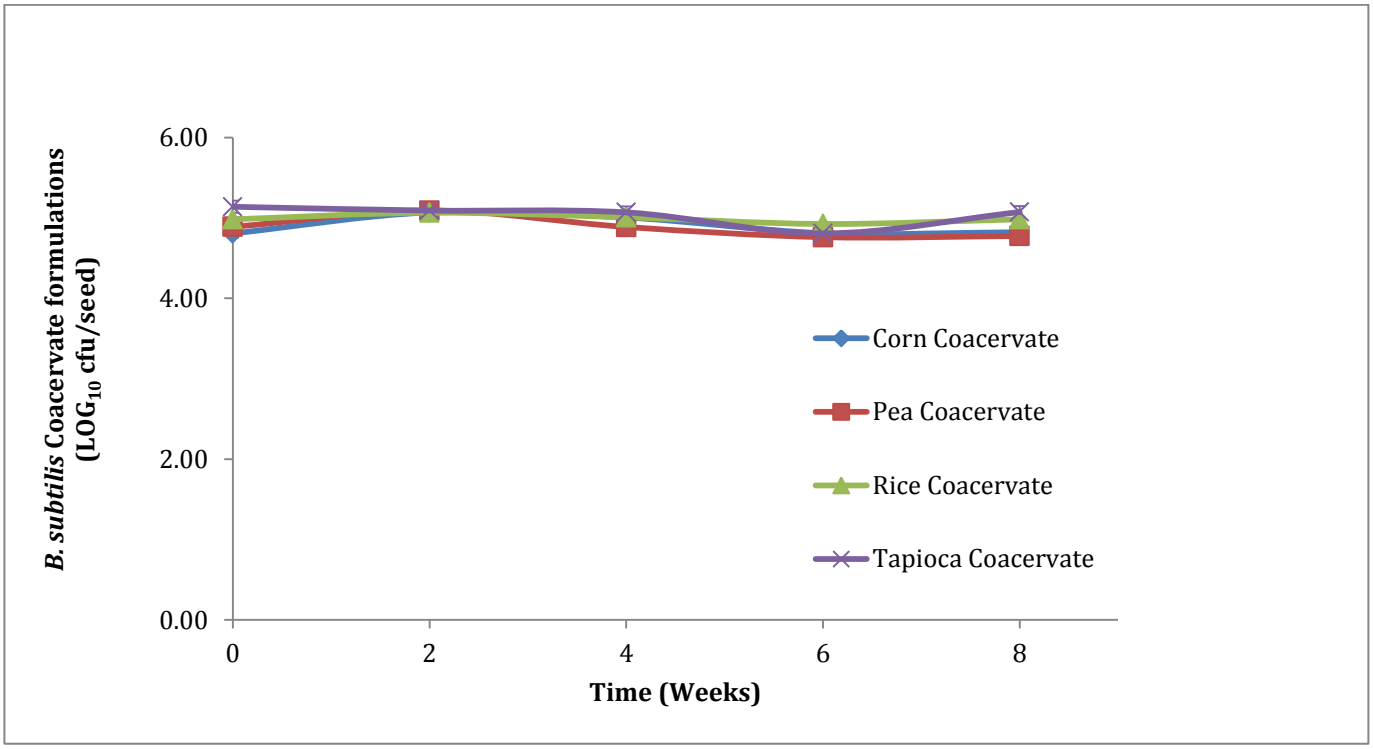

Fig. 2. Shelf-life study of canola seed treated with the *B. subtilis* complex coacervate formulation. Standard error ranged from 0.03 – 0.07 cfu/g seed.

The effect of the *B. subtilis*-complex coacervate formulation on germination following Weststar canola seed treatment was examined at 10, 18 and 25 °C in cone-tainers in plant growth chambers. There was no significant difference in seed germination with canola treated with the complex coacervate formulations at 10, 18 and 25 °C and the 2 watering (2and 6 mL/day) prepared with corn, rice, pea or tapioca starches as compared to the control, non-treated seed (entire data set not shown). Similarly, the granular and drench formulation did not affect canola germination at the three temperature and 2 watering regimes. The results of emergence of Westar canola at 18°C in wet (6 mL water/day) or dry (2 mL water/day) conditions are presented in Fig. 3. Predictability of seed germination under favorable conditions is important, consequently, a benign response to seed germination following seed coat application of the formulated biopesticide is a key observation and satisfies one of the key desires of seed treatments.

