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Survey & evaluation of soil microbes for clubroot control on canola

(Final report - Gary Peng, AAFC Saskatoon)

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Executive Summary

Clubroot of canola, caused by the protist pathogen *Plasmodiophora brassicae* (Pb), is an emerging threat to canola production in western Canada. The disease has been found in more than 450 fields in Alberta, and the pathogen was confirmed present in a Saskatchewan field in 2009. This study was initiated to assess microbial biofungicides as well as indigenous soil microorganisms for control of clubroot on canola.

Selected biofungicides were initially applied as a soil drench and the fungicides Allegro and Ranman were also included for comparisons. Promising products were further evaluated at varying concentrations and drench volumes, as a seed treatment, and later in combination with resistant cultivars in controlled and field conditions to determine the most effective treatments or treatment combinations.

In controlled environment, the biofungicides Serenade and Prestop, and synthetic fungicides Allegro and Ranman were highly effective under moderate disease pressure, reducing the severity of clubroot on canola by 85–100%. Product rates appeared to be important while soil-drench volumes were less critical. These products, however, can sometimes be less efficacious or ineffective under extremely high disease pressure. Seed treatment showed the potential of this delivery approach but more work is required on microbial formulation to pack more products on seeds or stimulate the microbial activity in the soil. Several resistant canola cultivars/lines reduced the impact of clubroot significantly. Resistance and fungicides may be used in combination for synergy to enhance clubroot control and aid in resistance stewardship.

Additionally, a total of 5,152 microbial isolates were obtained from the rhizosphere or interior of canola roots collected from Alberta and Saskatchewan, and screened for potential clubroot control using a tiered system. About 390 isolates were selected and tested against clubroot on canola. Three fungal endophytes showed high efficacy (>75%) while several fungal and bacterial isolates reduced clubroot by more than 50%. These candidates will be assessed further for potential development.

Under field conditions, two canola trials were carried out in Alberta, one canola and one Chinese-cabbage trials (a model system) were conducted in Ontario during the 2009 crop season. In each trial, resistant (R) and susceptible (S) cultivars were used. A spring drought in Alberta caused poor and late crop emergence, and product efficacy was not determined for one trial and insignificant for the other. In Ontario, the canola trial suffered from poor emergence and uneven pathogen inoculum distribution in field plots, and therefore trial data were not reliable. The Chinese-cabbage trial received rain shortly after seeding and showed a moderate level of clubroot severity in untreated S control plots. The fungicide treatments reduced clubroot severity by 54% to 84% when compared to the untreated S-cultivar control and there was no substantial difference among the products used. The varying product efficacy in different trials may be caused by different environmental conditions, especially the soil moisture after seeding. Cultivar resistance was significant in all three trials assessed, often reducing clubroot damage by > 90% when compared to S cultivars.

The biofungicides Serenade and Prestop, and synthetic fungicides Allegro and Ranman showed efficacy against clubroot of canola in both controlled and filed conditions. The data from field trials are inconclusive due to the drought condition after seeding or poor seed quality, but continued research in microbial formulations and delivery approaches will likely further improve the performance and practicality of these products in canola cropping systems. There is a potential to use these products with R cultivars for enhanced clubroot control and resistance stewardship.

Highly efficacious microbes identified from indigenous canola roots represent a new source of clubroot control agents. Resources will be pursued to fast track biological assessment of these promising candidates and develop them as new treatments against clubroot on canola.

Background and Rationale

Since the first discovery near Edmonton in 2003 (Tewari et al. 2004), clubroot has been found in more than 15 counties in Alberta (Alberta Agriculture and Food 2008) and is becoming an emerging threat to the canola industry (Financial Post 2007). All commercial canola cultivars are highly susceptible (Strelkov et al. 2006). The pathogen builds up rapidly on susceptible crops and can also persist in soils for many years when a suitable host is absent.

Prior to 2009, there is generally a lack of practical control options against clubroot in canola. A resistant (R) canola cultivar became available commercially in 2009. However, clubroot resistance is generally single-gene based and is against only certain races of the pathogen. Therefore, resistance stewardship is important to the longevity of R cultivars. Although crop rotation reduces pathogen inoculum load in the soil (Klasse 1996) and alleviates disease impact on less susceptible varieties (Wallenhammar et al. 2000), rotation intervals are too long due to pathogen's ability to persist in the soil with resting spores and to infect many weed species in the mustard family. In western Canada, potential impact of the disease is huge due to the size and intensity of canola production. Most management strategies developed for other crop systems are impractical for canola due to prohibitive costs. Experiences show that finding resistant genes against multiple pathotypes will likely be difficult (Diederichsen et al. 2006; Hirai 2006). Fungicides occasionally provide disease suppression (Donald et al. 2006; McDonald et al. 2004) but the cost (up to $900/ha) and application methods (e.g. soil incorporation) make these practices unsuitable for canola. Soil liming (Tremblay et al. 2005), calcium cyanamide (Donald et al. 2006; McDonald et al. 2004),

and a phosphonate product (Abbasi and Lazarovits 2006) have been shown to reduce clubroot on cruciferous vegetables, but they are likely impractical under most circumstances in field crops; Calcium cyanamide costs at east $400/ha (Donald et al. 2004) and the water volume required for phosphonate drench is over 30,000 L/ha on muck soils.

Recent research on Chinese cabbage in Japan highlights the potential of using soil microbes to control clubroot (Arie et al. 1998, Narisawa et al. 1998). Microorganisms, especially those of plant endophytes or rhizosphere colonizers, may move with roots and potentially protect them. This mechanism may be particularly useful for protection against clubroot due to long infection period by the pathogen in the soil. Several biofungicides targeting other soil-borne diseases show the ability of root colonization and may be evaluated for control or suppression of the disease on crucifers. This trait of root colonization may facilitate efficient delivery of the biofungicides as "inoculants", through seed treatment or in-furrow application, to achieve long-term root protection. Some of these biofungicides have been registered or in the process of registration in Canada for greenhouse and horticultural crops or registered in the US, including Actinovate, Mycostop, Prestop, Root Shield, Serenade, SoilGard, and Taegro. The potential of these products for clubroot control is not known but most of them have showed a general ability to colonize plant roots, compete with or suppress other soil pathogens. If proved effective, some of these products may be used in canola crops quickly because of their registration status in Canada.

