DEPARTMENT OF AGRICULTURAL, FOOD AND NUTRITIONAL SCIENCE
FACULTY OF AGRICULTURAL, LIFE & ENVIRONMENTAL SCIENCES

410 Agriculture/Forestry Centre
Edmonton, Alberta, Canada T6G 2P5
Tel: 780.492.3239
Fax: 780.492.4265
afns-chair@ualberta.ca
www.afns.ualberta.ca

January 30th, 2016

Mr. Doug Walkey, BSc, MBA, PAg
Executive Director
Alberta Crop Industry Development Fund Ltd.
5030 50 St, Agriculture Bldg.
LACOMBE, AB T4L 1W8

RE: Project number: 2013F075R, "Development of a rapid quantitative detection method for sclerotinia stem rot inoculum to aid disease risk assessments and fungicide spray decisions"

Dear Mr. Walkey,

Please find enclosed signed original copies of the Final Report for the above Project. We have also included an Appendix containing scientific manuscripts stemming from the Project.

A statement of award and expenditures will be forwarded to ACIDF by the Research Services Office of the University of Alberta under separate cover.

I am currently on sabbatical leave in France, so I have asked my Research Associate, Dr. Tiesen Cao, to mail these documents to you on my behalf.

On behalf of the Research Team, I would like to thank ACIDF, the Alberta Canola Producers Commission, and the Western Grains Research Foundation for your support of this research.

Sincerely,

Stephen Strelkov, PhD
Professor, Plant Pathology

E-mail: stephen.strelkov@ualberta.ca
Telephone: 780-492-1969 (Temporary Tel.: 011 + 33 + 2 99 52 88 07)
Fax: 780-492-4265

cc. Ward Toma, Alberta Canola Producers Commission, 14560-116 Ave NW, Edmonton AB T5M 3E9
cc. Pat Flaten, Western Grains Research Foundation, 306-111 Research Drive, Saskatoon SK S7N 3R2

Date Received
For Administrative Use Only

Full Research Project Final Report

- This report must be a stand-alone report, *i.e.,* must be complete in and of itself. Scientific articles or other publications cannot be substituted for the report.
- One electronic copy and one signed original copy are to be forwarded to the lead funding agency on or before the due date as per the investment agreement.
- A detailed, signed income and expenditure statement incurred during the entire funding period of the project must be submitted along with this report. Revenues should be identified by funder, if applicable. Expenditures should be classified into the following categories: personnel; travel; capital assets; supplies; communication, dissemination and linkage; and overhead (if applicable).
- For any questions regarding the preparation and submission of this report, please contact ACIDF

Section A: Project overview

1. Project number: 2013F075R
2. Project title: Development of a rapid quantitative detection method for sclerotinia stem rot inoculum to aid disease risk assessments and fungicide spray decisions
3. Research team leader: Stephen Strelkov
4. Research team leader's organisation: University of Alberta
5. Project start date (yyyy/mm/dd): 2013/04/01
6. Project completion date (yyyy/mm/dd): 2015/12/31
7. Project final report date (yyyy/mm/dd): 2016/01/31

Section B: Non-technical summary (max 1 page)

Provide a summary of the project results which could be used by the funders for communication to industry stakeholders (*e.g.*, producers, processors, retailers, extension personnel, etc.) and/or the general public. This summary should give a brief background as to why the project was carried out, what were the principal outcomes and key messages, how these outcomes and key messages will advance the agricultural sector, how they will impact industry stakeholders and/or consumers, and what are the economic benefits for the industry.

Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum,* is an economically important disease of canola commonly managed by routine application of fungicides. Petal infestation is an important stage of the disease cycle in canola and has been the focus of previously developed Sclerotinia stem rot risk assessment methods. Quantitative (q)PCR analysis can provide a more rapid and

accurate assessment of petal infestation levels. Primers and a hydrolysis probe were designed to amplify a 70-bp region of a *S. sclerotiorum*-specific gene SS1G_00263. A hydrolysis probe-based qPCR assay was developed that had a detection limit of 8.0×10^{-4} ng of *S. sclerotiorum* DNA and only amplified *S. sclerotiorum* DNA. Evaluation of petals collected at five sampling points in each of 10 commercial canola fields on each of two sampling dates (corresponding to 20-30% bloom and 40-50% bloom) revealed infestation levels ranging from 0 to 3.3×10^{-1} ng *S. sclerotiorum* DNA per petal. The relationship between petal infestation levels and final stem rot incidence in canola was explored further in two additional studies. In the first study, conducted over 2 years, petal infestation was compared with disease incidence in 34-35 commercial canola fields distributed across Alberta, Saskatchewan and Manitoba. In the second study, these parameters were compared over 3 years in 9-11 fields located in central Alberta. In the fields sampled across the Prairies, no consistent relationship was observed between petal infestation and stem rot incidence at harvest. However, no information was available on whether or not these fields received a fungicide treatment, which may have reduced the ability to detect any correlations. In contrast, sampling points in the second study were located in fungicide-free check strips, and the incidence of stem rot was generally found to increase with increasing petal infestation. The strength of the relationship varied across the study years, and was strongest when canola petals were analyzed at full bloom and in years when disease pressure was high. The qPCR assay may serve as the basis for a risk assessment system, as well as representing a useful tool for the study of the epidemiology of Sclerotinia stem rot of canola. It can quantify the level of petal infestation, thereby providing a measure of disease risk when timely fungicide application decisions need to be made. It is important to emphasize, however, that a forecasting system based on qPCR quantification of petal infestation should be linked to environmental conditions, as well as cropping history, seeding date and crop canopy conditions, which may influence stem rot development and the need to spray a fungicide.

Section C: Project details

1. Project team (max ½ page)

Describe the contribution of each member of the R&D team to the functioning of the project. Also describe any changes to the team which occurred over the course of the project.

The project leader, Dr. Strelkov, guided the development of the quantitative (q)PCR-based methodology to detect and measure *Sclerotinia sclerotiorum* in canola tissues. The main collaborator, Dr. Turkington, contributed to the design and coordination of all of the field-related components of the research, including establishment of sampling sites, development of sample collection protocols, and sampling regimes. Barbara Ziesman is a graduate student who conducted the research as part of her studies. She developed methodologies, compiled data, and analyzed the results, receiving feedback, guidance and suggestions as needed from Drs. Strelkov and Turkington. The composition of the research team did not change over the course of the project.

2. Background (max 1 page)

Describe the project background and include the related scientific and development work that has been completed to date by your team and/or others.

Sclerotinia stem rot, caused by *S. sclerotiorum*, is one of the most important biotic stresses influencing long-term production and economic stability for canola producers. The wide host range of the stem rot pathogen, its production of wind-borne ascospores, and the sporadic nature of disease development limit the effectiveness of many management strategies (Bom and Boland 2000). Stem rot on canola is primarily managed by the routine application of fungicides, typically without any indication of disease risk. To reduce the negative economic and environmental effects associated with the application of fungicides, a reliable stem rot forecasting system is desirable. Although producers and industry have access to existing risk assessment tools, such as weather-based forecast maps, checklists and petal testing, there has been limited acceptance or widespread use of these tools (McLaren et al. 2004). Weather maps are regional and not field-specific forecasts, while checklists are qualitative in nature. Petal testing is the only available risk assessment tool providing a direct assessment of inoculum levels, but is under-used due to the time required from sample collection to availability of results. The development of a qPCR-based system to accurately measure the amount of *S. sclerotiorum* inoculum on canola petals could overcome some of these limitations, helping to provide forecasts for disease risk while taking into account environmental conditions.

There have been several previously published reports of qPCR detection systems for *S. sclerotiorum*. Rogers et al. (2009) developed a qPCR assay that could quantify *S. sclerotiorum* DNA, but was unable to differentiate low levels of *S. sclerotiorum* DNA from a high background of *B. cinerea* DNA. Yin et al. (2009) developed a qPCR assay that was able to amplify *S. sclerotiorum* DNA with a high level of sensitivity, but did not include any species closely related to *Sclerotinia* in their study, making it difficult to assess the method specificity. In addition, no Canadian isolates of the fungus were analyzed. Most recently, Almquist and Wallenhammar (2015) developed a hydrolysis probe-based qPCR assay for the detection of *S. sclerotiorum* DNA in air samples and infected leaves and petals of oilseed rape (*B. napus*) in central Sweden.

The ideal PCR-based forecasting system for deployment in Canadian canola production systems should be quantitative, highly sensitive, and selective for *S. sclerotiorum*. Most importantly, the results obtained with this forecasting system should be positively correlated with final stem rot levels in the field. A recent study (Liang et al. 2013) identified a hypothetical protein from *S. sclerotiorum* that appears to be unique to this fungus. The gene encoding this protein was selected as a target for the development of a qPCR-based assay for the detection and quantification of *S. sclerotiorum* in canola, which then was used to analyze petal infestation levels and related to final stem rot severity in commercial fields.

3. Objectives and deliverables (max 1 page)

State what the original objective(s) and expected deliverable(s) of the project were. Also describe any modifications to the objective(s) and deliverable(s) which occurred over the course of the project.

The specific objectives of the research project included:

a) Development and refinement of a rapid quantitative method for pathogen detection in canola flowers

b) Understanding of the relationship between the amount of pathogen on the petals and final stem rot levels in commercial fields

c) Assessment of correlations between pathogen detection, weather-based forecasts and final stem rot levels

The long-term goal is for this information to serve as the basis for a commercially available test for stem rot forecasting.

4. Research design and methodology (max 4 pages)

Describe and summarise the project design, methodology and methods of laboratory and statistical analysis that were actually used to carry out the project. Please provide sufficient detail to determine the experimental and statistical validity of the work and give reference to relevant literature where appropriate. For ease of evaluation, please structure this section according to the objectives cited above.

a) Development and refinement of a rapid quantitative method for pathogen detection in canola flowers

DNA isolation from field-collected canola petals. To validate the qPCR assay, canola petals were collected from 10 commercial fields in the Edmonton, AB, region in 2012. The fields were sampled once at 20-30% bloom and again at 40-50% bloom, as determined by visual assessment (Harper and Berkenkamp 1975). The incidence of Sclerotinia stem rot also was assessed at the end of the growing season for future reference (see below). Petals were collected at each of five sampling sites within each field. At each sampling site, the top 10-20 cm of the inflorescences from each of 20 plants were collected at random, placed in a plastic bag, and stored in a cooler on ice for transport back to the laboratory. DNA was isolated according to the protocol of Liang et al. (2013) from a randomly chosen subsample of 20 petals from each sampling site, and quantified with a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA) as above.

Development of *S. sclerotiorum*-specific primers. Forward and reverse primers and a hydrolysis probe were designed using Primer 3 Express (Applied Biosystems, Carlsbad, CA, USA) based on the 468-bp sequence of a single-copy gene (SS1G_00263) encoding the hypothetical secreted protein ssv263 (Liang et al. 2013) from *S. sclerotiorum*. The forward (SSBZF) and reverse (SSBZR) primers were designed to amplify a 70-bp product in a region of SS1G_00263 that exhibits the greatest difference with an orthologue in *B. cinerea*. The hydrolysis probe (SSBZP) was labeled with a non-fluorescent quencher-mini groove binder (NFQ-MGB) on the 3' end, and with the reporter dye FAM (6-carboxyfluorescein) on the 5' end. The probe and primer sequences were used to query the GenBank databases using the Basic Local Alignment Search Tool (BLAST) in order to identify any similar sequences from other organisms that could lead to false positives.

Development of the exogenous positive internal control. To identify false negatives resulting from failed DNA extraction or inhibition of the PCR, an exogenous internal control was included in the analysis of all samples. The *ToxB* gene from *Pyrenophora tritici-repentis*, causal agent of tan spot of wheat, was selected as the exogenous internal control since it is unlikely to be found in canola petals. The pSilent1 plasmid (Nakayashiki et al. 2005) containing a 432-bp fragment of *ToxB* from *P. tritici-repentis* (Aboukhaddour et al. 2012) was used as the template for the design

of the primers and hydrolysis probe. The *ToxB*-specific primers (ToxBF and ToxBR) and a probe (ToxBP) were designed using Primer 3 Express (Applied Biosystems). The *ToxB*-specific hydrolysis probe was labelled with NFQ-MGB on the 3' end and with the reporter dye VIC on the 5' end.

qPCR analysis. All qPCR analyses were conducted in a ViiA7 Real-Time PCR System (Life Technologies) using the Universal FastStart Master (Rox) mix (Roche, Indianapolis, IN, USA) in a MicroAmp® Fast Optical 96-well reaction plate (Applied Biosystems), which was sealed with MicroAmp optical adhesive film (Applied Biosystems). The reaction conditions included a hot start at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec as per the manufacturer's instructions for the master mix. Each of the primer/probe sets was analyzed separately and reactions were not multiplexed. Each sample was analyzed in triplicate for quantification of the target gene, while samples were analyzed in duplicate for assays of the internal control. If the standard deviation of the mean C_q obtained for a set of triplicates of a given sample was > 0.5, the sample was reanalyzed, and if an outlier was present, it was removed. Similarly, if the standard deviation of the mean C_q obtained for duplicates of the *ToxB* internal control was > 0.5, the sample was re-extracted.

Specificity testing. The specificity of the SS1G_00263 primer and probe set was confirmed by separately testing 100 ng of DNA from 13 fungal species and the canola host as templates in the qPCR analysis. In addition, amplification of a total of eight isolates of *B. cinerea* and seven isolates of *S. sclerotiorum* was compared. No amplification of any product, or a C_q value > 2 cycles below that of the smallest standard, were considered to be indicative of no detection. The reproducibility of the standard curve was assessed by calculating the standard deviation at each point, for 4 replications included in separate 96-well plates. Plasmids containing the *ToxB* insert were analyzed with the SS1G_00263-specific primer set to ensure that SSBZF/SSBZR did not amplify the internal control. Amplicons obtained with the SSBZF/SSBZR primer set were sequenced to confirm the identity of the product.

Sensitivity testing. To test the limit of detection (LOD) of the assay, standard curves were generated with 1:10 serial dilutions of purified *S. sclerotiorum* DNA. All standard curves tested consisted of five serial dilutions ranging from 8.0 ng to 8.0×10^{-4} ng or from 5.0 ng to 5.0×10^{-4} ng. Each standard was included in triplicate in the same 96-well plate as the samples being analyzed. The LOD was regarded as the lowest quantity of DNA that could be detected with confidence in all three of the triplicates. The efficiency and R^2 of the standard curves were determined for each replication. To determine if inclusion of the internal control and the presence of *B. napus* DNA reduced the LOD for *S. sclerotiorum*, fungal DNA was quantified in the presence or absence of the *ToxB* internal control and *B. napus* DNA. The mean C_q and standard deviation of the mean for each point in the dilution series were compared between the spiked and non-spiked samples.

Evaluation of canola petals. Total genomic DNA isolated from samples consisting of 20 canola petals each was diluted by a factor of 1:5 with nuclease free water (Life Technologies) prior to qPCR analysis with the SS1G_00263- and *ToxB*-specific primers and probes. DNA was analyzed from samples collected at five sampling sites from each of 10 commercial canola fields at each of two sampling dates as described above. Results are expressed on a per petal basis by first

accounting for the dilution, then by dividing the estimate by 20. The qPCR estimates were averaged over each field on each sampling date and the standard deviation of the mean was calculated for each field.

