Seedborne transmission of clubroot of canola: evaluation of significance and control

CARP Project No. 2008-14

Final Report

August 13, 2010

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KEY FINDINGS

1. Using both conventional and quantitative PCR techniques, the clubroot pathogen [*Plasmodiophora brassicae*] was found on canola, pea, and wheat seeds, as well as potato tubers, harvested from clubroot-infested fields.

2. Quantifiable levels of infestation were found on seven of 46 samples analyzed (15.2%) and ranged from $<1.0 \times 10^3$ to 3.4×10^4 resting spores per 10 g of seeds; the vast majority of resting spores (80 to 100%) on these samples were viable, as determined by Evan's blue staining.

3. The levels of infestation found were in some instances higher than that required to cause clubroot symptoms in greenhouse bioassays.

4. One unregistered [Nebijin SSC (flusulfamide)] and four registered [Dynasty 100 FS (azoxystrobin), Vitavax RS (carbathiin + thiram), Prosper FX (clothianidin + carbathiin + trifloxystrobin + metalaxyl), and Helix XTra (thiamethoxam + difenconazole + metalaxyl + fludioxonil)] seed treatments were applied to canola seeds artificially infested with clubroot spores, and the treated seeds were planted in pots in a greenhouse, where the incidence of clubroot galls was assessed at maturity. The relative effectiveness of the products tested was as follows: Nebijin SSC = Dynasty > Vitavax RS > Prosper FX > Helix Xtra > Untreated Control.

5. While seedborne dissemination of clubroot is of secondary significance relative to spread on farm machinery and equipment, it could (under the right conditions) lead to the establishment of localized, minor infections, which could become more pronounced with repeated cropping of a susceptible host.

6. The findings support the recommendation that farmers avoid planting of common, untreated seed harvested from clubroot-infested fields.

Please refer to the complete report for full details and interpretation of these findings.

EXECUTIVE SUMMARY

Clubroot, caused by *Plasmodiophora brassicae*, is an important disease of crucifers worldwide and has recently emerged as a major constraint to canola (*Brassica napus*) production in Alberta, Canada. Efforts to minimize the spread of clubroot have focused largely on the sanitation of field equipment, as pathogen resting spores can be disseminated on infested soil carried on machinery. There have also been historical and anecdotal reports of the transmission of clubroot as a seedborne contaminant. However, these reports are limited, and the risk associated with seedborne transmission of *P. brassicae* is not well understood. Using quantitative PCR, pathogen DNA was detected and quantified on canola, pea, and wheat seeds, as well as potato tubers, harvested from clubroot-infested fields. Quantifiable levels of infestation were found on seven of the 46 samples analyzed, and ranged from $<1.0 \times 10^3$ to 3.4×10^4 resting spores per 10 g of seeds; the vast majority (80 to 100%) of resting spores on these samples were viable, as determined by Evan's blue staining. The levels of infestation found were in some instances higher than that required to cause clubroot symptoms in greenhouse bioassays. In order to assess the efficacy of seed treatments for eradicating seedborne inoculum, one unregistered [Nebijin SSC (flusulfamide)] and four registered [Dynasty 100 FS (azoxystrobin), Vitavax RS (carbathiin + thiram), Prosper FX (clothianidin + carbathiin + trifloxystrobin + metalaxyl), and Helix XTra (thiamethoxam + difenconazole + metalaxyl + fludioxonil)] seed treatments were applied to

canola seeds artificially infested with spores of *P. brassicae*, and the treated seeds were planted in pots in a greenhouse, where the incidence of clubroot galls was assessed at maturity. The relative effectiveness of the products tested was as follows: Nebijin SSC = Dynasty > Vitavax RS > Prosper FX > Helix Xtra > Untreated Control. While seedborne dissemination of clubroot appears to be of secondary significance to its spread on farm machinery and equipment, it might (under the right conditions) lead to the establishment of localized, minor infections, which could become more pronounced with repeated cropping of a susceptible host. Therefore, farmers should avoid planting of common, untreated seed harvested from clubroot-infested fields.

ACKNOWLEDGEMENTS

We would like to thank the Canadian Seed Growers' Association, Alberta Canola Producers Commission, and Alberta Crop Industry Development Fund Ltd. for their generous support of this research. We are also grateful to Ms. K. Dunfield (University of Alberta) for technical assistance, and Mr. D. Burke (Alberta Agriculture and Rural Development [AARD]) for helpful discussions regarding resting spore staining techniques. We also wish to thank Mr. M. Hartman (AARD) for providing the seed samples from the seed cleaning plant, and various farmers for providing seeds harvested from their clubroot-infested fields.

INTRODUCTION

The obligate parasite *Plasmodiophora brassicae* is the causal agent of clubroot, an important disease of the Brassicaceae family. Infection by this pathogen can result in considerable yield and quality losses in susceptible crops, accounting for a 10-15% reduction in yields on a global scale (Dixon 2009). Clubroot has recently emerged as a major constraint to canola (*Brassica napus*) production in the province of Alberta, Canada (Howard et al., 2010). While only 12 clubroot-infested fields were found when the disease was first identified in central Alberta in 2003 (Tewari et al., 2005), more than 450 infested fields were reported by 2009 (Strelkov et al., 2010), with the outbreak spreading to southern and eastern regions of the province.

The life history of *P. brassicae* consists of primary and secondary phases occurring in the root hairs and cortex of the host, respectively (Buczaki, 1983). Clubroot development is initiated by the germination of pathogen resting spores and encystment of primary zoospores in the root hairs, followed by the formation of primary plasmodia and release of secondary zoospores that infect the root cortex. Gall formation is a consequence of this cortical infection and the resulting hyperplasia and hypertrophy of the affected root tissues. Resting spores are formed within the galled roots, and are dispersed by various means after root decomposition (Buczaki, 1983). The resting spores can remain viable in the soil for many years, with the potential to infect any nearby host (Wallenhammer, 1996).