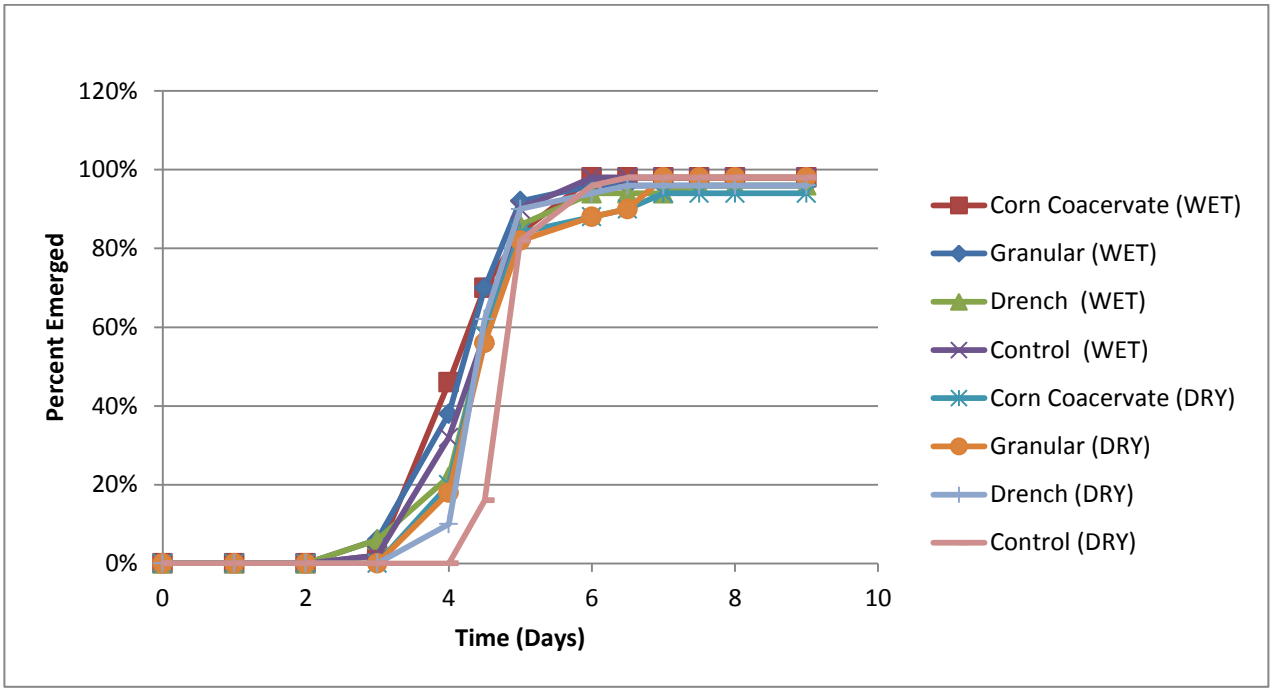

Fig. 3. Emergence of Westar Canola at 18°C in wet (6 mL water/day) or dry (2 mL water/day) conditions.

The effect of the 4 seed coat applied formulations (Fig. 4), granular and drench formulations on canola shoot growth in the absence (disease free) of the clubroot pathogen, *P. brassicae,* in the greenhouse was recorded weekly to 4 weeks (Fig. 5). The results suggest a small increase in shoot height following application of any one of the formulations including the seed coat applied formulations without the biopesticide *B. subtilis*. This may be due to the natural microbial flora consuming dispersed formulation ingredients in the canola rhizosphere and secreting plant growth promoting compounds that are then taken up by canola.

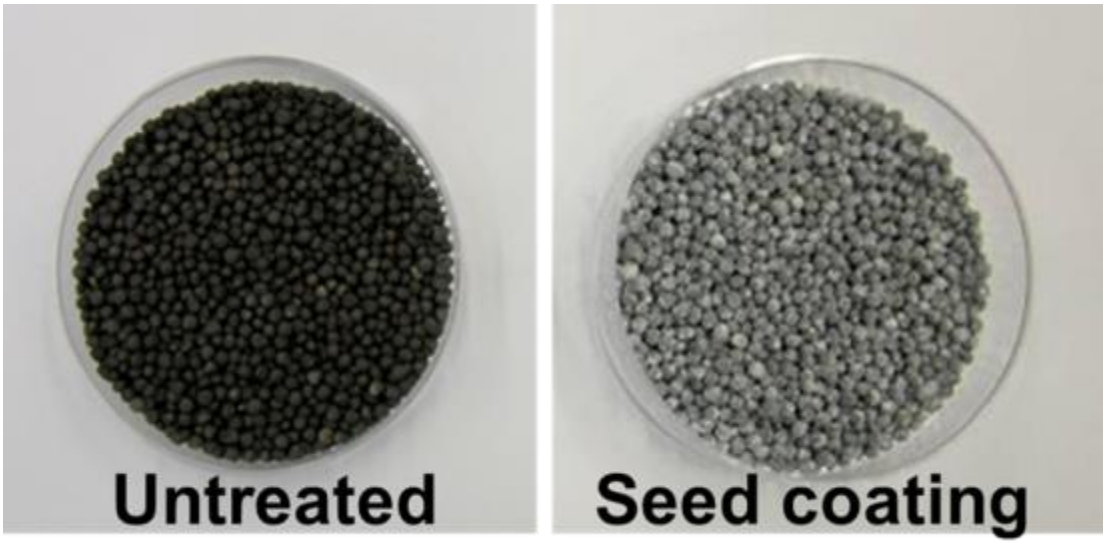

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Fig. 4. Coating of canola seed with the biofungicide Serenade (*B. subtilis*) for protection against clubroot.