Assessment of the SsF and SsR primers. Isolates of *S. sclerotiorum* collected from canola fields in central Alberta were subjected to qPCR analysis with the primers SsF and SsR developed by Yin et al. (2009). Quantitative PCR assays were conducted in a Step One Plus Real-Time PCR System (Applied Biosystems). After the reaction was complete, a melting point analysis was conducted to confirm the presence of a single amplification product that had a melting temperature (T_M) consistent with the predicted T_M of 84°C. The specificity of the SsF/SsR primer set also was evaluated as described above for the other primer and probe sets.

b) Understanding of the relationship between the amount of pathogen on the petals and final stem rot levels in commercial fields

Field selection and sample collection. Two experiments (Exp. 1 and Exp. 2) were conducted to evaluate the relationship between petal infestation level and final stem rot disease incidence. Experiment 1was conducted over two years (2011 and 2012) by monitoring petal infestation and subsequent disease incidence in 35 (in 2011) or 34 (in 2012) commercial canola fields in Alberta (near Edmonton and Lacombe), Manitoba (Brandon, Morden and Carman) and Saskatchewan (Melfort and Saskatoon). Experiment 2 was conducted in commercial canola fields located near Edmonton, Alberta, in 2011, 2012 and 2013. Nine fields were included in 2011, 10 fields in 2012, and 11 fields in 2013. The same central Alberta fields were monitored for Exp. 1 and Exp. 2 in 2011 and 2012. Experiment 1 was focussed on a Prairies-wide evaluation of the relationship between petal infestation and stem rot incidence, while Exp. 2 examined this relationship within a smaller regional scale.

With the exception of the fields near Edmonton, the fields in Exp. 1 were selected at random. For the fields sampled in Manitoba and Saskatchewan, field histories were not known, and no information was available as to whether or not fungicides were applied for stem rot control during the growing season. In each field, there were 5 sampling sites spaced 50 m apart and at least 75 m from the field edge. In Exp. 2, the sampling sites were situated in fungicide-free check strips. There were five sampling sites per field arranged 50 m apart in a linear fashion.

Canola petals were collected at early flowering (10-20% bloom) and full flowering (40-50% bloom). In 2012, the first sampling of the fields near Edmonton was delayed until 20-30% bloom. Samples were collected between 1100 h and late afternoon, with the exception of the fields near Edmonton in 2012 where sampling occurred early in the morning due to high temperatures during the flowering period. At each sampling site, the top 20-30 cm of 20 randomly selected inflorescences were collected and placed into clean labelled plastic bags and kept on ice in a cooler during transport back to the laboratory. In the laboratory, the samples were maintained at 4°C prior to processing, which was carried out within 24 hours of sample collection.

Estimates of *S. sclerotiorum* infestation. Petals were assessed for *S. sclerotiorum* infestation by qPCR analysis in both Exp. 1 and Exp. 2, as well as by the traditional petal plate test in Exp. 2.

The qPCR analysis was conducted as described above. The petal plate test was conducted as described by Turkington et al. (1988). Briefly, petals were plated on Petri dishes containing solid potato dextrose agar amended with 25 ppm ampicillin and 25 ppm streptomycin and incubated for 4-5 days in darkness at room temperature (approximately 20-24°C). The percentage of petals infected with *S. sclerotiorum* was determined through visual assessment, and the petal infestation estimates were averaged for each field on each sampling date.

Sclerotinia stem rot assessment. Sampling sites were revisited prior to swathing, in order to assess Sclerotinia stem rot incidence and severity in the canola crops. Twenty randomly selected plants were evaluated at each sampling site, for a total of 100 plants per field. The plants were rated for stem rot of severity on a 0-5 scale as described by Kutcher and Wolf (2006). In Exp. 1, disease incidence was defined as the percentage of plants that had any symptoms of stem rot on any organ (i.e., rated as 1 or greater). In Exp. 2, disease incidence was adjusted to include only plants with a severity rating of 2 or higher. The adjusted disease incidence values excluded infections rated as 1 (pod infections), since upper canopy (i.e., pod) infections would be expected to be a minor contributor to yield loss. Statistical analysis for Exp. 2 indicated that the adjusted disease incidence values improved the R^2 values slightly, but did not change the significance of the regression models. As a result, the calculations for disease incidence in Exp. 1 were not adjusted.

Statistical analysis. The relationship between qPCR estimates of petal infestation and final stem rot levels was determined with quadratic regression using proc reg in SAS (Statistical Analysis System) software. Assumptions of regression were tested and no transformations were required. Scatter plots indicated a non-linear regression. This was confirmed by the lower Akiake information criterion (AIC) and Bayesian information criterion (BIC) values for quadratic regression models over simple linear regression models, indicating a better fit. The AIC/BIC values were calculated for both linear and quadratic models using proc mixed and the residual maximum likelihood (REML) method. Regression analysis was conducted on the average qPCR value for each field at each of the sampling dates individually. Analysis was carried out separately for the three sampling years because of differences in the respective environmental conditions. The year to year variation in the strength of the statistical relationship was used to assess whether or not qPCR-based estimates of petal infestation could consistently indicate stem rot risk without consideration of other factors. Regression models were considered significant when the slopes of the independent variables and the overall models were significant at $P = 0.05$.

In Exp. 2, the relationship between Sclerotinia stem rot disease incidence and percent petal infestation (PPI), determined with the plate test, was assessed by simple linear regression using proc reg in SAS. Regression analysis with non-transformed disease incidence did not meet the assumptions of regression. Disease incidence values were Arcsine transformed as discussed by Turkington and Morrall (1993), and the resulting residuals more closely met the assumptions of regression. Thus, arcsine transformed disease incidence (TDI) was used for all regressions with percent petal infestation (PPI) values. Simple linear regression models were confirmed to have lower AIC and BIC values than the quadratic regression models, indicating a better fit for the linear models. As with the qPCR results, regression analysis was conducted separately for each of the three sampling years. Regression models were considered significant when the slopes of the independent variables and the overall models were significant at $P = 0.05$.

Correlation analysis with proc reg in SAS was used in Exp. 2 to determine the relationship between the qPCR-based and petal plate test estimates of petal infestation level. In all three years, there was a slight deviation from normality for at least one variable. As a result, Spearman Rank Correlation was used.

c) Assessment of correlations between pathogen detection, precipitation and final stem rot levels

In Exp. 1, the data were further sorted in three different ways: by province, by average regional annual precipitation, and by average annual regional precipitation from April to August ('summer precipitation'). The aim of this analysis was to determine which method accounted for the most variation and produced the stronger regression model. Precipitation data were obtained from Environment Canada. The data were separated into three main groups based on average regional annual precipitation: >500 mm (Carman and Morden), between 400-500 mm (Brandon, Lacombe and Edmonton), and <400 mm (Melfort and Saskatoon). Summer precipitation was averaged for the months of April through August and was used to separate the data into two main groups: >300mm (Morden, Edmonton, Lacombe, Brandon and Carman) and <300mm (Melfort and Saskatoon).

5. Results, discussion and conclusions (max 8 pages)

Present the project results and discuss their implications. Discuss any variance between expected targets and those achieved. Highlight the innovative, unique nature of the new knowledge generated. Describe implications of this knowledge for the advancement of agricultural science. For ease of evaluation, please structure this section according to the objectives cited above.
NB: Tables, graphs, manuscripts, etc., may be included as appendices to this report.

a) Development and refinement of a rapid quantitative method for pathogen detection in canola flowers

Amplicon generated with SSBZF/SSBZR. The amplicons generated from six isolates of *S. sclerotiorum* with the SSBZF/SSBZR primer set targeting the gene SS1G_00263 were sequenced and confirmed to be of the expected 70-bp size. Moreover, the sequences were identical to each other, and a query of the GenBank database using BLASTN revealed 100% identity with accession number XM_001598127.1 from *S. sclerotiorum* (e-value = 3e-31).

Specificity testing. While the SS1G_00263-specific primers consistently amplified a 70-bp product from DNA of each of the *S. sclerotiorum* isolates evaluated, they did not generate an amplicon from any of the other 13 species tested, including the closely related *B. cinerea, S. trifoliorum*, or *S. minor*. Similarly, no amplicon was obtained from DNA of the host canola plant. Specificity testing with the *ToxB*-specific primers did not yield an amplicon from *S. sclerotiorum* or any of the non-target organisms tested.

Sensitivity testing. The lowest reliable LOD was 8.0×10^{-4} ng *S. sclerotiorum* DNA. Although the assay could detect quantities of *S. sclerotiorum* DNA as low as 5.0×10^{-4} ng, such detection could not be accomplished with a standard deviation of the mean $C_q < 0.5$. As such, the lowest

point of the standard curve was set to 8.0×10^{-4} ng DNA. To compare replicates of the standard curve, the C_q values were averaged for a set of comparisons and the standard deviation of the mean for each point on the curve was calculated. An average standard deviation of 0.19 was obtained for the mean C_q over all five points on the curve, among four replicates of the standard curve in separate 96-well plates; none of the points had a standard deviation > 0.5. For the four replicates of the standard curve generated in separate 96-well plates, the PCR efficiencies ranged from 88.90 to 90.25 and the R^2 values ranged from 0.9928 to 0.9999.

Curves generated with the SS1G_00263-specific primers in the presence or absence of the internal control and *B. napus* DNA were compared to identify any potential reduction in the quantification of *S. sclerotiorum* DNA. The C_q values were averaged for each dilution point in the two dilution series and the standard deviation of the C_q values for the replicates was calculated. Each point on the standard curves was compared individually and no single point had a standard deviation of the mean C_q > 0.54.

Quantification of *S. sclerotiorum* in canola petals. Total genomic DNA extracted from 20 petals from each of five sampling sites in each of 10 commercial canola fields on two different sampling dates (100 DNA samples in total) was used as a template for qPCR analysis. The petal infestation estimates for individual sampling sites ranged from 0 ng to 3.3×10^{-1} ng *S. sclerotiorum* DNA per petal. The amount of *S. sclerotiorum* DNA in each of the 5 sampling sites per field was averaged to give a single petal infestation estimate for each field on each sampling date. On the first sampling date, when the canola was at 20-30% bloom, average infestation in the 10 fields ranged from a mean (± standard deviation) of 6.0×10^{-3} ng ± 7.0×10^{-3} ng to 3.4×10^{-2} ng ± 2.8×10^{-2} ng *S. sclerotiorum* DNA per petal (C_q values = 30.5-36.1). On the second sampling date, at 40-50% bloom, average infestation in the 10 fields ranged from a mean (± standard deviation) of 1.0×10^{-3} ng ± 1.0×10^{-3} ng to 8.0×10^{-2} ng ±1.4×10^{-1} ng *S. sclerotiorum* DNA per petal (C_q values = 28.9-36.1).

In addition to the analysis with the SS1G_00263-specific primer and probe set, all samples were analyzed with the *ToxB* (internal control)-specific primer and probe set in separate, singleplex qPCR assays. The mean and standard deviation of the mean C_q value for duplicates was calculated for early bloom and late bloom sample sets to get an estimate of the amount of variation. At 20-30% bloom, the overall mean C_q (± standard deviation) was 29.6 ± 1.08, and at 40-50% bloom, the overall mean C_q (± standard deviation) was 27.6 ± 0.70.

Assessment of the SsF and SsR primers. The primers SsF and SsR (Yin et al. 2009) were evaluated to determine their suitability for quantifying *S. sclerotiorum* DNA in canola petals under Canadian conditions. The primers amplified a product of approximately 225-bp from all isolates of *S. sclerotiorum*, and did not amplify DNA of *S. minor*, *Aspergillus niger*, *Cladosporium* sp., *Mucor* sp., *Alternaria alternata*, or *Leptosphaeria maculans*. However, the primers were found to amplify products from DNA of *S. trifoliorum*, as well as from all isolates tested of *B. cinerea* and *B. fuckeliana*, *Rhizopus* sp., *Trichoderma* sp., *Rhizoctonia solani*, *Penicillium* sp., and *Fusarium graminearum*. The amplicons obtained from *B. cinerea*, *S. trifoliorum* and *Trichoderma* sp. all had temperature peaks between 84 and 85°C, which was within the range expected for the product from *S. sclerotiorum*. For the products amplified from DNA of *B. fuckeliana*, *Rhizopus* sp., *R. solani*, *Penicillium* sp., and *F. graminearum*, the melting

curve analysis revealed the presence of multiple temperature peaks associated with non-specific amplification. The sensitivity of the SsF/SsR primer set was similar to that of the SSBZF/SSBFR primers, with a consistent LOD of 8.0×10^{-4} ng DNA.

b) Understanding of the relationship between the amount of pathogen on the petals and final stem rot levels in commercial fields

Experiment 1. Sclerotinia stem rot disease incidence and the qPCR estimates of petal infestation for individual fields were variable over the sampling years and between locations. In both 2011 and 2012, the disease incidence ranged from 0 to 92% among the sampled fields, but the mean disease incidence (MDI) for all fields, presented as MDI ± standard deviation, was higher in 2012 (27.2% ± 29.5%) than in 2011 (11.9% ± 17.7%). The mean amount of *S. sclerotiorum* DNA per petal for all fields, as measured by qPCR analysis, was highest at full bloom in both 2011 (0.013 ng/petal ± 0.0018 ng/petal) and 2012 (0.068 ng/petal ± 0.18 ng/petal). There was variation between sampling sites, years and individual fields for disease incidence and the amount of *S. sclerotiorum* DNA per petal. When the data were separated by province, none of the relationships between the qPCR results and disease incidence were significant at either sampling date in 2011. The coefficient of variation (CV) for these regression models ranged from 94.14 to 144.05. In 2012, however, the relationship between the early bloom qPCR results and disease incidence for the fields in Alberta was found to be significant, with the early bloom qPCR results accounting for 59.1% of the variation in disease incidence ($R^2 = 0.591$; $P = 0.0073$; CV 42.694). The regression models for the Alberta fields at full bloom and for the Saskatchewan and Manitoba fields at both sampling dates were not significant, with CVs ranging from 51.05 to 111.67.

Experiment 2. Sclerotinia stem rot incidence, PPI and the estimates of petal infestation obtained by qPCR analysis were variable over the three years of the study. Mean stem rot incidence across all fields, presented as the mean ± standard deviation, was lower in 2011 (7.8% ± 6.6%) than in 2012 and 2013 (64% ± 23.2% and 39.36% ± 24.8%, respectively). For both sampling dates, estimates of petal infestation determined by qPCR analysis also were lowest in 2011 (early bloom: 0.005 ng/petal ± 0.005 ng/petal; full bloom: 0.017 ng/petal ± 0.013 ng/petal). On a field level, qPCR estimates of petal infestation at full bloom were higher for all 9 fields in 2011, for five of 10 fields in 2012, and for 5 of 11 fields in 2013. As in Exp. 1, there was a high amount of variation in the levels of petal infestation, as determined by qPCR analysis, in samples from some of the fields.