The goal of this project is to evaluate a diverse group of soil microorganisms and microbial biofungicides products for potential clubroot control with the following two objectives: 1) to assess selected microbial fungicide products for clubroot control on canola; 2) to isolate and evaluate rhizosphere and endophytic inhabitants of canola roots indigenous to the Canadian prairies for clubroot control. Overall, it is hoped that effective microbial products or agents can be used as a component in integrated clubroot control on canola.

Materials and Methods

Study I. Evaluate efficacy of microbial fungicides in controlled conditions

A conetainer system (**Figure 1**) was used to product canola plants for clubroot control efficacy trials carried out in the level-II pathology containment at AAFC Saskatoon, and in 4" pots in greenhouse at the Crop Development Centre north, Alberta Agriculture and Rural Development. These systems allow canola roots and plants to develop normally for 6-8 wks, proving sufficient time for clubroot to develop after inoculation.

Canola plants

Seeds of a commercial Roundup-ready canola cultivar (Fortune RR in containment trials and cv. 34-6S RR in greenhouse trials) were sown in a soilless mix [one part sand to approximately twelve parts of 1:2 sphagnum peat moss:vermiculite, amended with 1% (w/v) of 16-8-12 (N:P:K) control-released fertilizer] in individual conetainers. Plants were kept at 23/18ºC (day/night) in a growth cabinet in the containment or the greenhouse with 14 h supplementary daily lighting.

Plasmodiophora brassicae (Pb) inoculum

Galls of canola clubroot were collected from multiple fields in central Alberta infested with several pathotypes (races) of the pathogen, air dried, and stored at -15ºC until use. To extract resting spores, about 3 g of dry galls were soaked in 150 ml distilled water for 2 h to

soften the tissue and macerated in a Waring blender at high speed for 2 min. The resulting slurry was filtered through 4 layers of nylon cloth and spore concentrations in resulting suspensions were estimated using a hemacytometer.

Inoculation and disease assessment

About one wk after seeding, each plant was inoculated with 1-5 ml of Pb inoculum at 10^6 or 10^7 resting spores/ml. Inoculated plants were watered with acidified water (pH 6.3) and the soil kept saturated for the first week. After the first week, the plants were watered with tap water (pH 8.0 – 8.5) when required. Clubroot development was rated 3 wks after inoculation using a 0-3 rating 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-sized galls on more than 2/3 of roots (**Figure 2**). Disease severity index (DSI) was calculated based on the weight of each rating class observed

DSI (%) = $\sum$ (rating scale x No. of plants in the scale) x100 / (total No. of plants in the rep) x3

Figure 1. A conetainer system used for efficacy screening against clubroot root on canola

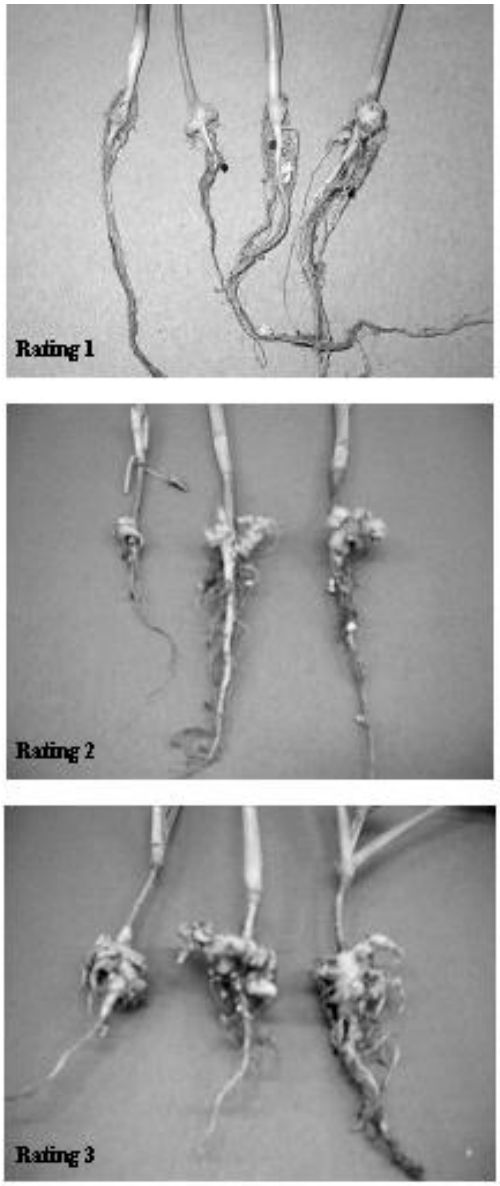

Figure 2. A pictorial key for assessment of clubroot severity

Expt I. Disease response to Pb inoculum dose

This experiment was to determine the impact of pathogen inoculum pressure and identify a range of Pb doses that would cause a moderate severity of clubroot for screening of biocontrol efficacy. Previous experiences indicated that extremely high disease pressure could overwhelm biocontrol treatments. Pb inoculum at the concentrations of 0 (control), 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 resting spores/ml were drench applied to the growth medium around 1-wk old canola seedlings at 1 to 5 ml per plant, and inoculated plants were assessed 3 to 5 wks after inoculation for clubroot development using the 0-3 scale.