While clubroot-resistant cultivars of *B. napus, Brassica oleracea* and *Brassica rapa* are available, their extensive use is often limited by the short durability and race or pathotype-specificity of the resistance (Diederichsen et al., 2009; Voorrips, 1995). An effective clubroot management plan, therefore, requires the use and integration of various tools for disease control (Diederichsen et al., 2009; Donald and Porter, 2009). Sanitary practices, crop rotation, chemical control and other means are promoted to effectively manage clubroot disease (Donald and Porter, 2009). In Alberta and the neighboring provinces of Canada, efforts to contain the outbreak have focused largely on the sanitation of field equipment, as *P. brassicae* resting spores can be disseminated on infested soil carried on farm machinery (Cao et al., 2009; Howard et al., 2010).

While soilborne transmission of clubroot on tools and equipment represents one of the principal modes of pathogen dispersal (Cao et al., 2009; Donald and Porter, 2009), the dissemination of *P. brassicae* resting spores as external seed contaminants has also been suggested. Eriksson (1930) described an account of clubroot on turnips (*B. rapa* var. *rapa*) in Sweden that was reportedly caused by seedborne inoculum, while Gibbs (1930) also attributed several cases of clubroot to seedborne infestations. More recently, it was suggested that seeds of white mustard (*Sinapis alba*) imported from Canada might have been the source of two cases of clubroot in New South Wales, Australia (Hind-Lanoiselet and Parker, 2005). To our knowledge, however, only one early study (Warne 1943) has explicitly examined the

question of seedborne transmission of *P. brassicae*. This research was limited to the sowing of untreated and surface-sterilized seeds of two swede (*B. napus*) cultivars in pots, with subsequent assessment of the roots for clubroot symptom development. While restricted in its scope, this study did provide indirect evidence for the presence of *P. brassicae* inoculum on some of the seeds (Warne 1943).

Given the concern associated with clubroot on Canadian canola crops, it is important to properly understand the extent of the risk associated with seedborne transmission of this disease. We hypothesize that infestation of seeds and tubers by *P. brassicae* may represent a secondary mode of inoculum dispersal, and that the level of inoculum found on seeds harvested from clubroot-infested fields could be sufficient to cause disease symptoms. In this context, the objectives of the present study were to: (1) develop a reliable, quantitative PCR (qPCR)-based assay to measure *P. brassicae* resting spores present in soil and dust associated with seeds and potato tubers, (2) estimate the levels of inoculum found on seeds of common field crops and potato tubers grown on clubroot-infested fields in Alberta, (3) assess the viability of this inoculum through staining and microscopic observation, (4) compare resting spore levels found on seeds and tubers to those required to cause disease under greenhouse conditions, and (5) evaluate different seed treatments for their ability to eradicate this inoculum. Knowledge of whether or not seedborne dissemination of *P. brassicae* represents a viable mechanism for clubroot spread is critical to properly understand the epidemiology of

this pathogen in agricultural systems, as well as for the development of effective strategies to limit the spread of clubroot in Canada and elsewhere. Seedborne dissemination could undermine the effectiveness of the rigorous sanitation and exclusion practices currently employed by many in the agricultural industry.

MATERIALS AND METHODS

Inoculum of *Plasmodiophora brassicae*

Galled roots of *Camelina sativa*, which had been inoculated with population SACAN03-1 of *P. brassicae* (Séguin-Swartz et al., 2009), were used as the source of inoculum for all components of this study. SACAN03-1 is a highly virulent population of the pathogen originally collected from infected canola plants in central Alberta (Strelkov et al., 2006). This population has been classified as pathotype 3, 16/15/12, or P_2 on the differentials of Williams (1966), the European Clubroot Differential series (Buczacki et al., 1975), and Somé et al. (1996), respectively (Strelkov et al., 2006). Clubroot galls were stored at -20°C until needed. The resting spores were isolated by homogenization of infected root tissue with a blender, filtration of the homogenate through a 450 μm sieve, and centrifugation in a 50% sucrose gradient as per the protocols of Castlebury et al. (1994) and Cao et al. (2007). The resulting spore pellets were re-suspended in 5% (v v^{-1}) glycerol, and the resting spore concentrations

were quantified with a hœmocytometer (VWR) and adjusted as needed with 5% (v v^{-1}) glycerol.

Artificial infestation of canola seeds

Seeds of the clubroot-susceptible canola cv. 34-65RR were surface-sterilized by soaking in 1% (v v^{-1}) bleach for 5 minutes and rinsing with sterile distilled water (sd-H_2O), after which they were air dried. Aliquots (10.5g) of the surface-sterilized seeds were placed in labeled, sterile 50 mL tubes (Corning). The approximate number of seeds per g was estimated by weighing three replicates of 100 seeds each, and a 2 mL volume of a resting spore suspension (1.3×10^9 resting spores per mL) was added to one of the aliquots to infest the seeds at a concentration of 1×10^6 resting spores per canola seed. Following addition of the spore suspension, the seeds were vortexed and shaken vigorously to ensure uniform distribution of the inoculum. The remaining resting spore suspension was serially diluted with 5% (v v^{-1}) glycerol and used to infest additional 10.5 g seed aliquots at concentrations of 1×10^5, 1×10^4, 1×10^3,1×10^2,1×10^1, and 1×10^0 resting spores per canola seed, as described above. Untreated, surface-sterilized seeds were used as a control. The artificially infested seed samples were placed in a glass seed desiccator over silica desiccant and stored at 4°C until dry.