Mean PPI, as assessed by the petal plate test of Turkington et al. (1988), was similar across both sampling dates in 2011 (early bloom: 35.9% ± 22.3%; full bloom: 40.1% ± 6.84%) and 2012 (early bloom: 34.3% ± 17.1%; full bloom: 41.2% ± 15.1%). In contrast, PPI was generally higher in 2013 (early bloom: 43.85% ± 18.2%; full bloom: 51.0% ± 18.5%). A comparison of petal infestation levels, as determined by qPCR analysis or the petal plate test, found similar results in 2011 and 2012: both methods indicated that petal infestation levels were lower at early bloom than at late bloom. In 2013, however, the different methods yielded different results. Petal infestation was found to be lower at early bloom when evaluated by the petal plate test, and lower at late bloom when evaluated by qPCR analysis.

Relationship between qPCR-based petal infestation estimates and disease incidence. In each year of Exp. 2, three non-linear regression models were analyzed to determine the relationship between qPCR estimates of petal infestation and final stem rot disease incidence. In all three years, disease incidence increased as the amount of *S. sclerotiorum* DNA per canola petal increased. However, the strength of the relationship varied with year and was not always significant. In all years, there was a significant amount of variation in both the final disease incidence and the petal infestation estimates in the fields sampled. In 2011, none of the three regression models were significant at P = 0.05. The field with the highest stem rot incidence also had a very low level of petal infestation (as determined by qPCR) and appeared to be an outlier. If this field is removed from the analysis, the relationship between the qPCR results at full bloom and disease incidence becomes significant ($R^2 = 0.9194$; P = 0.0018; CV = 27.21). In 2012, there was no significant statistical relationship between the qPCR estimates and disease incidence at early bloom. However, there was a significant relationship between disease incidence and qPCR estimates at full bloom (P = 0.0165; $R^2 = 0.6904$; CV = 22.91).

In 2013, there was no significant statistical relationship at either early or full bloom when all fields were included in the analysis. As in 2011, there was one outlier. When this outlier was removed, the relationship between the qPCR estimates at full bloom and stem rot disease incidence became significant ($R^2 = 0.6047$; P = 0.039; CV = 40.79). In 2013, there was a wider range of seeding dates (May 9-May 20), with some canola fields seeded later than in 2011 and 2012. When only those fields seeded on or prior to May 15 were included in the analysis, the regression became highly significant at full bloom ($R^2 = 0.9204$; P = value 0.0063; CV = 20.83).

The amount of variation in the data set was high in all years, as indicated by the coefficients of variation, which ranged from 37.22 to 89.056 for all non-significant regressions.

Relationship between PPI and disease incidence. In 2011 and 2013, there was not a significant statistical linear relationship between PPI and disease incidence at either early bloom or late bloom, even in 2013 when only early seeded fields were included in the analysis. In 2012, the relationship was significant at full bloom ($R^2 = 0.682$; P = 0.003; CV = 16.189).

Relationship between PPI and qPCR estimates of petal infestation. Correlation analysis was used to determine the relationship between PPI and the amount of *S. sclerotiorum* DNA per canola petal as determined by qPCR analysis. In 2011, the only significant correlation was at early bloom ($R^2 = 0.71$; P = 0.03). In 2012, the correlation was not significant at early bloom but was significant at full bloom ($R^2 = 0.80$; P = 0.0056). In 2013, there was no significant correlation at either early or full bloom.

c) Assessment of correlations between pathogen detection, precipitation and final stem rot levels

When the data from Exp. 1 were separated by average regional annual precipitation, the relationships were not significant for the locations with >400 mm precipitation in 2011, or for none of the groups in 2012. The CV for these models ranged from 51.046 to 159.16. The regression analysis for locations with >500 mm of annual precipitation at the late bloom sampling date in 2011 could not be conducted using a quadratic model because of the large

number of fields with a disease incidence of 0%. For this group of locations, the simple linear regression was analyzed and found not to be significant. The relationship between late bloom qPCR results and disease incidence for fields with average regional annual precipitation was found to be significant, with the late bloom qPCR results accounting for 57.6% of the variation in disease incidence ($R^2 = 0.576$; $P = 0.0356$; CV = 95.559).

When the data were separated by average regional summer precipitation, none of the regression models were significant in 2011. In 2012, the regression models for the group of locations with average summer precipitation levels > 300 mm were significant at both early bloom ($R^2 = 0.263$; $P = 0.035$; CV = 95.116) and late bloom ($R^2 = 0.244$; $P = 0.0459$; CV 96.301). The CV values for the non-significant regression models ranged from 95.56 to 129.022 in 2011 and from 84.295 to 99.600 in 2012.

d) Discussion

This study focussed on the development of a qPCR-based assay for the quantification of *S. sclerotiorum* DNA in canola petals, to enable rapid and accurate estimates of infestation levels. The assay targets a 70-bp region of a single-copy gene encoding the hypothetical secreted protein ssv263 (Liang et al. 2013). While the target region of the gene shares 71% similarity with an orthologue in *B. cinerea*, specificity testing indicated that there was no amplification of DNA from any of six *B. cinerea* isolates tested. The qPCR assay also did not amplify DNA from any of the 13 other species evaluated in this study, including *S. minor, S. trifoliorum* and the host plant, *B. napus*. These results indicate that the assay is highly specific for *S. sclerotiorum*, and can be used to estimate pathogen biomass in canola petals.

The sensitivity tests revealed a consistent LOD of 8.0×10^{-4} ng *S. sclerotiorum* DNA. Rogers et al. (2008) reported that an ascospore of *S. sclerotiorum* is equivalent to about 3.5×10^{-4} ng of DNA, suggesting a LOD of 2.3 ascospores per petal for the current assay. When adjusted to account for the dilution of DNA from canola petals during extraction and analysis, the LOD would correspond to 1.5×10^{-3} ng *S. sclerotiorum* DNA or 4.3 ascospores per petal. An issue with the use of ascospores as the basis for a standard curve to quantify petal infestation, however, is that this approach will not reflect increases in pathogen biomass associated with spore germination. Ascospores have been shown to begin germination as early as 3 hours after release from the apothecium under favorable environmental conditions (Willets and Wong 1980). Therefore, it is likely that any assay examining the amount of *S. sclerotiorum* DNA on canola petals is measuring DNA from the ascospores as well as from hyphae developing from germinated spores. As such, quantification of *S. sclerotiorum* on petals is more meaningful and accurate when expressed as the total amount of pathogen DNA. The specificity of the assay was not affected by the presence of *B. napus* DNA or of an internal control in the samples. These are important considerations if the assay is to be used to measure infestation of petals collected in the field.

Yin et al. (2009) developed a qPCR assay to measure *S. sclerotiorum* DNA in canola petals using intercalating dye (SYBR Green) detection technology that detected between 0.0252 ng-0.111 ng pathogen DNA per mg of canola petal tissue. Evaluation of the primers developed by Yin et al. (2009) on DNA extracted from Canadian isolates of *S. sclerotiorum* and other fungal species,

using a different master mix but the same dye detection technology, indicated sensitivity similar to that of the assay described in this report. However, the specificity of the primers seemed considerably lower and amplicons were obtained from various non-target species including *S. trifoliorum, B. cinerea, F. graminearum, Rhizopus* sp., and *Penicillium* sp. In their original report, Yin et al. (2009) found no amplification of DNA other than from *S. sclerotiorum*, but only evaluated one isolate of *B. cinerea* (*B. fuckeliana*) and did not test *S. minor, S. trifoliorum*, or *Rhizopus* sp. They did test *R. solani, Penicillium* sp. and *F. graminearum*, but did not detect any product from the isolates evaluated. This lower specificity represents a challenge for application of the earlier protocol (Yin et al. 2009) in the analysis of field samples, and hence for its use as the basis for a stem rot risk assessment system in Canada. While it may be possible to identify non-specific amplification products based on a melting curve analysis, this would complicate the procedure and introduce the possibility of additional errors. Moreover, the quantification of field samples containing a range of different fungal species in addition to *S. sclerotiorum* may be difficult. The similar sensitivity, but increased specificity of the assay developed in this study with the primers SSBZF/SSBZR and probe SSBZP may reflect the use of a hydrolysis probe instead of an intercalating dye. An intercalating dye, such as SYBR green, binds to any double-stranded DNA and releases a fluorescent signal (Ririe et al. 1997). This potentially can result in fluorescent amplification of non-target double-stranded DNA that must be further differentiated through melting curve analysis (Smith and Osborn 2008). The use of a hydrolysis probe ensures that a fluorescent signal is produced only when there is an exact match between the probe and the target (Smith and Osborn 2008). This increased level of specificity reduces the likelihood of false positives when analysing diverse field samples, without compromising sensitivity. As a result, the newly developed qPCR assay may represent a more reliable method to quantify *S. sclerotiorum* DNA in canola petals.

When the hydrolysis probe-based qPCR assay was used to quantify *S. sclerotiorum* in field-collected canola petals, considerable variation was observed in the amount of petal infestation in different fields and at different crop stages. Environmental conditions in 2012 were conducive to stem rot development in the sampled fields. In five of 10 fields, infestation levels were highest at 40-50% bloom, while in the remaining five fields, the infestation levels were highest at 20-30% bloom. This is consistent with the findings of Turkington and Morrall (1993), who reported changes in petal infestation from early to late bloom and at different locations when culturing petals on growth medium. Similarly, using qPCR analysis, Almquist and Wallenhammer (2015) also observed differences in *S. sclerotiorum* incidence at different stages of flowering. The evaluation of petals by qPCR analysis over the flowering period could provide an indication of when inoculum levels are highest, and thus when the crop may benefit most from fungicide application. The variation in the amount of *S. sclerotiorum* DNA between locations may reflect differences in inoculum level or in the timing of ascospore release, crop seeding date and growth stage, canopy density, or microclimatic conditions. It also indicates the importance of assessing petal infestation and risk potential for a particular field as opposed to an assessment of risk based on regional conditions. The inclusion of an internal control helped to identify outliers resulting from possible human error or other artifacts, such as inhibition of the PCR, thereby improving the accuracy of qPCR-based estimates of petal infestation.

In all fields and locations included in this study, there was variation in both the amount of *S. sclerotiorum* DNA detected by qPCR analysis and in the final disease incidence. There also was

significant year to year variation in these parameters, most likely reflecting differences in environmental conditions that in turn influence carpogenic germination of the sclerotia and the timing of ascospore release. Similarly, the differences observed between fields could also be a result of regional differences in environmental conditions, but they may also reflect differences in crop stand density, seeding date, seeding rate and crop history, all of which can influence stem rot development (Turkington and Morrall 1993; Jurke and Fernando 2008; Twengstrom et al. 1998). In addition to field to field variation, variation across sampling sites within particular fields also was observed. This variation likely reflected differences in crop stand and microclimate at different points within the same field. Five sites were sampled per field, since this was reported to be an accurate sampling size for evaluating the incidence of petal infestation (Turkington et al. 1988). The large amount of variation observed in this study, however, indicates that a larger number of sampling sites may be needed to provide a more reliable estimate of petal infestation in a particular field.

The amount of *S. sclerotiorum* DNA quantified per canola petal varied across the flowering season. These findings are consistent with previous reports that found that inoculum pressure is not consistent across the flowering period or between fields (Almquist and Wallenhammar 2015; Turkington and Morrall 1993). Differences in the level of infestation over the growing season will influence the strength of the statistical relationship between quantifications of petal infestation and stem rot incidence when evaluated across several fields. Monitoring changes in petal infestation over the flowering period may serve to identify the best timing for fungicide application in a particular field, but such an approach would have to be balanced by cost and other practical considerations. Nonetheless, closer monitoring of infestation levels on a temporal scale could prove useful in better understanding the epidemiology of Sclerotinia stem rot of canola.

The relationship between PPI as determined by the petal plate test and petal infestation as assessed by qPCR analysis was not always linear. An analysis of correlation indicated a significant amount of shared variation between the two methods only at early bloom in 2011 and at full bloom at 2012. Similarly, Almquist and Wallenhammar (2015) reported no correlation between the results of the petal plate test and a qPCR-based *S. sclerotiorum* detection method. There are several possible explanations for these differences. For example, the presence of a few highly infected canola petals in a sample would be reflected in a higher concentration of *S. sclerotiorum* DNA in the qPCR analysis, but would not be reflected in the petal plate test results (Almquist and Wallenhammar 2015). Furthermore, while both the number of ascospores and the amount of mycelium present in a sample can be measured by qPCR analysis, the petal plate test can only indicate whether or not a petal is infected. Finally, when levels of *S. sclerotiorum* infestation are low on infected petals, other fungal species (e.g. *Rhizopus* spp., *Mucor* spp., *Trichoderma* spp.) that are present on the petal tissue may outgrow colonies of *S. sclerotiorium*, thus masking the presence of *S. sclerotiorum*. Given the increased sensitivity of the qPCR-based method, detection and direct measurement of *S. sclerotiorum* by qPCR analysis are more likely to be influenced by the environment in which those petals were produced.

The strength of the statistical relationship between qPCR-based measurement of petal infestation and final stem rot incidence in the field is critical to assessing the suitability of the former for predicting the risk of disease. In Exp. 1, the strength of the relationship between the qPCR results

and disease incidence across the Prairies was variable across the three years of the study. Moreover, when the data were analyzed separately based on the amount of annual or summer precipitation in a particular region, the strength of the relationship was not increased. It is important to note that, with the exception of the fields in the Edmonton region, there was no information regarding fungicide application in the fields included in Exp. 1. This could have had a large impact on any potential correlation. For instance, a canola field in which there was heavy petal infestation may have been treated with fungicide, preventing or greatly reducing stem rot development. As such, the results of Exp. 1 must be treated with caution. In Exp. 2, all sampling was carried out in check-strips that were not treated with fungicide. Therefore, the results of this experiment may provide a better indication of the true relationship between disease incidence and petal infestation as measured by qPCR analysis. Indeed, in Exp. 2 the relationship between these two parameters was stronger, with stem rot incidence generally found to be greater in fields where petal infestation (*S. sclerotiorum* DNA per canola petal) also was higher. Despite this stronger relationship, however, the correlations were not always significant. This highlights the possible influence of other factors in stem rot development.

There was a wider range of seeding dates in 2013 than in 2011 or 2012, and this was found to influence the statistical relationship in Exp. 2. When only the early seeded fields were included in the analysis, the relationship was significant at full bloom, which was consistent with the results in 2012. These findings indicate that the relationship between disease incidence and the amount of *S. sclerotiorum* DNA per canola petal is strongest at full bloom and/or in years when disease pressure is high. The variation across the sampling years also indicates that measures of environmental conditions may need to be included in the model using multiple regression analysis, in order to fully account for year to year variation in weather. More broadly, comparison of the results obtained in Exp. 1 and Exp. 2 suggests that qPCR-based assessments of stem rot risk are more reliable on a smaller regional scale, and that differences in environment between locations should be considered when setting risk assessment thresholds.