Expt II. Efficacy trials in the containment at AAFC Saskatoon

Plants of canola cv. Fortune RR were produced in individual conetainers in growth cabinets (18–23ºC) and the following biofungicide/fungicide treatments were applied:

Treatments:

1. Control (blank)
2. Pathogen (check)
3. Mycostop® - Verdera Oy
4. Prestop® - Verdera Oy
5. Root Shield® - BioWorks Inc.
6. Actinovate® - Natural Industries Inc.
7. Taegro™ - Novozyme (registered in USA only)
8. Serenade® ASO - Agraquest Inc.
9. SoilGard®12 G – Certis USA (registered in USA only)
10. *Heteroconium chaetospira* Hc-M4006 (non-registered, provided by Dr. Narisawa, Japan)
11. Allegro 500F – ISK Biosciences Corp (fungicide)
12. Ranman® - ISK Biosciences Corp (fungicide)

Product rates and application timing: biofungicides (treatment 3-9) were applied at 5x label-rate concentrations and chemical fungicides (11 and 12) at 1x label rates. The treatment 10 was a fungal endophyte that had demonstrated high efficacy against clubroot on Chinese cabbage in Japan. Conidia of the fungus were produced on barley grains and conidial suspensions at10^6 spores/ml used. All treatments were applied as a soil drench 7-10 d after seeding at 50 ml/plant to saturate the growth medium. Biofungicide products/agents were applied 3 d prior to inoculation with the pathogen to allow the microorganisms to establish on canola roots whereas the two chemical fungicides were applied one h after the pathogen. Pb inoculum at10^6 resting spores/ml was applied at 5 ml/plant, the plants were assessed 3 wks after inoculation.

Experimental design and data analysis: the trials used a CRD with 7 plants per treatment. A total of 3 trials (repetitions) were carried for each product between July 25 and October 10, 2008. Data for individual trials (repetitions) were analyzed based on the disease rating (0-3), but data over 3 repetitions were analyzed using DSI calculated for each repetition. All data were subjected to ANOVA and, if significant (P=0.05), LSD was performed.

Expt III. Efficacy trials in greenhouse (CDC North, Edmonton)

Trials were conducted in a research greenhouse (20–22°C) at Crop Development Centre (CDC) North, Alberta Agriculture and Rural Development in Edmonton in collaboration with Drs. Strelkov and Hwang. The experimental protocol differed only slightly from the growth cabinet trials; canola cv 34-6S RR was seeded in Pb-infested field soil in 4-in pots or a non-infested soilless growth medium later inoculated with Pb inoculum. The treatments were applied as a soil drench at 50 ml per pot as follows:

Treatments

1. Pathogen control (check)
2. Mycostop® - Verdera Oy
3. Prestop® - Verdera Oy
4. Root Shield® - BioWorks Inc.
5. Actinovate® - Natural Industries Inc.
6. Serenade® ASO - AgraQuest Inc.
7. Allegro 500F – ISK Biosciences Corp
8. Ranman® - ISK Biosciences Corp
9. Calcium Cyanamide (another fungicide)

The biofungicides (treatments 2–6) were applied at 5x label rate and fungicides (treatments 7–9) at 1x label rate 1 week after seeding. The Pb inoculum was applied to non-infested growth medium 1 h after the treatment application. Inoculated plants were kept in the greenhouse for 8 weeks to allow the development of clubroot symptoms.

Two trials were conducted between October 2008 and February 2009. In Trial 1, the infested field soil was mixed with soilless growth medium at a ratio of 1:1 (v/v) and the Pb inoculum (10^7 spores /ml) was applied to non-infested growth medium at 2 ml /pot. The Pb inoculum doses were reduced in Trial 2 by mixing the infested field soil with the soilless medium at a ratio of 1:2 or applying Pb inoculum at 1 ml /pot to non-infested growth medium. The experimental design was a RCBD with 4 replicates. Within a block, every treatment (including pathogen control) was applied to 10 plants.

Expt IV. Effect of seed treatment (CDC, Edmonton and AAFC Saskatoon)
These trials were conducted at CDC north (two greenhouse trials, seeded in 4-in pots) and AAFC Saskatoon (one trial using conetainers in a growth cabinet). Canola seeds were immersed in a product solution/suspension for 5 min and air dried for 1 h prior to seeding. In the greenhouse trials, seeds were treated with biofungicides at 5x and fungicides at 1x label rate concentrations and planted into Pb-infested field soil (mixed with soilless growth medium at 1:1 and 1:2 rates in Trials 1 and 2, respectively) or a non-infested soilless growth medium later inoculated with Pb inoculum (10^7 spores/ml, 2 ml and 1 ml/pot for Trials 1 and 2, respectively). In the growth cabinet trial, all products were prepared at 10x label rate concentrations, and treated seed were seeded to a non-infested soilless growth medium later inoculated with Pb inoculum (10^7 spores/ml, 5 ml/plant). Treated plants were kept for 8 weeks for greenhouse trials and 3 wks for the growth cabinet trial, and then assessed for clubroot development. The eight products used in Experiment III were tested in greenhouse trials but only Serenade, Prestop, Allegro and Ranman were evaluated for seed treatment in the growth cabinet trial, based on earlier soil-drench efficacy under the same condition.

Expt V. Effect of product concentration and drench volume (AAFC Saskatoon)
Canola plants were produced in conetainers and the following treatments were applied:

Treatments
1. Control (blank)
2. Pathogen (check)
3. Prestop® - Verdera Oy
4. Serenade® ASO - Agraquest Inc.
5. Allegro 500F (fungicide) – ISK Biosciences Corp
6. Ranman® (fungicide) - ISK Biosciences Corp

The biofungicides were applied at 1x and 5x label rate concentrations at 5 d after seeding and fungicides at 1x label rate at 8 d after seeding. All products were applied as a soil drench at 50 ml and 25 ml /plant, respectively. The Pb inoculum (10^7 resting spores /ml) was applied at 5 ml /plant at 8 d after seeding, 1 h prior to the fungicides. Inoculated plants were kept in growth cabinets for 3 weeks before rating.

Study II. Survey and evaluate indigenous soil microbes for clubroot control

Sample collection

During the early spring of 2008, the emergence of canola crops were late on the prairies due to cold temperatures and a lack of soil moisture. To overcome the delays caused by the field condition, soil samples were collected initially from various soil zones in Saskatchewan and used to grow canola plants in greenhouse for the isolation purpose. *Brassica napus* and *B. rapa* were planted in over 20 field soil samples in the greenhouse (22/17°C,14-h light), and roots were sampled after 10 to 30 d after planting.