Seeds and potato tubers assessed for clubroot infestation

A total of 45 different seedlots and one lot of potato tubers were assessed for the occurrence and quantity of *P. brassicae* inoculum via qPCR and viability staining. These included two canola, two barley and three wheat samples from crops grown on clubroot-infested fields in central Alberta; these samples (which ranged in mass from 50 g to 2 kg) were kindly donated by area farmers shortly after harvest, and had not been commercially cleaned. In addition, 100 g aliquots of commercially cleaned seedlots of rye (one sample), oat (two samples), barley (six samples), wheat (six samples) and pea (15 samples) were collected from a seed cleaning plant in Sturgeon County, Alberta, located in the centre of the clubroot outbreak, and provided for the study by Mr. M. Hartman (Alberta Agriculture and Rural Development, Lacombe). The selection of seeds from the seed cleaning plant was random and no information was available as to which seedlots, if any, actually came from clubroot-infested fields. One lot of potatoes (consisting of six small tubers) and four (100 g) samples of peas grown on a clubroot-infested field in Newell County, southern Alberta, were also included in the analysis, as were an additional four (100 g) pea seed samples grown on an infested field in Leduc County, central Alberta (R.J. Howard, unpublished data).

Seed/tuber washes and DNA extraction

The wash protocol used to collect resting spores from seed or tuber surfaces was based on MacNeil et al. (2004) with some modifications. A 10 g aliquot of seeds was placed in a 250

mL Erlenmeyer flask and 25 mL sd-H_2O was added, or in the case of the potatoes, two tubers were placed in a generic 1 L Pyrex dish and 100 mL sd-H_2O was added. The flask or dish was then agitated on an orbital mixer at 150 rpm for 1 h. The seed wash was decanted into a 50 mL conical tube (Corning), while the tuber wash was transferred into a 250 mL polypropylene bottle (Nalgene). An additional 25 or 100 mL of sd-H_2O was added to each flask or dish containing the seeds or tubers, respectively, and these were agitated again for a brief period. The supernatants were decanted and pooled with the previously collected washes. Seed and tuber wash suspensions were centrifuged at 4,894 ×*g* for 20 min in a swinging bucket rotor. The supernatants were discarded, and the pellets re-suspended in the remaining supernatant and transferred to 2 mL Lysing E Matrix tubes (MP Biomedicals). The tubes were centrifuged at 14,000 rpm for 1 min and the supernatants discarded. Total DNA was then extracted from the resting spore pellets with a FastDNA® Spin Kit for Soil (MP Biomedicals) as per the manufacturer's instructions.

PCR analysis

Quantitative PCR analysis was performed on undiluted DNA extracted from the seed or tuber washes, or after dilution (1/2, 1/10, or 1/100 v v^{-1}) with sd-H_2O if the presence of PCR inhibitors was suspected. The primers DC1F (5'-CCTAGCGCTGCAT CCCATAT-3') and DC1R (5'-CGGCTAGGATGGTTCGAAAASYBR-3') were designed using Primer Express Software v. 3.0 (Rozen and Skaletsky, 2000) based on a partial 18S rRNA gene sequence

from *P. brassicae* available in GenBank (accession no. AF231027). All qPCR amplifications were conducted using a StepOnePlus Real Time PCR System (Applied Biosystems) in a 10 µL volume containing 2.5 µL of the template DNA solution, 2.5 µL of a 3.2 mM DR1F and DR1R primer mix, and 5 µL Dynamite qPCR Mastermix (Molecular Biology Service Unit, University of Alberta, Edmonton, Canada), which contains SYBR Green (Molecular Probes) as the detection dye. Reaction conditions consisted of an initial heat denaturation step at 95°C for 2 min, followed by 35 cycles of 95°C for 15 s and 60°C for 60 s. The melting point of each amplification product was determined at the end of the reaction.

A preliminary qPCR screen was performed on DNA extracted from a single subsample of each the 46 seed and tuber samples. Each sample that tested positive for the presence of *P. brassicae* in this initial screen was evaluated further by the extraction of DNA from three additional subsamples, which were subsequently analyzed by qPCR in three parallel reactions per subsample (for a total of nine qPCR amplifications per original seed or tuber lot). Infestation by the clubroot pathogen was further confirmed by conventional PCR as described previously (Cao et al., 2007), using the *P. brassicae*-specific primers TC1F (5′-GTGGTCGAACTTCATTAAATTTGGGCTCTT-3′) and TC1R (5′-TTCACCTACGGAACGTATATGTGCATGTGA-3′). The amplification products were resolved on 1% agarose gels stained with SYBR® Safe (Invitrogen, Carlsbad, CA) and visualized under UV light in a Syngene BioImaging System (Synoptics Inc.).

Standard curves for the quantification of *P. brassicae* resting spores were generated with DNA isolated from known quantities of spores, which had been purified on a sucrose gradient as noted above. Briefly, total DNA was extracted from 1×10^9 resting spores and serially diluted with sd-H_2O at 10-fold intervals down to a dilution of 1×10^{-2}; DNA from each dilution in the series was then used as a template in qPCR under the aforementioned conditions. Average C_T, linear regression coefficient (R^2), line equation, and PCR efficiency were calculated from four independent sets of serial DNA dilutions. In order to validate the seed wash, DNA extraction and qPCR protocols, as well as the standard curves, total DNA was extracted from washes of canola seeds that had been artificially infested at known concentrations ranging from 1×10^6 to 1×10^{-2} resting spores per seed; these DNA samples were subjected to qPCR as above. The average threshold cycle (C_T) value and 95% confidence interval for each infestation level were calculated based on analysis of four independent sets of artificially infested seeds.

The specificity of primers DC1F and DC1R was assessed by subjecting 5 ng and 50 ng aliquots of DNA from each of the 29 plant, fungal and bacterial species listed in Table 1 of Cao et al. (2007) to qPCR analysis as above. The average threshold cycle (C_T) value, average product melting temperature (T_M) and 95 % confidence intervals for both C_T and T_m were calculated for a minimum of two parallel qPCR reactions conducted with aliquots of DNA from each fungal or bacterial species.