The qPCR assay described in this study may serve as the basis for a risk assessment system, as well as representing a useful tool for the study of the epidemiology of Sclerotinia stem rot of canola. It can quantify the level of petal infestation, a key stage in the Sclerotinia stem rot disease cycle, thereby providing a measure of disease risk when timely fungicide application decisions need to be made. It is important to emphasize, however, that a forecasting system based on qPCR quantification of petal infestation should be linked to environmental conditions, as well as cropping history, seeding date and crop canopy conditions, which may influence stem rot development and the need to spray a fungicide.

6. Literature cited

Provide complete reference information for all literature cited throughout the report.

- Aboukhaddour, R., Kim, Y. M., and Strelkov, S. E. 2012. Mol. Plant Pathol. 13:318-326.
- Almquist, C., and Wallenhammar, A.C. 2015. Plant Pathol. 64:109-118.
- Bom, M., and Boland, G.J. 2000. Can. J. Plant Sci. 80:889-898.
- Harper, F. R., and Berkenkamp, B. 1975. Can. J. Plant Sci. 55:657-658.
- Jurke, C.J., and Fernando, W.G.D. 2008. Arch. Phytopathol. Plant Protect. 41:142-155.
- Kutcher, H.R., and Wolf, T.M. 2006. Crop Protect. 25: 640-646.

- Liang, Y., Yajima, W., Davis, M.R., Kav, N.N.V. and Strelkov, S.E. 2013. Can. J. Plant Pathol. 35:46-55.
- McLaren, D.L., Conner, R.L., Platford, R.G., Lamb, J.L., Lamey, H.A., and Kutcher, H.R. 2004. Can. J. Plant Pathol. 26:489-497
- Nakayashiki, H., Hanada, S., Nguyen, B. Q., Kadotani, N., Tosa, Y., and Mayama, S. 2005. Fungal Genet. Biol. 42:275-283.
- Ririe, K. M., Rasmussen, R. P., and Wittwer, C. T. 1997. Analytical Biochem. 245:154-160.
- Rogers, S.L., Atkins, S.D. and West, J.S. 2009. Plant Pathol. 58:324-331.
- Smith, C. J., and Osborn, A. M. 2008. FEMS Microbiol. Ecol. 67:6-20.
- Turkington, T.K., Morrall, R.A.A., and Baker, R.J. 1988. Can. J. Plant Pathol. 10:159-165.
- Turkington, T.K., and Morrall, R.A.A. 1993. Phytopathol. 83: 682-689.
- Twengstrom, E., Sigvald, R., Svensson, C., and Yuen J. 1998. Crop Prot. 17:405-411.
- Willetts, H.J. and Wong, J.A.-L. 1980. Bot. Rev. 46:101-165.
- Yin, Y., Ding, L., Lui, X., Yang,J., and Ma, Z. 2009. J. Phytopathol. 157:465-469.

7. Benefits to the industry (max 1 page; respond to sections *a)* and *b)* separately)

a) Describe the impact of the project results on Alberta's agriculture and food industry (results achieved and potential short-term, medium-term and long-term outcomes).

The qPCR-based assay for detection and measurement of *S. sclerotiorum* on canola petals is the first such method to be developed and validated for canola cropping systems in western Canada. The methodology is highly robust and could serve as the basis for a stem rot forecasting system. Indeed, there has been interest from several commercial testing laboratories in assessing the method, and it has been recently made publicly available. As discussed in this report, extensive analysis also was conducted to determine the relationship between petal infestation level, as measured by qPCR testing, and final stem rot incidence. While significant correlations were often detected, the relationship between inoculum level and disease incidence was found to be complex, and is influenced by parameters such as weather, crop density and seeding date. Establishment of defined action thresholds likely will require additional evaluation and multiple regression analysis. Nonetheless, the qPCR-based evaluation of canola infestation levels may provide a general indication of stem rot risk for risk-averse producers, which could be further refined with additional epidemiological data. The detection technology developed as part of this project represents a means to acquire these additional data, and also provides a tool for more in-depth study of the epidemiology of Sclerotinia stem rot. Routine use of risk assessment tools as part of an IPM approach will result in more informed fungicide-use decisions, leading to production systems with reduced input costs that address concerns regarding a safe, sustainable, and environmentally friendly food supply.

b) Quantify the potential economic impact of the project results (*e.g.,* cost-benefit analysis, potential size of market, improvement in efficiency, etc.).

Fungicide application is viewed as one of the main strategies for stem rot management, but application during flowering is a significant investment for canola producers. Also, because of the variability of stem rot from year to year, region to region and field to field, routine

application of fungicide may not be economical. It is estimated that up to 20% of canola acres are sprayed for stem rot each year at an estimated cost of $55 million. Spraying is typically required when disease incidence approaches 20%; however, surveys suggest that only 10-20% of fields actually have at least 20% infected plants. Routine use of risk assessment tools like qPCR-based detection and quantification of *S. sclerotiorum* will help rationalize fungicide application. Even just a 10% reduction in acres sprayed will reduce the amount of fungicide applied in the prairie ecosystem by as much as 44 million grams of active ingredient annually. The potential return per dollar invested as part of this project could be as much as $80, based on annual cost savings related to lower chemical and application costs; this excludes environmental benefits.

8. Contribution to training of highly qualified personnel (max ½ page)

Specify the number of highly qualified personnel (*e.g.*, students, post-doctoral fellows, technicians, research associates, etc.) who were involved in the project.

This project served as the basis for one graduate student program. Ms. Barb Ziesman begun as a M.Sc. student, but she transferred into the Ph.D. program in order to take full advantage of the training opportunities provided by the project. During the course of her involvement, she obtained expertise in experimental design, statistical analysis, molecular biology, and plant pathology and epidemiology. In addition, the project also served as the basis for the training of two students enrolled in the Women in Science, Engineering, Scholarship and Technology (WISEST) Program, as well as three summer students (University of Alberta undergraduates) who worked in the Plant Pathology Lab for two summers.

9. Knowledge transfer/technology transfer/commercialisation (max 1 page)

Describe how the project results were communicated to the scientific community, to industry stakeholders, and to the general public. Organise according to the following categories as applicable:

a) Scientific publications (*e.g.*, scientific journals); attach copies of any publications as an appendix to this final report
   - Ziesman, B.R., Turkington, T.K., Basu, U., and Strelkov, S.E. 2016. A quantitative PCR system for measuring *Sclerotinia sclerotiorum* in canola (*Brassica napus*). Plant Dis. (Accepted 13 December 2015). DOI: 10.1094/PDIS-05-15-0605-RE
   - Ziesman, B.R., Turkington, T.K., Basu, U., deMilliano, E.J., and Strelkov, S.E. 2014. Evaluation of a quantitative (q)PCR assay as the basis for a stem rot of canola (*Sclerotinia sclerotiorum*) risk assessment tool. Phytopathology 104(S3): S3.138 (Abstr.).
   - Ziesman, B.R., Turkington, T.K., Basu, U., and Strelkov, S.E. 2014. Initial validation of a quantitative PCR-based system for detection of *Sclerotinia sclerotiorum* on canola. Can. J. Plant Pathol. 36: 132. (Abstr.).
   - Ziesman B.R., Turkington, T.K., Basu, U., and Strelkov, S.E. 2013. Development of a quantitative PCR detection system for *Sclerotinia sclerotiorum* on canola petals. Can. J. Plant Pathol. 35: 520-521. (Abstr.).
   - Ziesman, B.R., Turkington, T.K., Basu, U., and Strelkov, S.E. 2013. Development of a quantitative PCR detection technique for *Sclerotinia sclerotiorum* on canola. Can. J. Plant Pathol. 35: 131-132 (Abstr.).

b) Industry-oriented publications (*e.g.*, agribusiness trade press, popular press, etc.) attach copies of any publications as an appendix to this final report

c) Scientific presentations (*e.g.*, posters, talks, seminars, workshops, etc.)

- Ziesman B.R., Turkington, T.K., Basu, U., and Strelkov, S.E. 2013. Initial validation of a quantitative PCR-based system for detection of *Sclerotinia sclerotiorum* on canola. Annual Meeting of the Plant Pathology Society of Alberta, November 5, 2013, Brooks, Alberta (Oral Presentation).

d) Industry-oriented presentations (*e.g.*, posters, talks, seminars, workshops, etc.)

- Canola Council of Canada (CCC) and Alberta Canola Producers Commission (ACPC), PeacePalooza field day at AAFC Beaverlodge organized by CCC and ACPC, June 25, 2015. Provided an outline of key strategies and recommendations to improve the management of sclerotinia stem rot of canola and other canola diseases.
- Canola Council of Canada and Alberta Canola Producers Commission, CanolaPalooza field day at AAFC Lacombe organized by CCC and ACPC, June 23, 2015. Provided an outline of key strategies and recommendations to improve the management of sclerotinia stem rot of canola and other canola diseases.
- Turkington, T.K., Buchwaldt, L., Orchard, D., Strelkov, S., Ziesman, B., and Lange, R. 2014. Assessing risk and spray timing for sclerotinia stem rot. Daily interactive workshops, held over three days. 2014 CanoLAB training event, Bolting and Beyond Session, Olds, Alberta, February 19-21, 2014.

e) Media activities (*e.g.*, radio, television, internet, etc.)

- Berg, M. 2014. Quickly predicting Sclerotinia is closer to reality. Top Crop Manager, Nov. 2014.
- King, C. 2014. Improving sclerotinia prediction: New tools are being developed to help canola growers with spray decisions. Top Crop Manager, March 2014.

f) Any commercialisation activities or patents

N.B.: Any publications and/or presentations should acknowledge the contribution of each of the funders of the project.

Section D: Project resources

1. Statement of revenues and expenditures:

a) In a separate document certified by the organisation's accountant or other senior executive officer, provide a detailed listing of all cash revenues to the project and expenditures of project cash funds. Revenues should be identified by funder, if

applicable. Expenditures should be classified into the following categories: personnel; travel; capital assets; supplies; communication, dissemination and linkage; and overhead (if applicable).

A statement of award and expenditures will be prepared and forwarded by the Research Services Office of the University of Alberta.

b) **Provide a justification of project expenditures and discuss any major variance (*i.e.*, ± 10%) from the budget approved by the funder(s).**

2. Resources:
Provide a list of all external cash and in-kind resources which were contributed to the project.

Total resources contributed to the project		
Source	**Amount**	**Percentage of total project cost**
Funders	$127,000	65%
Other government sources: Cash		%
Other government sources: In-kind	$68,000	35%
Industry: Cash		%
Industry: In-kind		%
Total Project Cost	**$195,000**	**100%**

External resources (additional rows may be added if necessary)		
Government sources		
Name (only approved abbreviations please)	Amount cash	Amount in-kind
Agriculture and Agri-Food Canada		$34,000.00
University of Alberta		$34,000.00
Industry sources		
Name (only approved abbreviations please)	Amount cash	Amount in-kind

Section E: The next steps (max 2 pages)

Describe what further work if any needs to be done.

a) Is new research required to deal with issues and opportunities that the project raised or discovered but were not dealt with within the current project?
b) Is there related work that needs to be undertaken to continue advancement of the project technology or practice?
c) Did the project identify any new technology or practice that needs to be developed?
d) What suggestions do you have that increase commercial use of results by farmers and/or companies. These may be:
   1. commercial uptake.
   2. further research toward commercial use.
   3. extension and information disbursement.

a) Additional study of the influence of environmental and other factors (such as seeding date) on the development of stem rot of canola would be valuable. The effect of these factors could be assessed through multiple regression analysis and incorporated into predictive models. Closer monitoring of petal infestation levels over the flowering period could prove useful in better understanding the epidemiology of Sclerotinia stem rot of canola, and thereby enable optimization of the timing of stem rot management practices.

b) An improved understanding of the influence of parameters other than inoculum level on stem rot development would enable refinement of qPCR-based methodologies as predictive tools. Eventually, this may allow identification of threshold levels of infestation that, when combined with specific environmental parameters, would notify growers that economically significant damage to the crop will occur unless control measures are taken.

c) The project developed a robust qPCR-based detection system for *S. sclerotiorum* in canola petals. The technology is robust and sufficient for monitoring levels of infestation, but additional epidemiological data is needed to refine understanding of the influence of factors other than inoculum load on stem rot development.

d) The methodology developed and knowledge obtained through this project is being made publicly available through the publication of peer-reviewed articles, presentation at scientific and industry meetings, and via open lines of communication with stakeholders. This will facilitate uptake of the technology by commercial and provincial diagnostic labs, as well as increased awareness among farmers and industry personnel that these new tools are available.

UNIVERSITY OF ALBERTA

Research Services Office
222 Campus Tower
8625 - 112 Street, Edmonton, AB T6G 2E1 Canada

Statement of Award & Expenditure

For the Period Ending - December 31, 2015

Name of Grantee - Project Role	Department	Reference Award Number	
Strelkov,Stephen - Principal Investigator	100300 - ALES AFNS General	2013F075R	
University Project Number	**Project/Grant Description**	**Start Date :**	**End Date :**
RES0017396	AgFC(ACIDF/ACPC/WGRF2013F075R)	April 1, 2013	December 31, 2015

	Reporting Period April 1, 2013 to December 31, 2015	
OPENING BALANCE	0.00	
AWARD		
Direct Costs	127,000.00	cr
Total Funds Available	127,000.00	cr
EXPENDITURE		
Salaries & Benefits		
Undergrad Stu Salary & Benefit		
Grad Student Salary & Benefits		
Graduate Student Salaries	41,843.28	dr
Graduate Student Benefits		
Postdoctoral Salary & Benefits		
Postdoctoral Fellows Salaries		
Postdoctoral Fellows Benefits		
Other Sal & Adj (all benefits)		
Other Salaries	48,389.02	dr
Other Benefits	4,581.88	dr
Professional & Technical Svcs		
Equipment	109.31	dr
Materials Supplies & Other Exp	30,012.06	dr
Travel	2,064.45	dr
Transfers Out		
Total Funds Expended	127,000.00	dr
Indirect Cost Expenses	0.00	
Total EXPENDITURE	127,000.00	dr
PROJECT/ GRANT BALANCE AT: December 31, 2015	0.00	

SIGNATURES

I hereby certify that the above statement is correct and that the expenditures conform to the general conditions imposed by the sponsoring agency, and were for the purpose for which the grant was made.

Project Manager – Role: Strelkov,Stephen-Principal Investigator

January 17th, 2016

Date

I certify that the expenditures summarized above were incurred wholly by and paid on behalf of the grantee, and that the vouchers are available for monitoring purposes.

Nafissa Aktary

Business Officer, Research Services Office

January 18, 2016

Date

NAFISSA AKTARY, MEd, BSc
Senior Financial Analyst
RESEARCH SERVICES OFFICE
The University of Alberta

Section F: Research Team Signatures and Employers' Approval

The team leader and an authorised representative from his/her organisation of employment MUST sign this form.

Research team members and an authorised representative from their organisation(s) of employment MUST also sign this form.

By signing as representatives of the research team leader's employing organisation and/or the research team member's(s') employing organisation(s), the undersigned hereby acknowledge submission of the information contained in this final report to the funder(s).