During early July and late September, 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.

Isolation of rhizosphere inhabitants

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 system for selection of biocontrol candidates

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. Three bioassays, progressively relating closer to interacting with clubroot pathogen/disease in more realistic field scenarios, were used to detect varying types of microbial interaction. In earlier tiered assays, an isolate of *Pythium ultimum* Trow was used as an indicator pathogen target for rapid efficacy evaluation because the clubroot pathogen could not be cultured on artificial media and trials involving Pb inoculum were not allowed outside the containment.

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 (**Figure 3**). 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.

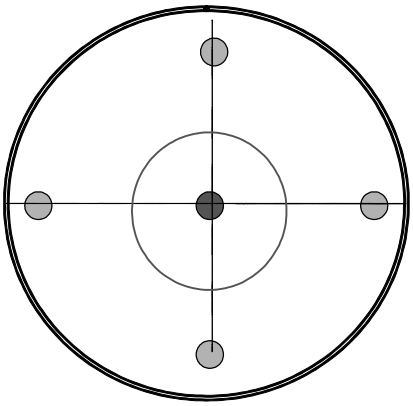

Figure 3. Candidate (grey) and pathogen (black) cultures in antibiosis/competition assay.

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 (200 ml of distilled water per plate), 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. Two canola seeds (cv. Fortune RR) were planted at about 2-cm depth and biocontrol treatments applied as a soil drench at 25 ml per conetainer. Inoculated plants were kept in a growth cabinet at 23/18ºC for 3-4 wks, and clubroot development rated using the 0-3 scale. Each conetainer was thinned to 1 at the 1st true-leaf stage. Untreated plants were used as controls for each trial. The experimental design was CRD with 7 replicates. Candidates consistently provided >50% efficacy were tested again in more repetitions.

Study III. Efficacy of selected fungicides/biofungicides in field conditions

In earlier trials under controlled conditions, several synthetic and biofungicides showed high efficacy against clubroot on canola, and these products helped prevent clubroot completely on R canola cultivars. This combined use of R cultivar and fungicides/biofungicides may be of value for enhanced efficacy of clubroot control as well as for resistance stewardship. Field trials were conducted to assess selected fungicide products for clubroot control under field conditions and to determine potential synergy of a fungicide with cultivar resistance in clubroot control.

The biofungicides Serenade (AgraQuest Inc.) and Prestop (Vedera Inc.), synthetic fungicides Allegro (ISK Biosciences Corp) and Ranman (FMC and ISK) were selected for field trials in Alberta and Ontario. Directly seeded Chinese cabbage was used in one of the trials in Ontario as a model system due to different pathogen race structure and climate conditions there. In Alberta, trials were set up in two different commercial fields with heavy clubroot infestation (predominant pathogen race: pathotype 3). In Ontario, trials were carried out on the Muck Soil Research Station, University of Guelph. The predominant pathogen race there is the pathotype 6 which tends to be less virulent to certain canola cultivars.

For each trial, both R and S cultivars were used to evaluate if there is synergy between the cultivar resistance and fungicide treatment. Due to different pathogen race structures in Alberta and Ontario, the following R and S cultivars were used:

In Alberta:

Canola:	08N825R (R) and 45H26 (S)

In Ontario:

Canola:	Pioneer 041 (R) and 042 (S)
Chinese cabbage:	Yuki (R) and Mirako (S)

All plots were seeded at 6.5 kg/ha (2.5-cm depth) with about 21-cm row spacing, and arranged in a randomized complete block design with 4 replicates per treatment. Each replicate consisted of four 6-meter rows, with all products applied in furrow (**Figure 4**) at 500 L/ha using a calibrated backpack sprayer.

Figure 4. In-furrow application of fungicides during seeding.

Untreated plots were used as controls. Fungicide products were applied at the following rates, and at these rates, the products showed significant efficacy against clubroot on canola in controlled conditions.

Product	**Rate (/ha)**
Prestop	1.4 Kg
Serenade AOS	13.0 L
Allegro 500F	3.0 L
Ranman	0.54 L

Clubroot incidence and severity were assessed 6 to 8 weeks after seeding (when canola was at mid- to late flowering) by destructive sampling of up to 25 plants from the central 4-meter area of each plot. The clubroot severity was rated for individual plant using the 0-3 scale and index of disease (ID) calculated over each replicate plot using the protocol described earlier.

For statistical analysis, ID was subjected to analysis of variance (ANOVA) to determine if there was substantial impact of cultivar and/or fungicide, and when significant, LSD (P=0.05) was applied to detect differences among fungicide products.

Results

Study I. Evaluate efficacy of microbial fungicides in controlled conditions

Expt I. Disease response to Pb inoculum dose

Inoculation of canola seedlings with Pb resting spores resulted in clubroot symptoms within 3 weeks (**Table 1**). Disease incidence and severity generally responded to the Pb inoculum dose applied; concentrations higher than 10^5 spores/ml caused clubroot symptoms on more than 50% of the plants and a moderate level of disease was caused by inoculation with higher than 10^6 spores /ml. As a result, Pb resting spore suspensions ranging from 10^6 to 10^7 spores /ml were used for inoculation in efficacy evaluation trials.

Table 1. Incidence and severity of canola clubroot symptoms caused by *Plasmodiophora brassicae* (Pb) at different inoculum concentrations [a].

Pb spore concentration (ml)	10^7	10^6	10^5	10^4	10^3	10^2
Disease incidence (%)	67	93	50	44	61	24
Disease severity index (DSI)	39	42	17	15	21	8

[a] Applied at 1 ml per plant.