DNA sequencing

The amplicon generated with primers DC1F and DC1R from DNA of *P. brassicae* population SACAN03-1 was subjected to sequence analysis to confirm that the expected product was obtained. The amplification products from two independent qPCR assays were visualized on agarose gels and purified with a Qiaquick Gel Extraction Kit (Qiagen) as per the manufacturer's instructions. The purified amplicons were then sequenced in the forward and reverse directions using primers DR1F and DR1R, respectively, with sequencing performed on a 3740 DNA Analyzer (Applied Biosystems) at the Molecular Biology Services Unit, University of Alberta, using a BigDye® Terminator Sequencing Kit (Applied Biosystems).

Viability staining

The viability of *P. brassicae* resting spores on seed and tuber samples was assessed using an Evan's blue staining protocol developed by Tanaka et al. (1999). Briefly, Evan's blue stain (20 mg mL^{-1}) (Sigma-Aldrich) was mixed in equal proportions with 500 µL of the concentrated seed and tuber washes described above. After a 15 to 30 min incubation period, the seed wash/Evan's blue solution was diluted 1:4 in 5% (v v^{-1}) glycerol and a 30 µL aliquot transferred to a glass microscope slide. Thirty resting spores from each sample were examined for cytoplasmic staining (an indication of non-viability) using bright-field microscopy on a Carl Zeiss Primo Star light microscope (Carl Zeiss) and the numbers of dead and viable spores were recorded.

Seed bioassays

In order to determine the infestation level at which visible clubroot symptoms could be obtained, two separate bioassays were conducted with artificially infested canola seeds. The seeds were sown either in 39 × 26 × 15 cm plastic trays (bioassay 1) or 7.5 cm diameter plastic pots (bioassay 2) filled with, respectively, 2 or 0.5 kg of Sunshine #4 Mix/LA7 soil medium (SunGro Horticulture). Seeds were sown at a density of 120 seeds m^{-2} in both bioassays, as this corresponds to the recommended seeding rate for canola in Alberta (Alberta Agriculture and Food, 2007). In the trays, 12 seeds were sown as two discrete rows, whilst in the pots three seeds were spaced out equally over the entire surface area. The trays in bioassay 1 were kept in a greenhouse at an average temperature of 20 ± 2°C, under natural light supplemented by artificial light and a 16 h photoperiod. The pots in bioassay 2 were maintained in a growth cabinet with a 16 h photoperiod at 21°C/18°C (day/night). Trays and pots were kept saturated with water for the first two weeks after sowing. Thereafter, the plants were watered and fertilized as required. Treatments in both bioassays were arranged in a randomized complete block design.

Six weeks after sowing, the plants were gently uprooted, washed with water and scored for clubroot symptom development on a 0 to 3 scale as per Kuginuki et al. (1999), where: 0, no galling; 1, a few small galls; 2, moderate galling; and 3, severe galling. These scores were used to calculate an index of disease (ID) for each treatment according to the

formula of Horiuchi and Hori (1980) as modified by Strelkov et al. (2006). Any galls that were not obviously symptoms of clubroot were subjected to conventional PCR analysis (Cao et al., 2007) to confirm *P. brassicae* infection. The 95% confidence interval for each treatment was calculated and all data were plotted using R software (R Development Core Team, 2008).

Seed treatments

The canola cv. 34-65RR was used in all of the seed treatment experiments. Canola seed samples (5 g) were thoroughly mixed with 1.5 g of *P. brassicae*-infested soil (containing 1×10^8 resting spores per g of soil) and 500 µL of water in 50 mL Falcon tubes, so as to evenly coat the seeds with the wet soil. The soil-coated canola seeds were then vortexed with 500 µL of a solution containing one of the following products: Helix Xtra (thiamethoxam + difenconazole + metalaxyl + fludioxonil, 1500 mL per 100 kg of seeds), Dynasty 100 FS (azoxystrobin, 200 mL per 100 kg of seeds), Nebijin™ SSC (flusulfamide, 4 mL per 100 kg of seeds), Prosper FX (clothianidin + carbathiin + trifloxystrobin + metalaxyl, 1400 mL per of 100 kg seeds), and Vitavax RS (carbathiin + thiram, 1250 mL per100 kg of seeds); seeds coated with infested soil but treated with 500 µL of water alone were included as controls. Since Nebijin™ SSC is not registered as a canola seed treatment, the upper limit of the recommended dosage for potato tubers was used. The other products were applied at the recommended rates for canola. Categorical data were analyzed for statistical significance

using the catmod procedure of SAS 8.2 software (SAS Institute, Cary, NC). To facilitate comparisons, indices of disease (Strelkov et al., 2007) are also presented along with the categorical statistical analysis.

RESULTS

Validation of qPCR

Sequence analysis of the amplicon obtained with primers DC1F/DC1R in two separate qPCR assays revealed it to be 90 bp in size with 100% homology to 13 *P. brassicae* accessions deposited in GenBank, confirming that the correct product was amplified. The experimentally determined average (± 95% confidence intervals) T_M of this amplicon was 85.18 ± 0.12°C. Primer specificity was assessed using genomic DNA from a collection of 29 plant, fungal and bacterial species as the template in qPCR. Non-specific amplification of DNA from these species was on average not observed until a threshold cycle (C_T) of greater than 29.76 ± 0.56 when using 5 ng of template, or 28.28 ± 1.04 with 50 ng of template. A two-tailed t-test indicated a significant difference ($p = 0.006$) between these two C_T values and, therefore, 28.28 ±1.04 was taken as the lower limit of detection. Similarly, reactions in which the C_T was less than 11 were not regularly reproducible, and were also excluded from the analysis. Thus, only those samples with a C_T value between 11 and 28.28, and which had a melting point consisting of a single peak that was comparable to the

experimentally verified T_M of 85.18 °C, were considered accurate for inoculum quantification purposes.