Team Leader's Organisation

Team Leader	
Name: **Stephen Strelkov**	**Title/Organisation:** **University of Alberta**
Signature:	**Date:** **January 30, 2015**
Team Leader's Employer's Approval	
Name: Ruurd T. Zijlstra Chair, AFNS	**Title/Organisation:**
Signature:	**Date:** FEB - 2 2016

Research Team Members (add more lines as needed)

1. Team Member	
Name: **Kelly Turkington**	**Title/Organisation:** **Agriculture and Agri-Food Canada**
Signature:	**Date:**
Team Member's Employer's Approval	
Name:	**Title/Organisation:**
Signature:	**Date:**

Section F: Research Team Signatures and Employers' Approval

The team leader and an authorised representative from his/her organisation of employment MUST sign this form.

Research team members and an authorised representative from their organisation(s) of employment MUST also sign this form.

By signing as representatives of the research team leader's employing organisation and/or the research team member's(s') employing organisation(s), the undersigned hereby acknowledge submission of the information contained in this final report to the funder(s).

Team Leader's Organisation

Team Leader	
Name: **Stephen Strelkov**	**Title/Organisation:** **University of Alberta**
Signature:	**Date:** **January 30, 2015**
Team Leader's Employer's Approval	
Name:	**Title/Organisation:**
Signature:	**Date:**

Research Team Members (add more lines as needed)

1. Team Member	
Name: **Kelly Turkington**	**Title/Organisation:** **Agriculture and Agri-Food Canada**
Signature:	**Date:** January 30, 2016
Team Member's Employer's Approval	
Name: François Eudes, Ph.D. Director - RDT Alberta	**Title/Organisation:** François Eudes, Ph.D. Director - RDT Alberta
Signature:	**Date:** FEB 05 2016

Development of a Rapid Quantitative Detection Method for Sclerotinia Stem Rot Inoculum to Aid Disease Risk Assessments and Fungicide Spray Decisions

Project Number: 2013F075R

January 30th, 2016

APPENDIX: (1) Paper in press (*Plant Disease*) and (2) Draft manuscript being prepared for submission to a scientific journal

Research Team: Stephen Strelkov and Kelly Turkington

Graduate Student: Barbara Ziesman

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

1 # A quantitative PCR system for measuring *Sclerotinia sclerotiorum* in canola
2 # (*Brassica napus*)

3

4

5 **B. R. Ziesman,** Department of Agricultural, Food and Nutritional Science, University of
6 Alberta, Edmonton, AB, Canada T6G 2P5; **T. K. Turkington**, Lacombe Research Centre,
7 Agriculture and Agri-Food Canada, Lacombe, AB, Canada T4L 1W1; **U. Basu** and **S. E.**
8 **Strelkov**, Department of Agricultural, Food and Nutritional Science, University of Alberta,
9 Edmonton, AB, Canada T6G 2P5

10

11

12

13 Corresponding author: S. E. Strelkov, E-mail: stephen.strelkov@ualberta.ca

14

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

15 **Abstract**

16 Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum*, is an economically important disease of
17 canola (*Brassica napus*) commonly managed by routine application of fungicides. Petal
18 infestation has been demonstrated to be an important stage of the disease cycle in canola and has
19 been the focus of previously developed Sclerotinia stem rot risk assessment methods.
20 Quantitative (q)PCR analysis can provide a more rapid and accurate assessment of petal
21 infestation levels. Primers and a hydrolysis probe were designed to amplify a 70-bp region of a
22 *S. sclerotiorum*-specific gene SS1G_00263. A hydrolysis probe-based qPCR assay was
23 developed that had a detection limit of 8.0×10^{-4} ng of *S. sclerotiorum* DNA and only amplified
24 *S. sclerotiorum* DNA. Evaluation of petals collected at five sampling points in each of 10
25 commercial canola fields on each of two sampling dates (corresponding to 20-30% bloom and
26 40-50% bloom) revealed infestation levels ranging from 0 to 3.3×10^{-1} ng *S. sclerotiorum* DNA
27 per petal. This qPCR assay can be used to reliably quantify petal infestation and with further
28 research has the potential to serve as the basis for a Sclerotinia stem rot risk assessment tool or as
29 a means to study Sclerotinia stem rot epidemiology.

30

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

31 Sclerotinia stem rot, caused by the ascomycete fungus *Sclerotinia sclerotiorum* (Lib.) de
32 Bary, is an economically important disease of canola (*Brassica napus* L.) (Purdy 1979; Willets
33 and Wong 1980). Yield losses can be as high as 50%, with a yield reduction of 0.5% estimated
34 for every 1% increase in disease incidence (del Río et al. 2007). The fungus overwinters as
35 sclerotia, which can germinate carpogenically in the spring to produce apothecia (Abawi and
36 Grogan 1979; Bardin and Hwang 2001; Willets and Wong 1980). Mature apothecia will forcibly
37 release wind-borne ascospores (Abawi and Grogan 1979). The ascospores come into contact
38 with canola petals onto which they adhere, germinate and penetrate the petal tissue to initiate
39 infection (Jamaux et al. 1995; Purdy et al. 1979). Petal infestation has been shown to be an
40 important stage in the Sclerotinia stem rot disease cycle (Jamaux et al. 1995; Morrall and Dueck
41 1982). After senescence, the infected petals fall into the crop canopy and the fungus grows from
42 these petals, infecting the leaf and stem tissues. The first visible symptoms of disease consist of
43 browning on and around the cast petals. As the fungal infection spreads to the stems, the
44 characteristic symptoms of Sclerotinia stem rot appear, including bleached whitish-grey lesions
45 where the infected host stem tissues are very brittle, shredding and shattering when dry (Bolton
46 et al. 2006; Jamaux et al. 1995; Young and Werner 2012). Once the infection is well-established,
47 hard black survivai structures known as sclerotia are produced by the fungus inside the stem. The
48 sclerotia can be dislodged during harvest and serve as inoculum in subsequent years.

49 The development of Sclerotinia stem rot of canola is influenced by environmental
50 conditions, in particular temperature and moisture. For carpogenic germination of the sclerotia
51 to occur, continuous soil moisture, near saturation, for about 10 days and moderate temperatures
52 are required (Abawi and Grogan 1979; Schwartz and Steadman 1978; Wu and Subbarao 2008).
53 Ascospore release has been found to be highest at 20-25°C, with the ascospores being able to

54 survive for up to 2 weeks in the environment (Abawi and Grogan 1979; Clarkson et al. 2003).
55 Infection of stem and leaf tissue by *S. sclerotiorum* is favored in the presence of free water and
56 moderate temperatures (15-25°C), while at extreme temperatures (<4°C and >30°C) fungal
57 growth is restricted (Bolton et al. 2006; Willets and Wong 1980).

58 The wide host range of *S. sclerotiorum*, along with the influence of temperature and
59 moisture conditions on apothecial development and subsequent plant infection, have limited the
60 effectiveness of cultural stem rot management practices. Crop rotations that include four years
61 away from a susceptible host were found to be ineffective for Sclerotinia stem rot management,
62 as a consequence of the survival of viable sclerotia in the soil and the introduction of ascospores
63 from external sources (Morrall and Dueck 1982). Sclerotinia stem rot tolerant canola cultivars
64 have become available recently in Canada, but when the risk of disease is high even these
65 cultivars can suffer damage and require fungicide application (Canola Council of Canada 2014;
66 Pratt 2012). As a consequence, the application of fungicides is the primary management tool for
67 Sclerotinia stem rot of canola. To be effective, fungicides need to be applied during the key
68 stage for infection, i.e., at flowering and before the appearance of symptoms in the crop.
69 Therefore, the application of fungicide often must be made without any objective indication of
70 disease risk (del Río et al. 2007; Koch et al. 2007; Turkington et al. 2011).

71 In an effort to predict the likelihood of Sclerotinia stem rot development in a given canola
72 crop, a petal test was developed whereby field-collected petals were plated onto potato dextrose
73 agar (PDA) and incubated for 3 to 5 days (Morrall and Thomson 1991). The cultured petals
74 were then examined for growth and the resulting colonies were identified. The proportion of
75 petals that yielded colonies of *S. sclerotiorum* was taken as the percent petal infestation. Risk
76 assessments were provided based on the statistical relationship between petal infestation and

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

77 final Sclerotinia stem rot development in the field, and were calculated based on percent petal
78 infestation (Gugel and Morrall 1986; Turkington et al. 1991). The percent petal infestation was
79 used to identify the Sclerotinia stem rot risk level as low, moderate, or high based on the
80 relationship between this parameter and disease incidence (Turkington et al. 1991; Turkington
81 and Morrall 1993). The 3 to 5 day incubation period that is required for accurate estimates of
82 petal infestation represents a potential disadvantage of this system, since timely spray decisions
83 need to be made during a fairly narrow window of crop development (McLaren et al. 2004;
84 Turkington et al. 1991). A molecular approach, such as quantitative polymerase chain reaction
85 (qPCR)-based analysis, may represent an alternative method to determine petal infestation levels
86 without the time delay associated with incubation of the petals. Moreover, PCR-based methods
87 can lower the risk of human error associated with the misidentification of the fungal cultures
88 growing out of the infested petals.

89 In recent years, many qPCR-based methodologies have been developed to detect and
90 quantify plant pathogens including *S. sclerotiorum* (Almquist and Wallenhammar 2015; Freeman
91 et al. 2002; Parker et al. 2014; Rogers et al. 2008; Yin et al. 2009). Freeman et al. (2002)
92 established a touchdown PCR assay to quantify *S. sclerotiorum* DNA in airborne ascospores of
93 the pathogen and inoculated canola petals. The primers SSFWD and SSREV amplified a 278-bp
94 fragment of ribosomal DNA and were found to be specific to *S. sclerotiorum*, even in the
95 presence of a 40-fold excess of *Botrytis cinerea* DNA. However, this primer set was unable to
96 distinguish between the DNA of *S. sclerotiorum* and that of close relatives such as *S. minor* and
97 *S. trifoliorum*. Nonetheless, Almquist and Wallenhammar (2015) were able to use the primers of
98 Freeman et al. (2002) to develop a hydrolysis probe-based qPCR assay for detection of *S.*
99 *sclerotiorum* DNA in air samples and infected leaves and petals of oilseed rape (*B. napus*) in

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

100 central Sweden. Rogers et al. (2008) described an intercalating dye-based qPCR assay to
101 quantify airborne ascospores of *S. sclerotiorum* around oilseed rape crops near Rothamsted,
102 United Kingdom, using primers designed to amplify a region of the mitochondrial small subunit
103 rRNA and open reading frame 1 (ORF1) of the fungus. This assay could detect *S. sclerotiorum*
104 DNA with a high level of sensitivity and specificity, even in the presence of DNA from the
105 closely related fungus *B. cinerea*, except at 5×10^{-5} ng of DNA where the sensitivity of the
106 quantification of *S. sclerotiorum* DNA was reduced (Rogers et al. 2008). Parker et al. (2014)
107 developed another intercalating dye-based qPCR assay for the quantification of airborne
108 ascospores of *S. sclerotiorum*, as part of a model for forecasting Sclerotinia rot of carrot (*Daucus*
109 *carota* ssp. *sativus*) The assay targeted the group I intron of the mitochondrial small subunit
110 (MtSSu) rRNA, and could detect 93% of the *S. sclerotiorum* isolates tested, although the
111 quantification cycle (C_q) values varied for different isolates. While this assay may be useful for
112 the detection of airborne ascospores of *S. sclerotiorum* on a regional scale, 7% of the isolates
113 tested could not be detected, indicating the potential for false negatives. The variation in C_q
114 values observed for different isolates also is a cause for concern, since it is indicative of
115 differences in the ability to quantify isolates. This likely would result in inconsistencies in the
116 quantification of the same amount of inoculum, depending upon which isolate is tested,
117 particularly if the standard curve is based on another isolate. Moreover, on carrots there is no
118 initial petal infestation stage, with infection first occurring on senesced leaves in a closed
119 canopy, and the additional risk of postharvest infection (Kora et al. 2005).
120 Most relevant to the current study, Yin et al. (2009) developed an intercalating dye-based
121 qPCR assay for the quantification of *S. sclerotiorum* DNA on canola petals in China. The
122 primers (SsF and SsR) targeted a region amplified by the microsatellite primer M13 (Ma et al.

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

123 2003). The authors reported that this assay was very specific to *S. sclerotiorum* and could
124 quantify as little as 5.0×10^{-4} ng DNA of the fungus. The specificity of the primers SsF/SsR,
125 however, was not validated by testing with DNA of pure cultures of other *Sclerotinia* species
126 such as *S. minor* or *S. trifoliorum*. Analysis of infected canola petals resulted in quantifications
127 ranging from 2.5×10^{-2} ng to 1.1×10^{-1} ng *S. sclerotiorum* DNA per mg canola petal tissue. This
128 assay may have value as the basis for a risk assessment tool in canola production systems in
129 Canada. Nevertheless, because of evidence of a high level of genetic variation in the *S.*
130 *sclerotiorum* population (Carpenter et al. 1999; Sirjusingh and Kohn 2001), specificity testing
131 would be needed to ensure that Canadian isolates of the fungus can be detected with no false
132 positives. In addition, it would be important to ensure that there is no difference in the detection
133 and quantification of different *S. sclerotiorum* isolates.

134 The objective of the current study was to develop a *S. sclerotiorum*-specific qPCR assay
135 for the quantification of Canadian isolates of *S. sclerotiorum* on canola petals using a hydrolysis
136 probe. Development of this assay was focused on petals rather than airborne inoculum because
137 of the importance of petal infestation in the disease cycle of Sclerotinia stem rot of canola. In
138 addition, estimates of petal infestation may provide a better field-specific indication of disease
139 risk, whilst detection of airborne inoculum would be best for regional risk assessments. The
140 qPCR assay described in this study also was compared with the qPCR assay developed by Yin et
141 al. (2009), in order to evaluate the potential utility of each method for quantifying *S.*
142 *sclerotiorum* DNA on canola petals.

143 **Materials and Methods**

144 **DNA isolation from fungal mycelium.** Pure fungal cultures of *S. sclerotiorum* and the
145 other fungal species used for specificity testing (Table 1) were grown on Difco potato dextrose

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

146 broth (Dickinson and Company, Sparks, MD, USA) that was amended with 25 ppm ampicillin
147 (Life Technologies, Carlsbad, CA, USA) and streptomycin (Sigma Chemical Company, St,
148 Louis, MO, USA) in 200 ml Erlenmeyer flasks. The cultures were grown at room temperature
149 (approximately 20-24°C) under natural light provided by a north-facing window with gentle
150 agitation for approximately 7 days, until a large mass of mycelium had formed. The supernatant
151 was decanted and the mycelium washed with sterile water, frozen at -80°C, and lyophilized in a
152 freeze-drier. Approximately 20 mg of lyophilized tissue was homogenized to a powder in a 1.5
153 ml microcentrifuge tube with a hand-held plastic pestle, and the DNA was isolated with a Wizard
154 Genomic DNA purification kit (Promega, Madison, WI, USA) as per the manufacturer's
155 instructions, but with the addition of a phenol/chloroform/isoamyl alcohol (25:24:1, v:v:v)
156 purification step. Briefly, following the protein precipitation step, three
157 phenol/chloroform/isoamyl alcohol extractions were performed. In each extraction, 600 μl of
158 phenol/chloroform/isoamyl alcohol (25:24:1, v:v:v) was added and the solution mixed and
159 centrifuged. The DNA was quantified with a NanoDrop 2000c spectrophotometer (Thermo
160 Scientific, Waltham, MA, USA), and the 260 nm/280 nm and 260 nm/230 nm absorbance ratios
161 were calculated as an indication of DNA quality prior to qPCR analysis.