Expt II. Efficacy trials in the containment at AAFC Saskatoon

Based on three trial repetitions, the biofungicides Serenade, Prestop, and Mycostop reduced disease significantly in all trials (**Figure 4, 5**), with a mean reduction in DSI of 91%, 81%, and 61%, respectively, compared to the inoculated check (**Table 2**). The fungicides Allegro and Ranman were also highly effective, reducing DSI by 91%. Other biofungicides were moderately effective to ineffective. Serenade at 5× label rate concentration caused slight stunting of canola seedlings in two of the three trials.

Expt III. Efficacy trials in greenhouse (CDC North, Edmonton)

The disease pressure was extremely high in Trial 1, causing 100% DSI in pathogen checks in both Pb inoculum scenarios (naturally infested soil and artificial inoculation). Biofungicide efficacy was generally low under this high disease pressure, but the fungicides Allegro and Ranman were noticeably more effective, especially in artificial Pb inoculation (**Table 3**). In Trial 2, the reduction of Pb inoculum dose lowered DSI slightly, and all treatments were significantly more effective than in Trial 1. Serenade, Actinovate, and Prestop were the most efficacious biofungicides. All fungicides were highly efficacious, especially against the artificial Pb inoculation (**Table 3**). No negative effect on canola plants was seen with any of the products applied.

Expt IV. Effect of seed treatment (CDC, Edmonton and AAFC Saskatoon)

As in the previous trial in Edmonton, extremely severe disease occurred in the Trial 1 and no treatment was effective (**Table 4**). The disease pressure was lower in Trial 2, and all treatments were more efficacious, especially in growth medium artificially inoculated with Pb (**Table 4**). The fungicides Allegro and Ranman were more effective than other treatments. As expected, the efficacy of seed treatments was lower than that of soil drenches.

The efficacy of seed treatments in the trial in Saskatoon did not was low (**Table 5**). Only Allegro reduced disease (57%), and its efficacy was much lower than that in Trial 2 at Edmonton.

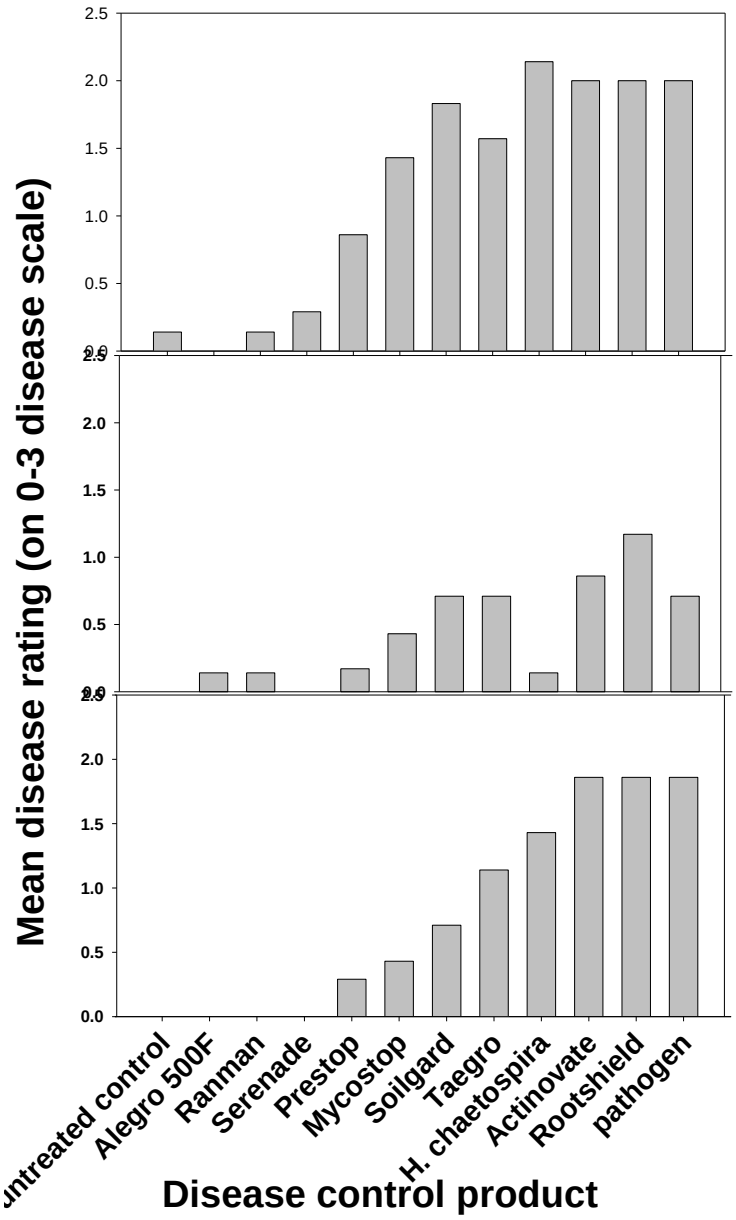

Figure 4. Efficacy of disease control products applied as a soil drench against clubroot on canola in three growth cabinet trials.