Reproducible linear standard curves could be generated from DNA extracted from between 1×10^8 to 1×10^3 resting spores of *P. brassicae* (Fig. 1). A good PCR amplification efficiency (E = 0.98) was confirmed from the slope of these standard curves, with a strong correlation ($R^2 = 0.99$) observed between the quantity of *P. brassicae* DNA and the corresponding C_T value (Fig. 1). The estimated numbers of resting spores calculated based on the qPCR analysis were on average 56% lower than the theoretical levels of infestation, as defined on the artificially infested canola seed samples over the established detection range (Fig. 1). The average confidence interval for C_T value in the artificially infested seedlots was 1.01, indicating that the extraction procedures were consistent over the established detection range (Fig. 1).

Quantification of *P. brassicae* inoculum on seed and tuber lots

Of the 46 seed and tuber lots analyzed in this study, resting spores of *P. brassicae* could be reliably and unequivocally quantified on seven (15.2%), all of which also tested positive by conventional PCR (Table 1). The highest level of infestation was detected on wheat seeds harvested from a clubroot-infested field in central Alberta (sample 1), which yielded an average C_T value of 19.94 ± 0.45 (equivalent to approximately 3.43×10^4 resting spores per 10 g of seeds). Potato tubers harvested from an infested field in southern Alberta (sample 4)

gave a C_T value of 24.63 ± 0.27 after a 10-fold dilution of the extracted DNA, which corresponded to approximately 1.40×10^4 resting spores per tuber (when corrected for this dilution). An average C_T value of 23.08 ± 0.91 (equivalent to 4.04×10^3 resting spores per 10 g of seeds) was obtained for a lot of canola seeds harvested from another infested field in central Alberta (sample 2). Resting spores of *P. brassicae* were also found on four separate lots of pea seeds, one originating from a seed cleaning plant in the center of the clubroot outbreak (sample 3), and three more originating from field plots in southern Alberta (samples 5, 6 and 7). The levels of infestation on these pea samples were fairly low, with C_T values ranging from 25.94 ± 9.54 to 28.00 ± 0.60 (or less than 1×10^3 resting spores per 10 g of seeds), but it is interesting to note that sample 3 had been commercially cleaned.

Viability staining

Resting spores of *P. brassicae* could be observed microscopically after Evan's blue staining of all seed and tuber washes that had tested positive by qPCR, serving to corroborate that analysis (Table 1). In those spores presumed to be non-viable (Tanaka et al., 1999), the cytoplasm became prominently stained with the Evan's blue dye. Based on the cytoplasmic staining reaction, the vast majority of resting spores examined appeared to be viable. Indeed, 100% of resting spores from pea sample 7 and 98% of spores from pea sample 3 were viable. Resting spores examined from pea samples 5 and 6 appeared to be 97% viable, while spore viability was 90% in the canola seed (sample 2) and potato tuber washes (sample 4). The

lowest viability was observed for resting spores from the wheat seed washes (sample 1), at 80% unstained spores (Table 1).

Seed bioassays

Canola plants were examined for the presence and severity of clubroot symptoms six weeks after sowing of the artificially infested seeds. At that time, mature inflorescences had developed on most plants and root development was extensive. The results from the bioassays are summarized in Fig. 2. In bioassay 1, in which the plants were grown in 39 × 26 × 15 cm plastic trays, no symptoms of disease were detected in the non-infested control (0 spores per seed) or at infestation levels of 1×10^1 or 1×10^2 resting spores per seed. A single small gall, however, was observed at 1×10^0 resting spore per seed, resulting in an ID of 1.85 ± 3.63% for this treatment. Given its small size and isolated occurrence, this gall was tested by conventional PCR with *P. brassicae*-specific primers (Cao et al., 2007) and confirmed to be positive for clubroot. In contrast, a fair amount of galling was found at infestation levels of 1×10^3 resting spores per seed (ID = 13.47 ± 2.64%) and 1×10^4 resting spores per seed (ID = 12.86 ± 12.13%). The most severe symptoms of clubroot were detected at infestation levels of 1×10^5 resting spores per seed (ID = 50.38 ± 15.59%) and 1×10^6 resting spores per seed (ID = 34.97 ± 8.66%) (Fig. 2).

Similar results were obtained in bioassay 2, in which the seeds were sown in 7.5 cm diameter plastic pots. No symptoms of clubroot were observed at 0, 1×10^0, 1×10^1 or 1 ×

10^2 resting spores per seed, while consistent galling was found at the higher infestation levels. At 1×10^3 resting spores per seed, an ID of 3.33 ± 6.53% was obtained. The disease severity increased considerably at an infestation level of 1×10^4 resting spores per seed, with an ID of 36.23 ± 0.25%. At an infestation level of 1×10^5 resting spores per seed, the ID was 37.73 ± 15.15%, while at 1×10^6 resting spores per seed, it was 42.99 ± 10.76% (Fig. 2).

Seed treatments

Pairwise comparisons revealed that all five fungicidal seed treatments significantly ($p <$ 0.0001) decreased clubroot symptom severity relative to the water-treated control, which had an ID = 68.1% six weeks after sowing (Table 2). The strongest decreases in clubroot severity were observed for the Dynasty 100 FS (ID = 13.2%) and Nebijin™ SSC (ID = 16.4%) treatments, followed by Vitavax RS (ID = 24.3%), Prosper FX (ID = 46.8%) and Helix Xtra (ID = 53.9%).

DISCUSSION

Given the persistence of *P. brassicae* resting spores in the soil, eradication of this pathogen is difficult if not impossible once it becomes established in a field. Therefore, much of the clubroot management effort in the Canadian outbreak on canola has been focused on exclusion of *P. brassicae* from disease-free fields (Howard et al., 2010). For such an approach to be successful, however, all mechanisms of pathogen dispersal must be

considered. The present assessment of seeds and tubers from clubroot-infested fields in Alberta supports our hypotheses that infestation of propagative materials by *P. brassicae* may represent a secondary mode of inoculum dispersal, and that the level of inoculum found on some samples exceeds that required to cause disease symptoms.