162 **DNA isolation from field-collected canola petals.** To validate the qPCR assay, canola
163 petals were collected from 10 commercial fields in the Edmonton, AB, region in 2012. These 10
164 fields were farmed by three different farmers and were known to have a history of Sclerotinia
165 stem rot. The fields were sampled once at 20-30% bloom and again at 40-50% bloom, as
166 determined by visual assessment (Harper and Berkenkamp 1975). These levels of flowering
167 correspond to growth stages 62-63 and 64-65, respectively, on the BBCH scale (Webber and
168 Bleiholder 1990; Lancashire et al. 1991). Sampling dates varied slightly in each field depending

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

169 on crop stage, but in general, sampling at 20-30% bloom was carried out in the first week of
170 July, followed by sampling at 40-50% bloom 3-4 days later. The incidence of Sclerotinia stem
171 rot also was assessed at the end of the growing season for future reference. Petals were collected
172 at each of five sampling sites within each field. The sampling sites were located 100 m from the
173 edge of each field and were situated 50 m apart along a fungicide-free check strip. At each
174 sampling site, the top 10-20 cm of the inflorescences from each of 20 plants were collected at
175 random, placed in a plastic bag, and stored in a cooler on ice for transport back to the laboratory.
176 A minimum of 80 flowers were selected randomly from the sampled inflorescences and stored in
177 an ultra-low temperature freezer (-80°C) until further processing.

178 DNA was isolated according to the protocol of Liang et al. (2013) from a randomly
179 chosen subsample of 20 petals from each sampling site. Petal samples were homogenized with a
180 TissueLyser II (Qiagen, Toronto, ON, Canada) and a single 5-mm stainless steel bead in a 2-ml
181 locking tube. The TissueLyser II adapters and petal samples were frozen at -80°C prior to
182 homogenization to prevent damage to the DNA. Prior to elution, the DNA pellets were dried in
183 a Vacufuge Plus (Eppendorf, Mississauga, ON, Canada) for 7 min at 45°C. The extracted DNA
184 was eluted with 30 µl of nuclease free water (Life Technologies, Carlsbad, CA, USA) and
185 quantified with a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA)
186 as above.

187 **Development of *S. sclerotiorum*-specific primers.** Forward and reverse primers and a
188 hydrolysis probe were designed using Primer 3 Express (Applied Biosystems, Carlsbad, CA,
189 USA) based on the 468-bp sequence of a single-copy gene (SS1G_00263) encoding the
190 hypothetical secreted protein ssv263 (Liang et al. 2013) from *S. sclerotiorum* (GenBank
191 accession no. XM_001598127). This gene is highly specific to *S. sclerotiorum* and orthologous

192 to a protein-encoding gene BC1G_00896 (GenBank accession no. XM_001560818) from *B.*
193 *cinerea* (Liang et al. 2013; Shah et al. 2009). The forward (SSBZF) and reverse (SSBZR)
194 primers were designed to amplify a 70-bp product in a region of SS1G_00263 that exhibits the
195 greatest difference with the *B. cinerea* orthologue. The hydrolysis probe (SSBZP) was labeled
196 with a non-fluorescent quencher-mini groove binder (NFQ-MGB) on the 3' end, and with the
197 reporter dye FAM (6-carboxyfluorescein) on the 5' end (Table 1). The amplified region shares
198 71% similarity with the orthologous gene in *B. cinerea*, but includes 20-bp pair mismatches, 17
199 of which are covered by the primer and probe sequences. The probe and primer sequences were
200 used to query the GenBank databases using the Basic Local Alignment Search Tool (BLAST) in
201 order to identify any similar sequences from other organisms that could lead to false positives.
202 **Development of the exogenous positive internal control.** To identify false negatives
203 resulting from failed DNA extraction or inhibition of the PCR, an exogenous internal control was
204 included in the analysis of all samples. The *ToxB* gene from *Pyrenophora tritici-repentis*, causal
205 agent of tan spot of wheat (Lamari and Strelkov 2010), was selected as the exogenous internal
206 control since it is unlikely to be found in canola petals. The pSilent1 plasmid (Nakayashiki et al.
207 2005) containing a 432-bp fragment of *ToxB* from *P. tritici-repentis* (Aboukhaddour et al. 2012)
208 was used as the template for the design of the primers and hydrolysis probe. The *ToxB*-specific
209 primers (ToxBF and ToxBR) and a probe (ToxBP) were designed using Primer 3 Express
210 (Applied Biosystems). The *ToxB*-specific hydrolysis probe was labelled with NFQ-MGB on the
211 3' end and with the reporter dye VIC on the 5' end.
212 For use as an internal control, every canola petal sample was spiked with 2×10^6
213 plasmids containing the *ToxB* gene prior to DNA isolation. Each sample was analyzed in two
214 separate singleplex qPCR reactions, one with the *ToxB* primers and probe set and another with

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

215 the *S. sclerotiorum*-specific primers and probe. To identify outliers and potential error, the
216 standard deviation of the C_q values obtained for the internal control in a set of samples was
217 calculated. If the standard deviation was close to or < 1.0 for a set of samples, the variation in
218 the internal control and the risk of false negatives as a result of failed DNA isolation or PCR
219 inhibition were regarded as low. Any samples that were outside of this range were discarded and
220 DNA was isolated again.

221 **qPCR analysis.** The terminology used to describe the qPCR analysis will be as
222 suggested in the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR
223 Experiments) guidelines (Bustin et al. 2009). All qPCR analyses were conducted in a ViiA7
224 Real-Time PCR System (Life Technologies) using the Universal FastStart Master (Rox) mix
225 (Roche, Indianapolis, IN, USA) in a MicroAmp® Fast Optical 96-well reaction plate (Applied
226 Biosystems), which was sealed with MicroAmp optical adhesive film (Applied Biosystems,).
227 Each qPCR was conducted in a total volume of 10 μl, including 0.1 μl of each forward and
228 reverse primer (50 μM SSBZF and 50 μM SSBZR, respectively), 0.03 μl of the hydrolysis probe
229 (100 μM SSBZP), 5 μl of the 2× master mix (Rox), 0.77 μl of molecular grade water (Life
230 Technologies), and 4 μl of the template DNA or negative control. For the quantification of the
231 internal control, the reaction mixture was as above except that 0.025 μl of the 100 μM ToxBP
232 hydrolysis probe and 0.775 μl of molecular grade water (Life Technologies) were added and the
233 primers ToxBF/ToxBR were substituted for SSBZF/SSBZR. The reaction conditions included a
234 hot start at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec as per
235 the manufacturer's instructions for the master mix. Each of the primer/probe sets was analyzed
236 separately and reactions were not multiplexed. Each sample was analyzed in triplicate for
237 quantification of the SS1G_00263 target, while samples were analyzed in duplicate for assays of

238 the internal control. If the standard deviation of the mean C_q obtained for a set of triplicates of a
239 given sample was > 0.5, the sample was reanalyzed, and if an outlier was present, it was
240 removed. Similarly, if the standard deviation of the mean C_q obtained for duplicates of the *ToxB*
241 internal control was > 0.5, the sample was re-extracted.

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

242 **Specificity testing.** The specificity of the SS1G_00263 primer and probe set was
243 confirmed by separately testing 100 ng of DNA from 13 fungal species and the canola host as
244 templates in the qPCR analysis (Table 1). In addition, amplification of a total of eight isolates of
245 *B. cinerea* and seven isolates of *S. sclerotiorum* was compared. No amplification of any product,
246 or a C_q value > 2 cycles below that of the smallest standard, were considered to be indicative of
247 no detection. The reproducibility of the standard curve was assessed by calculating the standard
248 deviation at each point, for 4 replications included in separate 96-well plates. Plasmids
249 containing the *ToxB* insert were analyzed with the SS1G_00263-specific primer set to ensure that
250 SSBZF/SSBZR did not amplify the internal control.

251 **Sequencing of PCR products.** Amplicons obtained with the SSBZF/SSBZR primer set
252 were sequenced to confirm the identity of the product. Conventional PCR was used to increase
253 the amount of amplicon obtained from pure mycelial DNA of six *S. sclerotiorum* isolates
254 (UAMH 6321, UAMH 4514, UAMH 9192, SSA-11, SSB-11, SSD-11) for sequencing purposes.
255 The PCR analysis was carried out in a 25 µl reaction volume, which consisted of 15.4 µl
256 molecular grade water (Life Technologies), 2 µl of 10× PCR buffer (no magnesium chloride)
257 (Invitrogen by Life Technologies, Carlsbad, CA, USA), 1 µl 2.5mM each dNTP, 1 µl 50 mM
258 $MgCl_2$, 0.2 µl of each 50 mM SSBZF and SSBZR, 0.2 µl Platinum Taq DNA Polymerase
259 (Intvitrogen), and 5 µl (100 ng) template DNA. The reaction conditions were 94°C for 3 min,
260 followed by 40 cycles of 94°C for 30 sec, 60°C for 20 sec and 72°C for 5 min, and ended with an

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

261 incubation at 72°C for 5 min followed by a constant 4°C. Polymerase chain reaction products
262 were resolved on a 2% agarose gel run at 90V for 50 min. The amplicon band was extracted
263 using a Qiaquick Gel Extraction Kit (Qiagen) and sent to the Molecular Biology Service Unit,
264 University of Alberta, Edmonton, AB, for sequencing. Sequencing was conducted on a 3730
265 Genetic Analyzer (Applied Biosystems) with Sanger cycle sequencing using fluorescently
266 labelled dye terminators and BigDye Terminator v1.1 chemistry.

267 **Sensitivity testing.** To test the limit of detection (LOD) of the assay, standard curves
268 were generated with 1:10 serial dilutions of purified *S. sclerotiorum* DNA. All standard curves
269 tested consisted of five serial dilutions ranging from 8.0 ng to 8.0×10^{-4} ng or from 5.0 ng to 5.0
270 $\times 10^{-4}$ ng. Each standard was included in triplicate in the same 96-well plate as the samples
271 being analyzed. The LOD was regarded as the lowest quantity of DNA that could be detected
272 with confidence in all three of the triplicates. The efficiency and R^2 of the standard curves were
273 determined for each replication. The reproducibility of the standard curve was assessed by
274 calculating the standard deviation at each point, for 4 replications included in separate 96-well
275 plates. A standard deviation of < 0.5 for the average Cq values of the 4 replications was
276 considered to indicate that the assay was reliable.

277 To determine if inclusion of the internal control and the presence of *B. napus* DNA
278 reduced the LOD for *S. sclerotiorum*, fungal DNA was quantified in the presence or absence of
279 the *ToxB* internal control and *B. napus* DNA. A 1:5 dilution series ranging from 4.0×10^1 ng to
280 5.0×10^{-4} ng *S. sclerotiorum* DNA was spiked with 2 µl *B. napus* DNA and 2×10^5 copies of the
281 plasmid containing the internal control. The *B. napus* DNA represented a 1:5 dilution of total
282 genomic DNA extracted from a set of 20 canola petals that were not infected with *S.*

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

283 *sclerotiorum*. The mean C_q and standard deviation of the mean for each point in the dilution
284 series were compared between the spiked and non-spiked samples.

285 **Evaluation of canola petals.** Total genomic DNA isolated from samples consisting of
286 20 canola petals each was diluted by a factor of 1:5 with nuclease free water (Life Technologies)
287 prior to qPCR analysis with the SS1G_00263- and *ToxB*-specific primers and probes. DNA was
288 analyzed from samples collected at five sampling sites from each of 10 commercial canola fields
289 at each of two sampling dates as described above. Results are expressed on a per petal basis by
290 first accounting for the dilution, then by dividing the estimate by 20. The qPCR estimates were
291 averaged over each field on each sampling date and the standard deviation of the mean was
292 calculated for each field. Any sample from which no *S. sclerotiorum* DNA could be amplified,
293 or which was below the lowest standard outside the range of the standard curve, was recorded as
294 0 ng *S. sclerotiorum* DNA per canola petal.

295 **Assessment of the SsF and SsR primer set.** Isolates of *S. sclerotiorum* collected from
296 canola fields in central Alberta were subjected to qPCR analysis with the primers SsF (5'
297 AGTCGAGGGACGGGTACTAA 3') and SsR (5' CTTGTCCTCATTGCCGTTT 3') developed
298 by Yin et al. (2009). The primers were evaluated using Dynamite qPCR Mastermix (Molecular
299 Biology Service Unit, University of Alberta, Edmonton, Canada) instead of the SYBR Premix
300 Ex *Taq* (TaKaRa Biotechnology Co. Ltd., Dalian, China) used by Yin et al. (2009), but both of
301 these master mixes rely on the intercalating dye SYBR Green as the basis of detection. The
302 reaction conditions consisted of an initial heat denaturation at 95°C for 2 min, followed by 40
303 cycles of 95°C for 15 sec and 60°C for 60 sec, as recommended by the manufacturer. These
304 differ slightly from the conditions used by Yin et al. (2009), but are optimized for the Dynamite
305 qPCR Mastermix utilized in the current analysis. Quantitative PCR assays were conducted in a

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

306 Step One Plus Real-Time PCR System (Applied Biosystems) in a 10 µl total reaction volume,
307 containing 5 µl Dynamite qPCR Mastermix, 0.8 µl molecular grade water, 0.1µl of each of 50
308 mM SsF and SsR, and 4 µl template DNA solution. After the reaction was complete, a melting
309 point analysis was conducted to confirm the presence of a single amplification product that had a
310 melting temperature (T_M) consistent with the predicted T_M of 84°C. The specificity of the
311 SsF/SsR primer set also was evaluated as described above for the other primer and probe sets.

312 **Results**

313 **Amplicon generated with SSBZF/SSBZR.** The amplicons generated from six isolates
314 of *S. sclerotiorum* with the SSBZF/SSBZR primer set targeting the gene SS1G_00263 were
315 sequenced and confirmed to be of the expected 70-bp size. Moreover, the sequences were
316 identical to each other, and a query of the GenBank database using BLASTN revealed 100%
317 identity with accession number XM_001598127.1 from *S. sclerotiorum* (e-value = 3e-31).

318 **Specificity testing.** While the SS1G_00263-specific primers consistently amplified a 70-
319 bp product from DNA of each of the *S. sclerotiorum* isolates evaluated, they did not generate an
320 amplicon from any of the other 13 species tested, including the closely related *B. cinerea, S.*
321 *trifoliorum,* or *S. minor* (Table 3). Similarly, no amplicon was obtained from DNA of the host
322 canola plant. Specificity testing with the *ToxB*-specific primers did not yield an amplicon from
323 *S. sclerotiorum* or any of the non-target organisms tested.