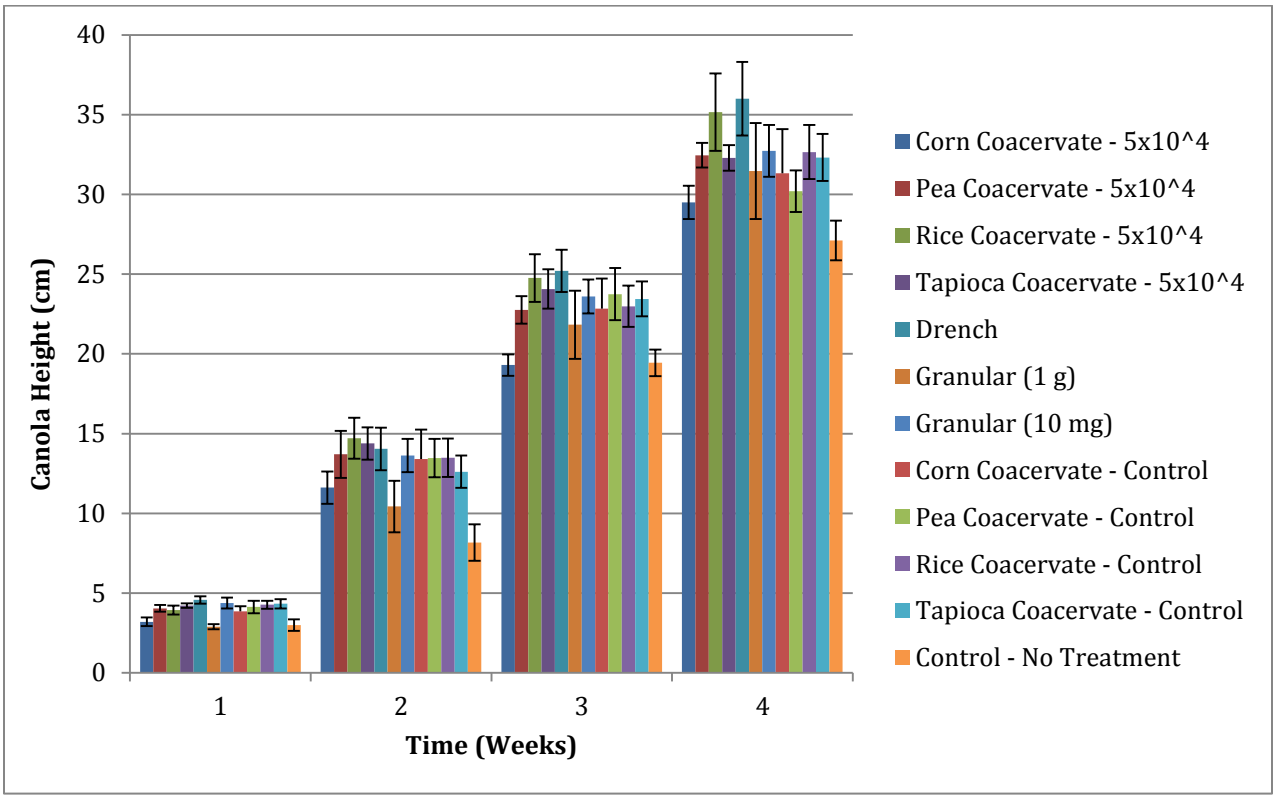

Fig. 5. Westar canola shoot growth in the absence of clubroot pathogen *P. brassicae*.

Plant growth room and field experiments were carried out to examine the effect of formulated biopesticide, *B. subtilis,* on disease severity by the clubroot pathogen, *P. brassicae*, on canola. Inconsistent results from the plant growth chamber studies (Fig. 6) were observed following application of 5 $\log_{10}$ cfu/seed B. subtilis in a seed coat applied or granular formulation (per g formulation basis) . Field studies showed no effect of seed coat treatment or soil application of the formulated biopesticide (data not shown).