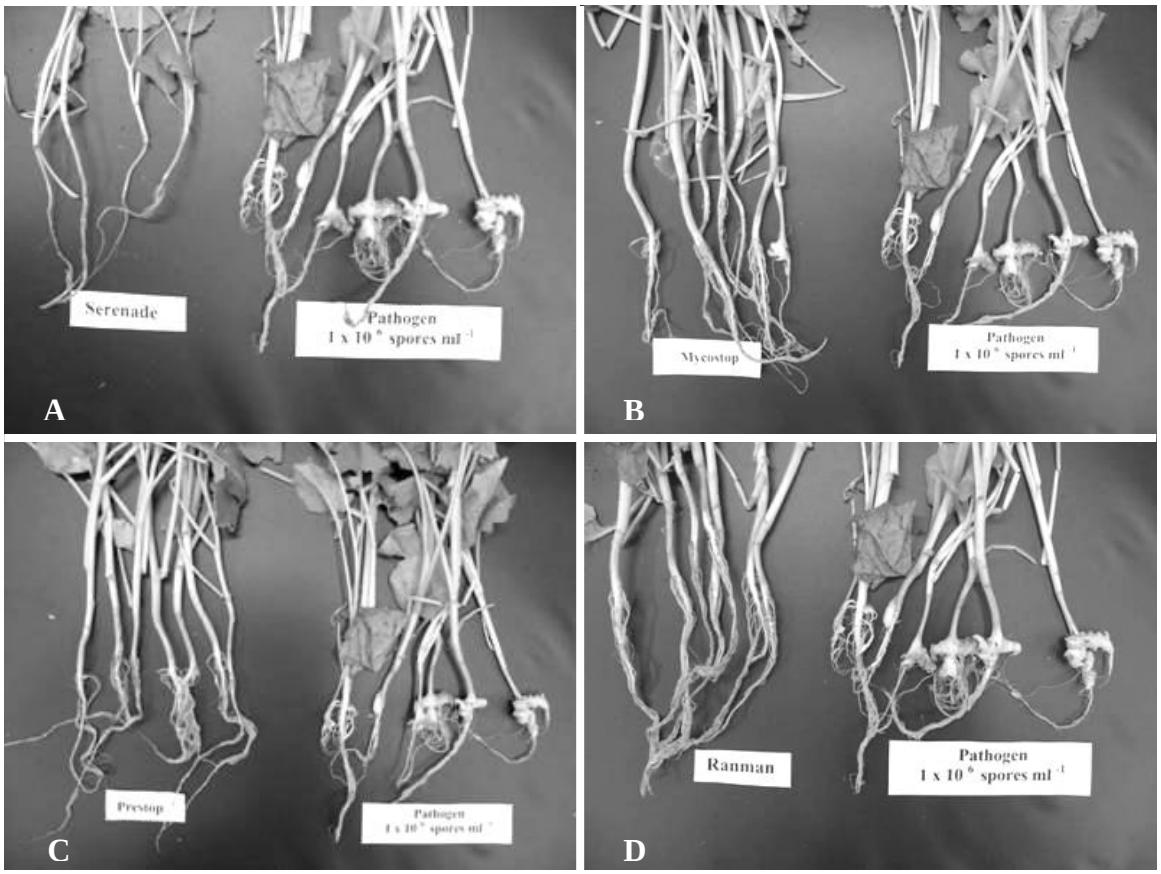

Figure 5. Efficacy of selected biofungicides and fungicides for control of clubroot on canola in controlled conditions. From A to D: Serenade, Mycostop, Prestop, and Ranman.

Table 2. Efficacy of selected products/agents against clubroot on canola (mean of 3 trials)

Treatment	Disease severity index (%)	Clubroot suppression (%)
Nontreated control	0 a	N/A
Serenade	3.2 ab	91.2
Ranman	3.2 ab	91.2
Allegro 500F	3.2 ab	91.2
Prestop	6.9 abc	81.1
Mycostop	14.3 abcd	60.8
Heteroconium chaetospira	19.1 bcde	47.8
SoilGard	23.8 cdef	34.8
Taegro	28.6 def	21.7
Pathogen control	36.5 efg	0.0
Actinovate	39.7 fg	- 8.7
Rootshield	46.6 g	- 27.6

Table 3. Effect of soil drench treatments on disease severity index (%) of clubroot on canola in two greenhouse trials (CDC, Edmonton)

Treatment	Trial 1		Trial 2	
	Pb-Infested field soil	Artificial Pb inoculation	Pb-Infested field soil	Artificial Pb inoculation
Pathogen control (check)	100 a	100 a	98.3 a	75.8 a
Mycostop	93.4 ab	93.3 ab	76.7 b	33.3 b
Root Shield	85.8 bc	90.8 abc	45.0 cd	22.5 c
Serenade	90.0 abc	87.5 bc	68.3 b	2.5 e
Prestop	85.0 c	87.5 bc	36.1 de	13.1 cd
Actinovate	90.0 abc	85.8 bc	49.1 c	8.4 de
Calcium cyanamide	17.5 d	82.5 c	26.7 ef	1.7 e
Allegro 500	28.4 d	0 d	22.9 f	0 e
Ranman	23.4 d	0 d	10.7 g	0 e

Table 4. Effect of seed treatment on disease severity index (%) of clubroot on canola in greenhouse trials (Edmonton)

Treatment	Trial 1		Trial 2	
	Pb-Infested field soil	Artificial Pb inoculation	Pb-Infested field soil	Artificial Pb inoculation
Pathogen control (check)	100 a	100 a	80.0 a	75.0 a
Mycostop	95.8 ab	96.7 ab	55.6 bcd	34.3 bc
Root Shield	99.2 a	95.0 abc	68.4 ab	27.5 c
Serenade	94.2 abc	92.5 abcd	49.5 cd	43.0 b
Prestop	91.7 abc	88.4 bcd	61.1 bc	33.2 bc
Actinovate	90.8 abc	100 a	58.7 bc	35.3 bc
Calcium cyanamide	85.8 c	88.4 bcd	31.2 e	25.5 c
Allegro 500	89.2 bc	84.4 cd	40.1 de	6.7 d
Ranman	75.0 d	82.5 d	33.7 e	6.7 d

Table 5. Effect of seed treatment on clubroot of canola in a growth cabinet trial (Saskatoon)

Treatment	Disease severity index (%)	Clubroot suppression (%)
Pathogen control (check)	33.3	N/A
Serenade	38.1	0
Prestop	50.0	0
Allegro 500	14.3	57.0
Ranman	33.3	0

Expt V. Effect of product concentration and drench volume (AAFC Saskatoon)
The disease pressure in Trial 1 was too low to assess rate-volume effects. Nevertheless, all treatments showed lower level of disease than the pathogen check (**Table 6**). In Trials 2 and 3, 5x label rate concentration for biofungicides was more efficacious than the 1x label rate. Drench volume (25 ml vs. 50 ml) had no effect on the efficacy of fungicides or biofungicides.

Table 6. Effect of product concentration and soil-drench volume on disease severity index (%) of clubroot of canola (AAFC Saskatoon).