324 **Sensitivity testing.** The lowest reliable LOD was 8.0×10^{-4} ng *S. sclerotiorum* DNA.
325 Although the assay could detect quantities of *S. sclerotiorum* DNA as small as 5.0×10^{-4} ng, such
326 detection could not be accomplished with a standard deviation of the mean $C_q < 0.5$. As such,
327 the lowest point of the standard curve was set to 8.0×10^{-4} ng DNA. To compare replicates of
328 the standard curve, the C_q values were averaged for a set of comparisons and the standard

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

329 deviation of the mean for each point on the curve was calculated. An average standard deviation
330 of 0.19 was obtained for the mean C_q over all five points on the curve, among four replicates of
331 the standard curve in separate 96-well plates; none of the points had a standard deviation > 0.5
332 (Fig. 1). For the four replicates of the standard curve generated in separate 96-well plates, the
333 PCR efficiencies ranged from 88.90 to 90.25 and the R^2 values ranged from 0.9928 to 0.9999.
334 Curves generated with the SS1G_00263-specific primers in the presence or absence of
335 the internal control and *B. napus* DNA were compared to identify any potential reduction in the
336 quantification of *S. sclerotiorum* DNA. The C_q values were averaged for each dilution point in
337 the two dilution series and the standard deviation of the C_q values for the replicates was
338 calculated. Each point on the standard curves was compared individually and no single point had
339 a standard deviation of the mean C_q > 0.54 (Fig 2).
340 **Quantification of *S. sclerotiorum* in canola petals.** Total genomic DNA extracted from
341 20 petals from each of five sampling sites in each of 10 commercial canola fields on two
342 different sampling dates (100 DNA samples in total) was used as a template for qPCR analysis.
343 The petal infestation estimates for individual sampling sites ranged from 0 ng to 3.3×10^{-1} ng *S.*
344 *sclerotiorum* DNA per petal. The amount of *S. sclerotiorum* DNA in each of the 5 sampling
345 sites per field was averaged to give a single petal infestation estimate for each field on each
346 sampling date (Fig. 3). On the first sampling date, when the canola was at 20-30% bloom,
347 average infestation in the 10 fields ranged from a mean (± standard deviation) of 6.0×10^{-3} ng ±
348 7.0×10^{-3} ng to 3.4×10^{-2} ng ± 2.8×10^{-2} ng *S. sclerotiorum* DNA per petal (C_q values = 30.5-
349 36.1). On the second sampling date, at 40-50% bloom, average infestation in the 10 fields
350 ranged from a mean (± standard deviation) of 1.0×10^{-3} ng ± 1.0×10^{-3} ng to 8.0×10^{-2} ng ±1.4
351 $\times 10^{-1}$ ng *S. sclerotiorum* DNA per petal (C_q values = 28.9-36.1).

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

352 In addition to the analysis with the SS1G_00263-specific primer and probe set, all
353 samples were analyzed with the *ToxB* (internal control)-specific primer and probe set in separate,
354 singleplex qPCR assays. The mean and standard deviation of the mean C_q value for duplicates
355 was calculated for early bloom and late bloom sample sets to get an estimate of the amount of
356 variation. At 20-30% bloom, the overall mean C_q (± standard deviation) was 29.6 ± 1.08, and at
357 40-50% bloom, the overall mean C_q (± standard deviation) was 27.6 ± 0.70.

358 **Assessment of the SsF and SsR primers.** The primers SsF and SsR (Yin et al. 2009)
359 were evaluated to determine their suitability for quantifying *S. sclerotiorum* DNA in canola
360 petals under Canadian conditions. The primers amplified a product of approximately 225-bp
361 from all isolates of *S. sclerotiorum*, and did not amplify DNA of *S. minor*, *Aspergillus niger*,
362 *Cladosporium* sp., *Mucor* sp., *Alternaria alternata*, or *Leptosphaeria maculans* (Table 3).
363 However, the primers were found to amplify products from DNA of *S. trifoliorum,* as well as
364 from all isolates tested of *B. cinerea* and *B. fuckeliana, Rhizopus* sp., *Trichoderma* sp.,
365 *Rhizoctonia solani, Penicillium* sp., and *Fusarium graminearum*. The amplicons obtained from
366 *B. cinerea, S. trifoliorum* and *Trichoderma* sp. all had temperature peaks between 84 and 85°C,
367 which was within the range expected for the product from *S. sclerotiorum*. For the products
368 amplified from DNA of *B. fuckeliana, Rhizopus* sp., *R. solani, Penicillium* sp., and *F.*
369 *graminearum*, the melting curve analysis revealed the presence of multiple temperature peaks
370 associated with non-specific amplification. The sensitivity of the SsF/SsR primer set was similar
371 to that of the SSBZF/SSBFR primers, with a consistent LOD of 8.0×10^{-4} ng DNA.

372 Discussion

373 This study focussed on the development of a qPCR-based assay for the quantification of
374 *S. sclerotiorum* DNA in canola petals, to enable rapid and accurate estimates of infestation

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

375 levels. The assay targets a 70-bp region of a single-copy gene encoding the hypothetical secreted
376 protein ssv263 (Liang et al. 2013). While the target region of the gene shares 71% similarity
377 with an orthologue in *B. cinerea*, specificity testing indicated that there was no amplification of
378 DNA from any of six *B. cinerea* isolates tested. The qPCR assay also did not amplify DNA from
379 any of the 13 other species evaluated in this study, including *S. minor, S. trifoliorum* and the host
380 plant, *B. napus*. These results indicate that the assay is highly specific for *S. sclerotiorum*, and
381 can be used to estimate pathogen biomass in canola petals.

382 The sensitivity tests revealed a consistent LOD of 8.0×10^{-4} ng *S. sclerotiorum* DNA.
383 Rogers et al. (2008) reported that an ascospore of *S. sclerotiorum* is equivalent to about $3.5 \times 10^{-}$
384 ^{4} ng of DNA, suggesting a LOD of 2.3 ascospores per petal for the current assay. When adjusted
385 to account for the dilution of DNA from canola petals during extraction and analysis, the LOD
386 would correspond to 1.5×10^{-3} ng *S. sclerotiorum* DNA or 4.3 ascospores per petal. An issue
387 with the use of ascospores as the basis for a standard curve to quantify petal infestation, however,
388 is that this approach will not reflect increases in pathogen biomass associated with spore
389 germination. Ascospores have been shown to begin germination as early as 3 hours after release
390 from the apothecium under favorable environmental conditions (Willets and Wong 1980).
391 Therefore, it is likely that any assay examining the amount of *S. sclerotiorum* DNA on canola
392 petals is measuring DNA from the ascospores as well as from hyphae developing from
393 germinated spores. As such, quantification of *S. sclerotiorum* on petals is more meaningful and
394 accurate when expressed as the total amount of pathogen DNA. The specificity of the assay was
395 not affected by the presence of *B. napus* DNA or of an internal control in the samples. These are
396 important considerations if the assay is to be used to measure infestation of petals collected in the
397 field.

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

398 Yin et al. (2009) developed a qPCR assay to measure *S. sclerotiorum* DNA in canola
399 petals using intercalating dye (SYBR Green) detection technology that detected between 0.0252
400 ng-0.111 ng pathogen DNA per mg of canola petal tissue. Evaluation of the primers developed
401 by Yin et al. (2009) on DNA extracted from Canadian isolates of *S. sclerotiorum* and other
402 fungal species, using a different master mix but the same dye detection technology, indicated
403 sensitivity similar to that of the assay described in this report. However, the specificity of the
404 primers seemed considerably lower and amplicons were obtained from various non-target
405 species including *S. trifoliorum, B. cinerea, F. graminearum, Rhizopus* sp., and *Penicillium* sp.
406 In their original report, Yin et al. (2009) found no amplification of DNA other than from *S.*
407 *sclerotiorum*, but only evaluated one isolate of *B. cinerea* (*B. fuckeliana*) and did not test *S.*
408 *minor, S. trifoliorum,* or *Rhizopus* sp. They did test *R. solani, Penicillium* sp. and *F.*
409 *graminearum*, but did not detect any product from the isolates evaluated. This lower specificity
410 represents a challenge for application of the earlier protocol (Yin et al. 2009) in the analysis of
411 field samples, and hence for its use as the basis for a Sclerotinia stem rot risk assessment system.
412 While it may be possible to identify non-specific amplification products based on a melting
413 curve analysis, this would complicate the procedure and introduce the possibility of additional
414 errors. Moreover, the quantification of field samples containing a range of different fungal
415 species in addition to *S. sclerotiorum* may be difficult. The similar sensitivity, but increased
416 specificity of the assay developed in this study with the primers SSBZF/SSBZR and probe
417 SSBZP may reflect the use of a hydrolysis probe instead of an intercalating dye. An
418 intercalating dye, such as SYBR green, binds to any double-stranded DNA and releases a
419 fluorescent signal (Ririe et al. 1997). This potentially can result in fluorescent amplification of
420 non-target double-stranded DNA that must be further differentiated through melting curve

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

421 analysis (Smith and Osborn 2008). The use of a hydrolysis probe ensures that a fluorescent
422 signal is produced only when there is an exact match between the probe and the target (Smith
423 and Osborn 2008). This increased level of specificity reduces the likelihood of false positives
424 when analysing diverse field samples, without compromising sensitivity. As a result, the newly
425 developed qPCR assay may represent a more reliable method to quantify *S. sclerotiorum* DNA in
426 canola petals.

427 When the hydrolysis probe-based qPCR assay was used to quantify *S. sclerotiorum* in
428 field-collected canola petals, considerable variation was observed in the amount of petal
429 infestation in different fields and at different crop stages. Environmental conditions in 2012
430 were conducive to stem rot development in the sampled fields. In five of 10 fields, infestation
431 levels were highest at 40-50% bloom, while in the remaining five fields, the infestation levels
432 were highest at 20-30% bloom. This is consistent with the findings of Turkington and Morrall
433 (1993), who reported changes in petal infestation from early to late bloom and at different
434 locations when culturing petals on growth medium. Similarly, using qPCR analysis, Almquist
435 and Wallenhammer (2015) also observed differences in *S. sclerotiorum* incidence at different
436 stages of flowering. The evaluation of petals by qPCR analysis over the flowering period could
437 provide an indication of when inoculum levels are highest, and thus when the crop may benefit
438 most from fungicide application. The variation in the amount of *S. sclerotiorum* DNA between
439 locations may reflect differences in inoculum level or in the timing of ascospore release, crop
440 seeding date and growth stage, canopy density, or microclimatic conditions. It also indicates the
441 importance of assessing petal infestation and risk potential for a particular field as opposed to an
442 assessment of risk based on regional conditions. The inclusion of an internal control helped to

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

443 identify outliers resulting from possible human error or other artifacts, such as inhibition of the
444 PCR, thereby improving the accuracy of qPCR-based estimates of petal infestation.

445 The qPCR assay described in this study may serve as the basis for a risk assessment
446 system, as well as representing a useful tool for the study of the epidemiology of Sclerotinia stem
447 rot of canola. It can quantify the level of petal infestation, a key stage in the Sclerotinia stem rot
448 disease cycle, thereby providing a measure of disease risk when timely fungicide application
449 decisions need to be made. To further investigate the possibility of developing a forecasting
450 system based on this technology, the relationship between the petal infestation estimates and
451 final stem rot incidence must be determined. The strength of the relationship between petal
452 infestation as assessed through qPCR analysis and final disease incidence can be investigated
453 with the use of regression analysis. It is also important to emphasize that a forecasting system
454 based on qPCR quantification of petal infestation should be linked to environmental conditions,
455 as well as cropping history, seeding date and crop canopy conditions, which may influence
456 Sclerotinia stem rot development and the need to spray a fungicide. Validation as a predictive
457 tool under field conditions is the focus of a follow-up study.

458 **Acknowledgements**

459 This work would not have been possible without the generous support of the Alberta
460 Crop Industry Development Fund, the Alberta Canola Producers Commission, the Western
461 Grains Research Foundation, and Agriculture and Agri-Food Canada and the Canola Council of
462 Canada via the Growing Forward program. We thank Dr. R. J. Howard (Alberta Agriculture and
463 Rural Development, Brooks, AB) for providing isolates of *B. cinerea*, Dr. R. Aboukhaddour
464 (University of Alberta) for providing the plasmid used to develop the internal control, and Ileana
465 Strelkov, Derek Rennie, Troy Locke and Dr. Tiesen Cao (University of Alberta) for technical

466 assistance. We also thank Emile deMilliano and Ryan Adams (Crop Production Systems, Ft.
467 Saskatchewan, AB) and several cooperating farmers for assisting with petal collection and
468 including a fungicide-free check strip in their crops.

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

469 **Literature Cited**

470 Abawi, G. S., and Grogan R. G. 1979. Epidemiology of diseases caused by *Sclerotinia* species.
471 Phytopathology 69:899-904.

472 Aboukhaddour, R., Kim, Y. M., and Strelkov, S. E. 2012. RNA-mediated gene silencing of *ToxB*
473 in *Pyrenophora tritici-repentis.* Mol. Plant Pathol. 13:318-326.

474 Almquist, C., and Wallenhammar, A.- C. 2015. Monitoring of plant and airborne inoculum of
475 *Sclerotinia sclerotiorum* in spring oilseed rape using real-time PCR. Plant Pathol. 64: 109-118.

476 Bardin, S. D., and Huang, H. C. 2001. Research on biology and control of *Sclerotinia* diseases in
477 Canada. Can. J. Plant Pathol. 23:88-98.

478 Boland, G. J., and Hall, R. 1994. Index of plant hosts of *Sclerotinia sclerotiorum*. Can. J. Plant
479 Pathol.16:92-108.

480 Bolton, M. D., Bart, T., Thomma, P. H. J., and Nelson, B. D. 2006. *Sclerotinia sclerotiorum*
481 (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. Mol. Plant Pathol. 7:1-
482 16.

483 Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R.,
484 Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., and Wittwer, C. T. 2009. The MIQE
485 quidelines: Minimum information for publication of quantitative real-time PCR experiments.
486 Clinical Chem. 55:611-622.

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

487 Canola Council of Canada. 2014. Canola encyclopedia: Sclerotinia stem rot. Canola Council of
488 Canada, Winnipeg, Manitoba, Canada. [Online] Available:
489 http://www.canolacouncil.org/canola-encyclopedia/diseases/sclerotinia-stem-rot/#variety-
490 selection-and-genetic-resistancetolerance. Verified: 15 October 2014.

491 Carpenter, M. A., Frampton, C., and Stewart, A. 1999. Genetic variation in New Zealand
492 populations of the plant pathogen *Sclerotinia sclerotiorum*. NZ J. Crop Hortic. Sci. 27:13-21.

493 Clarkson, J. P., Staverley, J., Phelps, K., Young, C. S., and Whipps, J. M. 2003. Ascospore
494 release and survival in *Sclerotinia* sclerotiorum. Mycol. Res. 107:213-222.