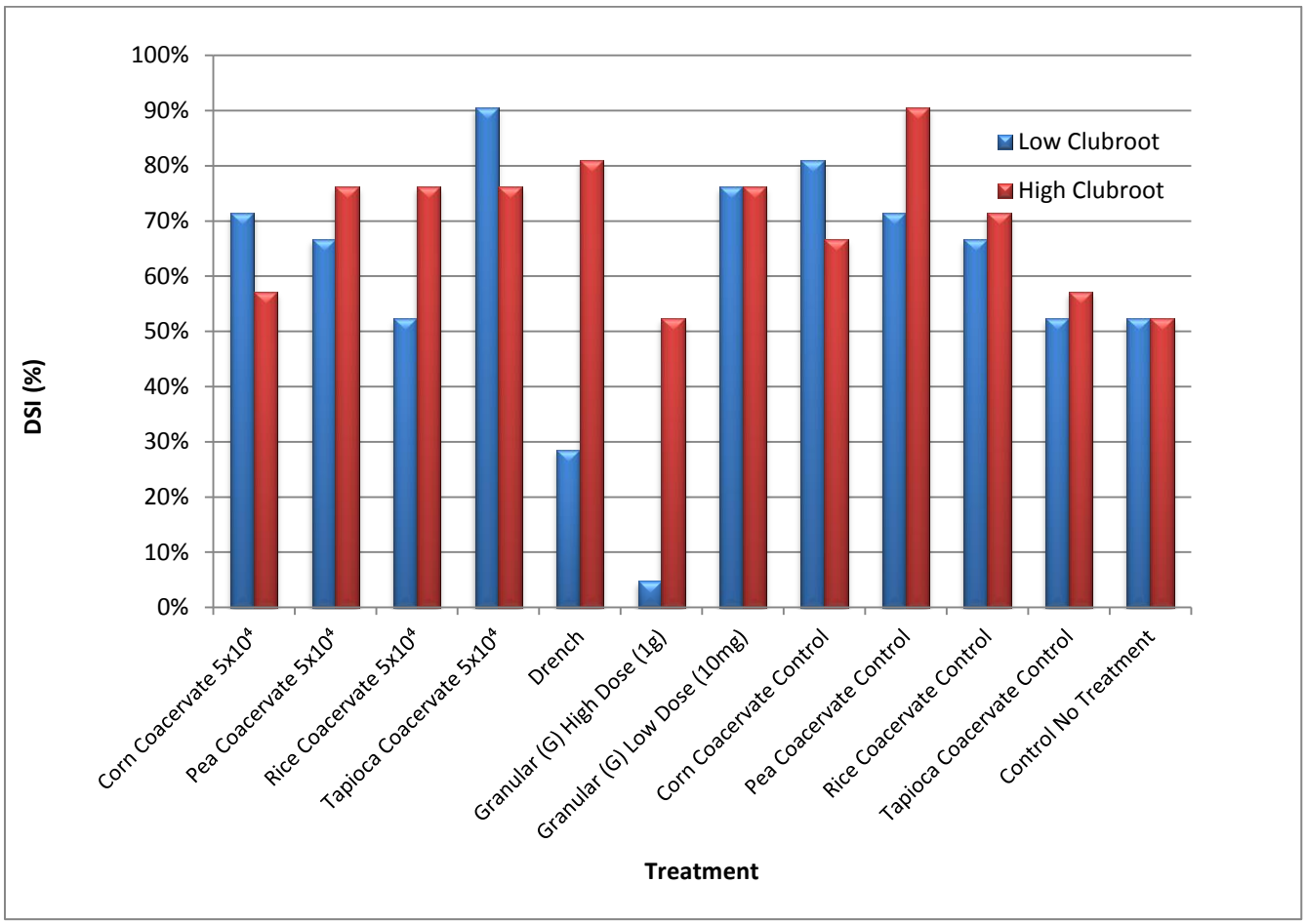

Fig. 6. Effect of biopesticide *B. subtilis* SER on disease severity index (DSI) of clubroot of canola at high (6 $\log_{10}$ spores/cone-tainer) and low (5 $\log_{10}$ spores/cone-tainer) applied as a seed coat, granule and liquid formulations.

Conclusions and New Discoveries

The following conclusions were drawn from this component of the study.

2. The viability of biopesticide *Bacillus subtilis* SER microencapsulated by complex coacervation followed by lyophilisation was stable for at least 16 weeks. This is a novel formulation for *B. subtilis*.
3. The viability of biopesticide *Bacillus subtilis* SER in novel soil applied granules was stable for at least 16 weeks.
4. The seed coat applied formulation of *Bacillus subtilis* SER did not affect canola seed germination or have a significant effect on canola shoot height up to 4 weeks after seeding as compared to the non-treated control.
5. Growth chamber and field studies with the seed coat formulation and granular formulation of Bacillus subtilis did not reduce disease severity index of clubroot disease of canola.

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