Although the infestation of seeds by *P. brassicae* has been suggested previously (Warne 1943), it has not been directly demonstrated, since the pathogen is an obligate parasite that cannot be cultured on axenic medium. In recent years, the detection of *P. brassicae* has been facilitated by the development of molecular techniques to identify the pathogen (Faggian and Strelkov, 2009). However, most of these approaches have been qualitative in nature, and if the risk posed by seedborne clubroot is to be properly evaluated, an assessment of the amount of inoculum present is also required. The qPCR protocol developed in the current study represents an important tool for conducting such a quantitative assessment. Analysis of the artificially infested seedlots revealed that the resting spore numbers calculated using the qPCR assessment were about 56% lower than the theoretical values, which likely reflected spore and/or DNA loss during the extraction procedures. Nevertheless, a strong linear correlation ($R^2 = 0.99$) was observed between the amount of *P. brassicae* DNA and the corresponding C_T value. Moreover, while the numbers of spores on the artificially infested seeds may have been somewhat underestimated, the small confidence intervals indicate that the data were consistent (Fig. 1). Therefore, the qPCR-based assay

appears to be a reliable method to estimate the numbers of *P. brassicae* resting spores on soil and dust associated with seeds and tubers, and we used this method to assess the levels of infestation on propagative materials from clubroot-infested regions in Alberta.

Quantifiable numbers of *P. brassicae* resting spores were found on seven of 46 field-grown seed and tuber lots analyzed by qPCR, including a commercially cleaned pea sample (Table 1). The number of resting spores found on three of the seven infested samples (representing wheat, potatoes and canola) was greater than that required to cause clubroot symptoms in greenhouse bioassays (Fig. 2). Indeed, the infestation level of 4.04×10^3 resting spores per 10 g of seed on one of the canola seedlots (sample 2) (Table 1) corresponds to about two resting spores per seed, at which an ID of $1.85 \pm 3.63\%$ was obtained in bioassay 1 (Fig. 2). The highest levels of inoculum, however, were found on wheat seeds (sample 1) and potato tubers (sample 4) harvested from clubroot-infested fields (Table 1). While these crops do not serve as hosts for *P. brassicae*, they may serve as vectors for the dispersal of this pathogen, particularly if they are more commonly contaminated with soil or dust than are seeds of canola. Of course, any resting spores introduced into a field along with a non-host crop would have to survive in the soil until canola or some other susceptible species was planted. Since resting spores of *P. brassicae* have been estimated to have a half-life of 3.6 years (Wallenhammar 1996), the longer the period prior to cropping of a susceptible host, the smaller the chance that a clubroot infestation could become established. Nevertheless, the

levels of inoculum found in some of the samples were not trivial, particularly if extrapolated to a field-scale. For instance, 3.43×10^4 resting spores per 10 g of wheat seeds (Table 1) would correspond to approximately 93 million resting spores per bushel (27.2 kg) of wheat. At a seeding rate of 2.5 bushels per acre (67.2 kg per hectare), more than 230 million resting spores could be introduced per acre (or 568 million per hectare). Whether any of these spores could successfully cause infection would depend not only on the length of time prior to the cropping of a susceptible host, as noted above, but also on the prevailing environmental conditions and the final distribution and concentration of the spores in the field.

The distribution of resting spores in a field as a result of seed infestation would likely be a function of the distribution of the resting spores within a particular seedlot. While the inoculation protocol employed in the current study likely resulted in a fairly homogenous distribution of resting spores on the artificially infested seeds, such uniformity cannot be assumed in naturally infested samples. Since the confidence intervals in the qPCR analysis of the artificially infested seedlots were small and the correlation co-efficient was high, variation observed in the C_T values of naturally infested seedlots likely reflected, at least in part, a heterogenous distribution of the inoculum (Fig. 1, Table 1). Thus, the mean infestation level does not necessarily capture the extremes that may occur within a particular seedlot, and some seeds could be infested at much higher (or lower) rates than the average. Such a situation could arise, for example, if there were small pieces of soil matter or plant

debris mixed with the seeds. In such a context, susceptible seedlings germinating in close proximity to these pieces of soil or plant debris would be more likely to become infected, given the proper conditions, since they would be adjacent to greater numbers of spores. Indeed, the incidental gall observed at 1×10^0 resting spore per seed in bioassay 1 suggests that clubroot development can also occur by stochastic means, even in seedlots infested at very low levels.

While the presence of *P. brassicae* can be confirmed and quantified by PCR-based protocols, these methods assess only the amount of pathogen DNA, and not necessarily the viability of the inoculum. The Evan's blue staining assay employed in this study was developed to assess the impact of physical damage, resulting from heat and ultraviolet (UV) irradiation, on resting spore viability (Tanaka et al., 1999), but was found not to be applicable for assessing the impact of chemical damage or ionic stress (Donald et al., 2002). Since the seedlots analyzed in the current report had not been chemically treated, the staining protocol was appropriate for assessing resting spore viability. The results suggested that the vast majority of *P. brassicae* resting spores recovered from the seed and tuber samples were viable (Table 1), and that any exposure to heat or other mechanical stresses during handling did not have a large impact on this inoculum.

Since all of the seed treatments examined in this study significantly ($p < 0.0001$) reduced the development of clubroot symptoms relative to the untreated controls, it is likely

that seed treatments represent one of the most effective approaches to mitigating the threat of seedborne dissemination of *P. brassicae*. Four of the five seed treatments evaluated are currently registered for canola, suggesting that many of the treatments already in place may be sufficient to eradicate or reduce seedborne clubroot inoculum from this crop. The fifth, Nebijin SSC (flusulfamide), is used for control of powdery scab on potatoes and clubroot on vegetable Brassicas. Given that the level of control provided by Dynasty was equally good, it may not be necessary to pursue registration of Nebijin SSC as a seed treatment for canola. Moreover, although some of the other registered treatments did not reduce clubroot levels as strongly as Nebijin SSC and Dynasty, it is possible that these other treatments also provide sufficient control for natural seedborne infestations of the disease. It is important to stress that the level of infestation used for the seed treatment study was very high, and was intended to represent a 'worst-case' scenario. No naturally infested seed or tuber samples were found to have such high numbers of resting spores.