Treatment	Trial 1	Trial 2	Trial 3
Water control	0.0	0 d	0 c
Pathogen control (check)	16.7	66.7 a	85.7 a
1x Prestop, 25 ml drench	9.5	47.6 bc	85.7 a
1x Prestop, 50 ml	4.8	57.1 ab	88.9 a
5x Prestop, 25 ml	4.8	14.3 d	NT
5x Prestop, 50 ml	4.8	9.5 d	NT [a]
1x Serenade, 25 ml	4.8	33.3 c	77.8 b
1x Serenade, 50 ml	0.0	50.0 abc	81.0 ab
5x Serenade, 25 ml	4.8	5.6 d	16.7 c
5x Serenade, 50 ml	0.0	5.6 d	13.3 c
1x Allegro, 25 ml	4.8	4.8 d	9.5 c
1x Allegro, 50 ml	9.5	5.6 d	0 c
1x Ranman, 25 ml	0.0	0 d	0 c
1x Ranman, 50 ml	0.0	4.8 d	14.3 c

[a] The 5x Prestop label rate concentration was not tested because the product was used up.

Study II. Survey and evaluate indigenous soil microbes for clubroot control

Isolation of microorganisms from canola roots

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.

Tiered screening system for selection of biocontrol candidates

Dual-cultured bioassay (Tier 1): All isolates obtained were screened against *Pythium ultimum* on medium agar. 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% (**Table 7**). 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.

Table 7. Inhibition of *Pythium ultimum* in duel-cultured bioassay.

Inhibition of *P. ultimum* colony[1]	Rhizosphere isolates		Endophytic isolates	
	Bacteria	Fungi	Bacteria	Fungi
0%	79%	10%	68%	15%
<25%	2%	0%	1%	0%
25-50%	18%	59%	28%	69%
>50%	1%	30%	3%	15%

[1] Based on the reduction of *P. ultimum* colony diameter against controls

Tier II. Pythium-canola seedling 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.

Tier III. Canola clubroot screening bioassay: 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 (**Table 8**). The efficacious microbial treatment also alleviated the negative impact of clubroot on the vigor of canola plants (**Figure 6**). All highly effective candidates are endophytes capable of colonizing canola roots. Several fungal and bacterial isolates reduced the disease by more than 50% (**Table 8**).

Figure 6. Severity of clubroot on canola treated with an endophytic fungus (right side) and water (left side). The growth medium was pre contaminated with clubroot resting

Table 12. Efficacy of clubroot control on canola by selected indigenous microorganisms

Isolates		Clubroot reduction (%)[1]		
		26-50	50-75	75-100
Fungi	Endophyte	7	1	3
	Rhizosphere	13	2	0
Bacteria	Endophyte	7	1	0
	Rhizosphere	5	0	0

[1] Compared to the pathogen control in the same trial.

Study III. Efficacy of selected fungicide/biofungicides in field conditions

The spring of 2009 was extremely dry in Alberta. There was no rain for 5 weeks after the seeding of first trial, and plant emergence was extremely sparse by early August. Therefore the trial was abandoned. The second trial received an inch of rain 4 weeks after seeding. Although the crop development was delayed, there were still a lot plants in each plot for assessment of clubroot severity. The index of the disease was high in untreated control plots of S cultivar; reaching an average of about 80% (**Figure 7**). The disease damage appeared slightly less severe with the fungicide treatments on the S cultivar but the difference was insignificant when compared to the control ($P > 0.05$, ANOVA). The R canola plants showed much less clubroot damage when compared to the S plants (**Figure 7, 8**) and the fungicide treatments did not further reduce clubroot substantially on the R cultivar.

The insignificance of fungicide treatments in the Alberta canola trial may be due to the drought condition experienced after seeding. Possibly by the time the rain came 4 weeks later, there would be little products left in the soil to fight against the pathogen. It is not clear, thought, how long these fungicides can last in the soil for clubroot control. The manufacturer suggested that the half life of the two synthetic fungicides is only a few days.

The canola trial in Ontario was initiated after the occurrence of extremely poor emergence in the Alberta trials. However, this trial encountered its own problems because the cultivars selected for the predominant local clubroot pathogen race (pathotype-6) showed poor emergence. These are not commercially available hybrids and the seeds had been stored for years. As a result, some plots had only a few plants, especially for the S cultivar. Additionally, the results were highly variable among different replicates (data not shown), a circumstance possibly attributable to uneven distribution of pathogen inoculum in the field plots. Therefore, no substantial effect was found with any of the fungicide treatment when compared to the untreated control (**Table 9**).

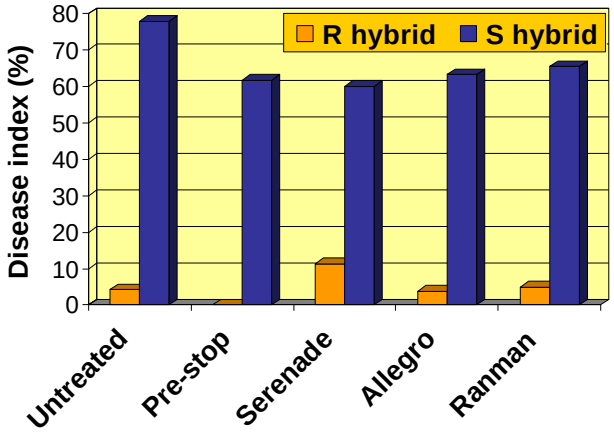

Figure 7. Average clubroot severity (4 reps) as affected by fungicides and canola cultivar resistance in the second field trial in Alberta (2009).

Figure 8. Plant height and vigour of susceptible and resistant canola hybrids affected by clubroot in the second field trial in Alberta (2009).