While the current results provide 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.

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.

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Table 1. Levels of seed and tuber infestation by resting spores of *Plasmodiophora brassicae*, as determined via qPCR with the *P. brassicae*-specific primers DR1F and DR1R.

Sample[a]	Crop	Description	C_T Value (± 95% Confidence Intervals)	qPCR Estimated Resting Spore Number	T_M (°C) (± 95% Confidence Intervals)	Conventional PCR Result[b]	Evan's Blue Viability Assay[c] (% viable; n=30)
1	Wheat	Farmer harvested, not commercially cleaned	19.94±0.45	3.43×10^4	85.24±0.03	Positive	80
2	Canola	Farmer harvested, not commercially cleaned	23.08±0.91	4.04×10^3	85.20±0.03	Positive	90
3	Pea	Farmer harvested, commercially cleaned	25.94±9.54	<1000	84.41±0.06	Positive	98
4[d]	Potato	Harvested from field plots, not commercially cleaned	24.63±0.27	1.40×10^4	85.29±0.05	Positive	90
5	Pea	Harvested from field plots, not commercially cleaned	27.56±0.47	<1000	84.95±0.16	Positive	97
6	Pea	Harvested from field plots, not commercially cleaned	26.70±2.17	<1000	85.03±0.21	Positive	97
7	Pea	Harvested from field plots, not	28.00±0.60	<1000	85.02±0.17	Positive	100

		commercially cleaned					
NA	Various	DNA from each of 28 plant, fungal or bacterial species as template	28.28 ± 1.04[e]	NA	83.77±0.15[e]	Negative	NA
NA	NA	No template included	NA	NA	76.47±7.49	Negative	NA

[a] Samples 1, 2 and 3 were collected from Sturgeon County, Alberta, Canada, while samples 4, 5, 6 and 7 were collected from the County of Newell, Alberta, Canada.

[b] Conventional PCR was performed with primers TC1F and TC1R as per Cao et al. (2007).

[c] The Evan's blue viability assay was performed as per Tanaka et al. (1999).

[d] Sample 4 was diluted 10-fold prior to quantification and the relative spore load was adjusted accordingly.

[e] This value represents the average from two independent qPCR amplifications using DNA extracted from each of 28 plant, fungal or bacterial species as a template.

Table 2. Contrasts among canola seed treatments for control of clubroot development in plants grown from seeds artificially infested with resting spores of *Plasmodiophora brassicae*

	Control ID[a] = 68.1%	Helix Xtra ID = 53.9%	Prosper FX ID = 46.8%	Vitavax RS ID = 24.3%	Nebijin™ SSC ID = 16.4%
Dynasty 100 FS ID = 13.2%	χ^2=557.54 *p*<0.0001	χ^2=268.72 *p* <0.0001	χ^2=254.51 *p* <0.0001	χ^2=26.41 *p* <0.0001	χ^2=2.54 *p*=0.1113
Nebijin™ SSC ID = 16.4%	χ^2=444.11 *p*<0.0001	χ^2=207.24 *p* <0.0001	χ^2=182.97 *p* <0.0001	χ^2=11.73 *p*=0.0006	
Vitavax RS ID = 24.3%	χ^2=293.20 *p* <0.0001	χ^2=119.79 *p* <0.0001	χ^2=90.90 *p* <0.0001		
Prosper FX ID = 46.8%	χ^2=71.73 *p* <0.0001	χ^2=6.98 *p*=0.0082			
Helix Xtra ID = 53.9%	χ^2=25.38 *p* <0.0001				

[a] ID = index of disease; canola seeds were artificially infested with a dusting of soil containing 1×10^8 *P. brassicae* resting spores per g soil, and then seeded in 6 cm × 6 cm × 6 cm plastic pots filled with Metro-Mix 290 (Scotts) potting medium. Clubroot disease severity was evaluated six weeks after planting as described in Strelkov et al. (2006).