Table 13. Clubroot damage on canola (Ontario)

Cultivar	Avg. ID (%)	Significance
Pioneer 042	14.6	
Pioneer 041	1.9	***
Treatment		
Allegro	14.8	ns
Serenade	10.7	ns
Untreated CK	7.0	ns
Ranman	5.0	ns
Pre-stop	3.7	ns

***: Significant at $P = 0.01$ (ANOVA); ns: not significant ($P = 0.05$)

On Chinese cabbage (a model system), the R cultivar Yuki was highly effective with only a few plants affected by clubroot 8 weeks after seeding (**Figure 9**). The disease level was moderate on the S cultivar Mirako and all fungicide/biofungicide treatments reduced the disease significantly when compared to the untreated control. The efficacy ranged from 54% to 84%, and there was no substantial difference among the four fungicide products tested. The condition was almost ideal for the performance of fungicides during this trial; the rain came two days after seeding, and the disease pressure was at a moderate level, which tended to manifest the efficacy of fungicides.

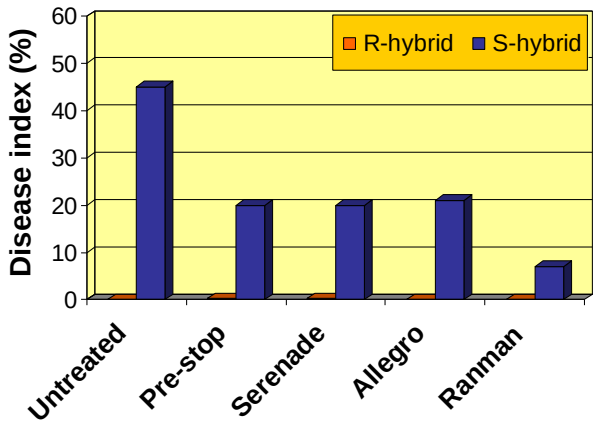

Figure 9. Average clubroot severity (4 reps) as affected by fungicides and cultivar resistance on Chinese cabbage (Ontario, 2009).

Across the trials, R cultivars showed much less clubroot damage when compared to S cultivars. In the case of Chinese cabbage, the R cultivar Yuki was so effective that would overshadow any potential efficacy of the fungicides.

The results of 2009 field trials showed that the selected fungicides have the potential to control clubroot in field conditions. However, the data are inconclusive for two canola trials due mostly to the drought condition in Alberta and poor canola germination in the Ontario trial. It is recommended that these trials be repeated using a similar protocol to determine the efficacy of field applications.

Improvements have been under way to address the problems encountered in 2009 field trials: irrigation equipment is being planned for future clubroot trials in Alberta to alleviate the impact of dry spring weather; the canola plots in Ontario have been harrowed repeatedly in vertical and horizontal directions to increase the uniformity of pathogen inoculum distribution. In addition, R and S canola seeds against the predominant clubroot pathogen races in Ontario will be sought from commercial sources in future trials to ensure acceptable seed quality in terms of germination and emergence.

Conclusions and recommendations

A total of 5,152 indigenous microbial isolates were screened for potential clubroot control using a tiered system, with a total of 390 selected candidates tested directly against clubroot on canola. Three endophytic fungal isolates, when applied as a soil drench, demonstrated high efficacy even under high clubroot pressure. These organisms are capable of colonizing canola roots, with the potential to provide long-term protection. Therefore, they should be further studied as potentially new agents against clubroot.

Existing products, including the biofungicides Serenade, Prestop and the synthetic fungicides Allegro, Ranman showed high efficacy under moderate clubroot pressure, reducing clubroot severity by 85–100% under controlled conditions. However, they can be significantly less effective or ineffective under high disease pressure conditions (disease index = 100%).

Results from seed-treatment trials were too variable to draw conclusions but there was an indication that this approach may work. More research is required on formulation that packs more products on the seed and stimulates the biofungicide agents in the soil.

In field plots, the efficacy of all products appeared variable due largely to weather (drought), seed quality (poor germination), and pathogen inoculum (uneven distribution) factors. But as shown in the Chinese-cabbage trial, these selected fungicides can work. Soil conditions and potential interactions of fungicide/biofungcide formulations with resistant canola cultivars need to be further studied for optimal microbial performance and clubroot control. Cultivar resistance was significant in all field trials, forming a foundation for integrated management of clubroot.

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Technology Transfer Activities

Talks

1. Alberta Plant Pathological Society and Canadian Phytopathological Society Saskatchewan Group joint meeting – Oct 22, 2008, Lloydminster, SK.
2. Soils and Crops – Feb. 26, 2009, Saskatoon, SK
3. Canola Summit – March 17-19, 2009, Saskatoon, SK
4. Clubroot Summit – April 29, 2009, Nisku, AB
5. International Clubroot Workshop – September 16-19, 2009, Kunming, China
6. Canadian Phytopathological Society Saskatchewan Group Annual Meeting – December 1, 2009, Saskatoon, SK.
7. Radio interview by “Call of the Land” in April, 2009, and broadcast at 13 local stations in Alberta.

Posters/Factsheet

1. At 2008 Canola Industry Meeting in Saskatoon (Dec. 10, 2008)
2. Western Canada Crop Production Show – Saskatoon (Jan 12-14, 2009)
3. Canadian Phytopathological Society Annual Meeting – June 22-25, 2009, Winnipeg, MB
4. Factsheet: Biocontrol of clubroot on canola – a new initiative. AAFC No. 10876, Cat. No. A52-140/2009E, ISBN 978-1-100-11930-4.

Publication

1. Peng, G, B.D. Gossen, S.E. Strelkov, S.F. Hwang and M.R. McDonald. 2009. Efficacy of selected biofungicides for control of clubroot on canola. Canadian Journal of Plant Pathol. 31:146-146 (abstract).
2. Peng, G, B.D. Gossen, S.E. Strelkov, S.F. Hwang and M.R. McDonald. 2009. Evaluation of biofungicides for control of clubroot on canola. Soil and Crops. University of Saskatchewan, CD Rom. 15pp.
3. Peng, G, B.D. Gossen, S.E. Strelkov, S.F. Hwang and M.R. McDonald. 2009. The potential for microbial control of clubroot on canola. In: Proceedings of Kunming International Clubroot Workshop, pp. 7-16. September 16-19, 2009, Kunming, China.
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