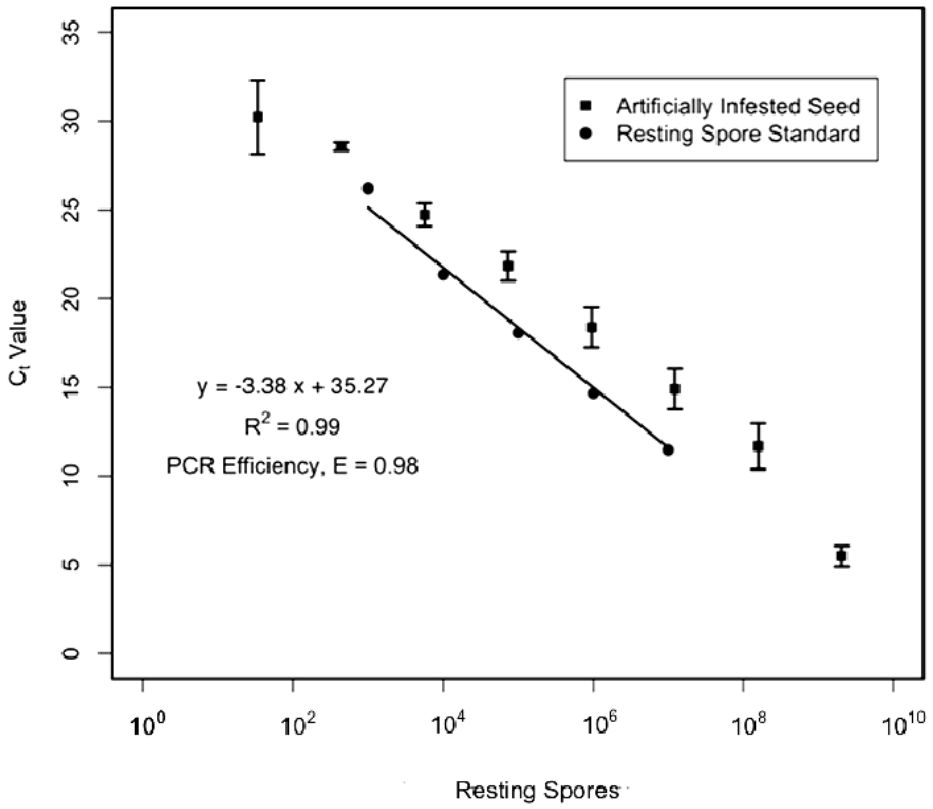

Fig. 1. Cycle threshold (C_T) values obtained using DNA extracted from different numbers of *Plasmodiophora brassicae* resting spores as a template in quantitative PCR. Standards were prepared from a serial dilution of 1×10^8 to 1×10^3 resting spores, and the averages from four replications of the experiment are shown (circles). Quantitative PCR was also performed using DNA extracted from artificially infested canola seeds as a template, with the averages from four replications also shown (squares). Bars indicate the 95% confidence intervals for each data point, and the line represents the linear regression of the resting spore standard over the reliable range of detection. The R^2 was calculated from the regression line as was the line formula. The PCR efficiency (E) was determined according to the formula: $E = 10^{-1/slope} - 1$.

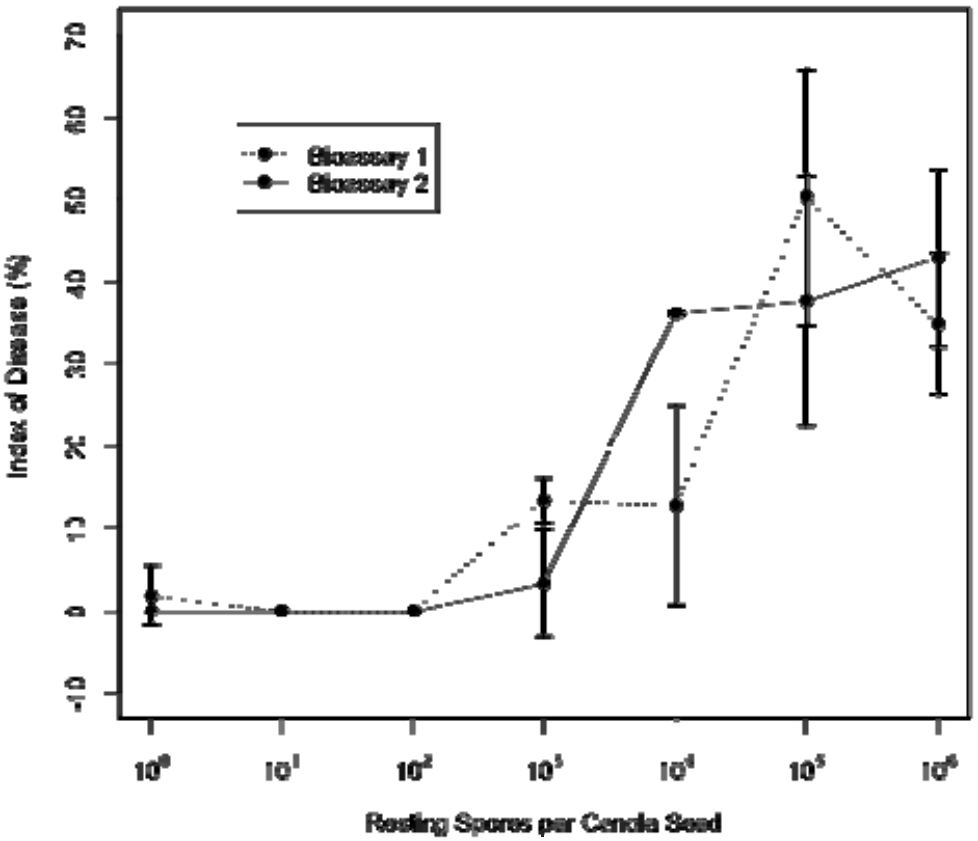

Fig 2. Average index of disease on canola seedlings grown from seeds artificially infested with resting spores of *Plasmodiophora brassicae* at different rates. Infested seeds were sown at a density of 120 seeds m^{-2} in 39 × 26 × 15 cm plastic trays (bioassay 1) or 7.5 cm diameter plastic pots (bioassay 2). The trays or pots were filled with Sunshine #4 Mix/LA7 soil medium (SunGro Horticulture) and maintained in a greenhouse or growth cabinet, respectively. Treatments were arranged in a randomized complete block design. Plants were rated for symptom development six weeks after seeding on a four-point scale, where: 0 represents no galling, 1 represents mild galling, 2 represents moderate galling, and 3 represents extensive galling. The individual scores were used to calculate indices of disease for the various treatments as per Strelkov et al. (2006). Bars indicate the 95% confidence intervals for each data point. Control plants inoculated at a rate of 0 resting spores per seed did not show any symptoms of *P. brassicae* infection in either bioassay (not shown).

COMMUNICATION, DISSEMINATION AND LINKAGE ACTIVITIES

Publications

Rennie, DC, Manolii, VP, Cao, T., Hwang, SF, Howard, RJ, Strelkov, SE. Direct evidence of seed and tuber infestation by *Plasmodiophora brassicae* and quantification of inoculum loads. *Manuscript completed and ready for submission to a refereed journal.*

Cao, T, Manolii, VP, Rennie, DC, Hwang, SF, Strelkov, SE. Efficacy of seed treatments for reducing clubroot [*Plasmodiophora brassicae*] disease severity on canola. *Manuscript under preparation.*

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