495 del Río, L. E., Bradley, C. A., Hensen, R. A., Endres, G. J., Hanson, B. K., McKay, K.,
496 Halvorson, M., Porter, P. M., Le Gare, D. G., and Lamey, H. A. 2007. Impact of sclerotinia stem
497 rot on yield of canola. Plant Dis. 91:191-194.

498 Freeman, J., Ward, E., Calderon, C., and McCartney, A. 2002. A polymerase chain reaction
499 (PCR) assay for the detection of inoculum of *Sclerotinia sclerotiorum*. Eur. J. Plant Pathol.
500 108:877-886.

501 Gugel, R. K., and Morrall, R. A. A. 1986. Inoculum disease relationships in sclerotinia stem rot
502 of rapeseed in Saskatchewan. Can. J. Plant Pathol. 8:89-96.

503 Harper, F. R., and Berkenkamp, B. 1975. Revised growth-stage key for *Brassica campestris* and
504 *B. napus*. Can. J. Plant Sci. 55:657-658.

505 Jamaux, I., Gelie, B., and Lamarque, C. 1995. Early stages of infection of rapeseed petals and
506 leaves by *Sclerotinia sclerotiorum* revealed by scanning electron microscopy. Plant Pathol.
507 44:22-30.

508 Koch, S., Dunker, S., Kleinhenz, B., Röhrig, M., and Von Tiedemann, A. 2007. A crop loss-
509 related forecasting model for Sclerotinia stem rot in winter oilseed rape. Phytopathology
510 97:1186-1194.

511 Kora, C., McDonald, M. R., and Boland, G. J. 2005. Epidemiology of sclerotinia rot of carrot
512 caused by *Sclerotinia sclerotiorum*. Can. J. Plant Pathol. 27:245-258.

513 Lamari, L., and Strelkov, S. E. 2010. The wheat/*Pyrenophora tritici-repentis* interaction:
514 progress towards an understanding of tan spot disease. Can. J. Plant Pathol. 32:4-10.

515 Lancashire, P. D., Bleiholder, H., Langelüddecke, P., Stauss, R., Van Den Boom, T., Weber, E.,
516 and Witzen-Berger, A. 1991. A uniform decimal code for growth stages of crops and weeds.
517 Ann. Appl. Biol. 119:561-601.

518 Liang, Y., Yajima, W., Davis, M. R., Kav, N. N. V., and Strelkov, S. E. 2013. Disruption of a
519 gene encoding a hypothetical secreted protein from *Sclerotinia sclerotiorum* reduces its virulence
520 on canola (*Brassica napus)*. Can. J. Plant Pathol. 35:46-55.

521 Ma, Z., Luo, Y., and Michailides, T. J. 2003. Nested PCR assays for detection of *Manilinia*
522 *fruticola* in stone fruit orchards and *Botryosphaeria dothidea* from pistachios in California. J.
523 Phytopathol. 151:312-322.

524 McLaren, D. L., Conner, R. L., Platford, R. G., Lamb, J. L., Lamey, H. A., and Kutcher, H. R.
525 2004. Predicting diseases caused by *Sclerotinia sclerotiorum*on canola and bean – a western
526 Canadian perspective. Can. J. Plant Pathol. 26:489-497.

527 Morrall, R. A. A., and Thomson, J. R. 1991. Petal test manual for *Sclerotinia* in canola.
528 University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

529 Morrall, R. A. A., and Dueck, J. 1982. Epidemiology of sclerotinia stem rot of rapeseed in
530 Saskatchewan. Can J. Plant Pathol. 4:161-168.

531 Nakayashiki, H., Hanada, S., Nguyen, B. Q., Kadotani, N., Tosa, Y., and Mayama, S. 2005.
532 RNA silencing as a tool for exploring gene function in ascomycete fungi. Fungal Genet. Biol.
533 42:275-283.

534 Parker, M. L., McDonald, M. R., and Boland, G. J. 2014. Evaluation of air sampling and
535 detection methods to quantify airborne ascospores of *Sclerotinia sclerotiorum*. Plant Dis. 98:32-
536 42.

537 Pratt, S. 2012. Sclerotinia resistant canola provides new option. The Western Producer, 20 July
538 2012. [Online] Available: http://www.producer.com/2012/07/sclerotinia-resistant-canola-
539 provides-new-option/. Verified: 15 October 2014.

540 Purdy, L. H. 1979. *Sclerotinia sclerotiorum* history, diseases and symptomology, host range,
541 geographic distribution and impact. Phytopathology 69:875-880.

542 Ririe, K. M., Rasmussen, R. P., and Wittwer, C. T. 1997. Product differentiation by analysis of
543 DNA melting curves during the Polymerase Chain Reaction. Analytical Biochem. 245: 154-160.

544 Rogers, S. L., Atkins, S. D., and West, J. S. 2008. Detection and quantification of airborne
545 inoculum of *Sclerotinia sclerotiorum* using quanitative PCR. Plant Pathol. 58:324-331.

546 Schwartz, H. F., and Steadman, J. R. 1978. Factors affecting sclerotium populations of, and
547 apothecium production by *Sclerotinia sclerotiorum*. Phytopathology 68:383-388.

548 Shah, P., Atwoood III, J. A., Orland, R., Mubarek, H. E., Podila, G. K., and Davis, M. R. 2009.
549 Comparative proteomic analysis of *Botrytis cinerea* secretome. J. Proteome Res. 8:1123-1130.

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

550 Sirjusingh, C., and Kohn, L. M. 2001. Characterization of microsatellites in the fungal plant
551 pathogen *Sclerotinia sclerotiorum.* Mol. Ecol. Notes 1:267-269.

552 Smith, C. J., and Osborn, A. M. 2008. Advantage and limitations of quantitative PCR (Q-PCR)-
553 based approaches in microbial ecology. FEMS Microbiol. Ecol. 67:6-20.

554 Turkington, T. K., Kutcher, H. R., McLaren, D., and Rashid, K. Y. 2011. Managing sclerotinia in
555 oilseed and pulse crops. Prairie Soils Crops 4:105-113.

556 Turkington, T. K., and Morrall, R. A. A. 1993. Use of petal infestation to forecast sclerotinia
557 stem rot of canola: the influence of inoculum variation over the flowering period and canopy
558 density. Phytopathology 83:682-689.

559 Turkington, T. K., Morrall, R. A. A., and Gugel, R. K. 1991. Use of petal infestation to forecast
560 Sclerotinia stem rot of canola: evaluation of early bloom sampling. 1995-1990. Can. J. Plant
561 Pathol. 13:50:59.

562 Weber, E., and Bleiholder, H. 1990. Erläuterungen zu den BBCH-Dezimal-Codes für die
563 Entwicklungsstadien von Mais, Raps, Faba-Bohne, Sonnenblume und Erbse - mit Abbildungen.
564 Gesunde Pflanzen 42:308-321.

565 Willets, H. J., and Wong, J. A.-L. 1980. The biology of *Sclerotinia sclerotiorum, S. trifoliorum,*
566 and *S. minor* with emphasis on specific nomenclature. Botanical Rev. 46:101-165.

567 Wu, B. M., and Subbarao, K. V. 2008. Effects of soil temperature, soil moisture and burial
568 depths on carpogenic germination of *Sclerotinia sclerotiorum* and *S. minor.* Phytopathology
569 98:1144-1152.

570 Yin, Y., Ding, L., Lui, X., Yang, J. and Ma, Z. 2009. Detection of *Sclerotinia sclerotiorum in*
571 *planta* by a real-time PCR assay. J. Phytopathol. 157:465-469.

572 Young, C. S., and Werner, C. P. 2012. Infection routes for *Sclerotinia sclerotiorum* in apetalous
573 and fully petalled winter oilseed rape. Plant Pathol. 61:730-738.

574

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

575 **Table 1.** List of species used to test the specificity of primers and hydrolysis probes developed for the detection and quantification of

576 *Sclerotinia sclerotiorum* and a *ToxB* internal control.

Species	Isolate	Origin	Source	Supplier
Sclerotinia sclerotiorum[ab]	UAMH 6321	Devon, AB	Potted *Garzania* sp.	University of Alberta Microfungus Collection and Herbarium (UAMCH)
S. sclerotiorum[b]	UAMH 4514	Central Alberta	Rapeseed	UAMCH
S. sclerotiorum[b]	UAMH 9192	Maryland, USA	Bean stem	UAMCH
S. sclerotiorum[b]	SSA-11	Edmonton, AB	Canola petals	B. Ziesman, University of Alberta (U of A)
S. sclerotiorum[b]	SSB-11	Edmonton, AB	Canola petals	B. Ziesman, U of A
S. sclerotiorum	SSC-11	Edmonton, AB	Canola petals	B. Ziesman, U of A
S. sclerotiorum[b]	SSD-11	Edmonton, AB	Canola petals	B. Ziesman, U of A
S. minor	CBS 207.25	Unknown	Unknown	Centraalbureau Voor Schimmelcultures (CBS), Royal Netherlands Academy of Arts and Sciences, Uppsalalaan, NL
S. trifoliorum	CBS 122377	Netherlands	Unknown	CBS
Botrytis cinerea	DR12-5	Unknown	Potato tuber tissue	R.J. Howard, Alberta Agriculture and Rural Development (AARD), Brooks, AB
B. cinerea	414JV	Unknown	Alfalfa	R.J. Howard, AARD
B. cinerea	DAOM 192631	Winnipeg, Manitoba	*Fragaria chiloensis*	Canadian Collection of Fungal Cultures (CCFC), Ottawa, Ontario

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

B. cinerea	DAOM 189076	Charlottetown, PEI	Potato tubers	CCFC
B. cinerea	DAOM 166439	Beaverlodge, AB	*Festuca rubra*	CCFC
B. cinerea	CGC5	PEI	Winter wheat	Canadian Grain Commission, Winnipeg, MB
Botryotinia fuckeliana[c]	UAMH 16	Unknown	Bean	UAMCH
B. fuckeliana	UAMH 1784	Beaverlodge, AB	Indoor air exchange strip from *Apis mellifera* equipment cleaning warehouse	UAMCH
Rhizoctonia solani	N/A	Unknown	Unknown	J.P. Tewari, U of A
Rhizopus sp.	N/A	Unknown	Unknown	J.P. Tewari, U of A
Trichoderma sp.	N/A	Unknown	Soil	J.P. Tewari, U of A
Penicillium sp.	N/A	Edmonton, AB	Canola petals	B. Ziesman, University of Alberta (U of A)
Aspergillus niger	N/A	Unknown	Unknown	J.P. Tewari, U of A
Cladosporium sp.	N/A	Edmonton, AB	Canola petals	B. Ziesman, U of A
Mucor sp.	N/A	Edmonton, AB	Canola petals	B. Ziesman, U of A
Alternaria alternata	N/A	Unknown	Unknown	J.P. Tewari, U of A
Fusarium graminearum	G-1	Unknown	Unknown	A. Tekauz, Agriculture and Agri-Food Canada, Winnipeg, MB
Leptosphaeria maculans	N/A	Unknown	Unknown	J.P. Tewari, U of A
Brassica napus	N/A	Edmonton, AB	N/A	B. Ziesman, U of A

577 [a] *S. sclerotiorum* UAMH 6321 was originally designated as *S. minor* in the UAMCH, but later identified as *S. sclerotiorum*.
578 [b] Amplicon obtained from the isolate sequenced to confirm identity of the PCR product.
579 [c] Teleomorph of *B. cinerea*; species names are given as provided by the original supplier.

580 **Table 2.** Primers and hydrolysis probes for detection and quantification of *Sclerotinia*
581 *sclerotiorum* and a *ToxB* internal control. The primer set SSBZF/SSBZR amplifies a 70-bp
582 fragment of the gene SSIG_00263 in *S. sclerotiorum* and was used in conjunction with SSBZP in
583 a hydrolysis probe-based assay to quantify *S. sclerotiorum* DNA. The primer set ToxBF/ToxBR
584 amplifies a 70-bp fragment of the *ToxB* gene from *Pyrenophora tritici-repentis* and was used in
585 conjunction with ToxBP to quantify the exogenous internal control in a hydrolysis probe-based
586 assay.

Primer/probe name	Sequence	Size (bp)
SSBZF	5'-GCTCCAGCAGCCATGGAA-3'	18
SSBZR	5'-TGTTGAAGCAGTTGACGAGGTAGT-3'	24
SSBZP	5'-CAGCGCCTCAAGC-3'	13
ToxBF	5'-CCATGCTACTTGCTGTGGCTAT-3'	22
ToxBR	5'-CGCAGTTGGCCGAAACA-3'	17
ToxBP	5'-CTCCCTGCTGCCCC-3'	13

587
588
589
590
591
592
593
594
595
596

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

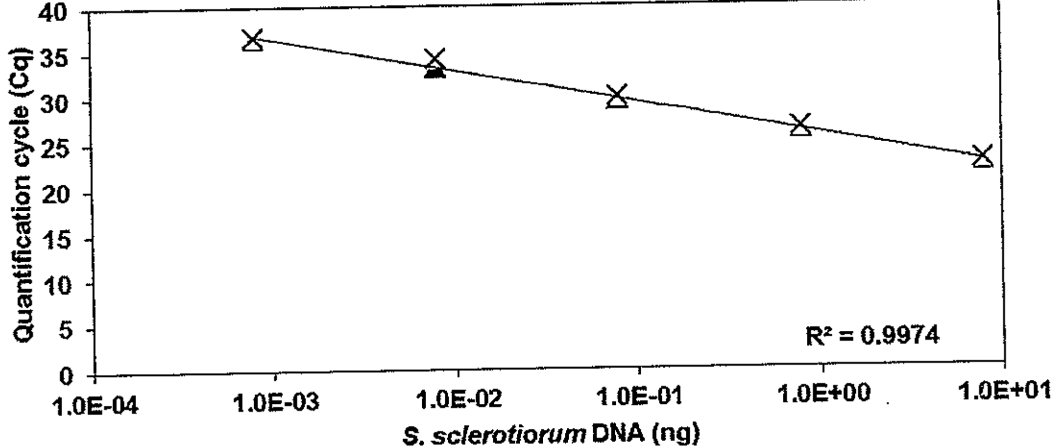
597 **Table 3.** Results of specificity testing with the SsF/SsR primer set (Yin et al. 2009) and
598 SSBZF/SSBZR primer set. The quantification cycle (C_q) value indicates the cycle number at
599 which fluorescence from amplification of a product exceeds background fluorescence.

Fungal species	C_q value (SsF/SsR)	C_q value (SSBZF/SSBZR)
Sclerotinia trifoliorum	27.673	ND[a]
Sclerotinia minor	ND	ND
Botryotinia fuckeliana UAMH 1784	33.219	ND
B. fuckeliana UAMH 16	35.017	ND
Botrytis cinerea DR12-5	34.954	ND
B. cinerea DAOM 192631	34.099	ND
B. cinerea DAOM 166439	35.017	ND
Rhizopus sp.	34.285	ND
Trichoderma sp.	30.617	ND
Rhizoctonia sp.	35.330	ND
Penicillium sp.	31.550	ND
Aspergillus niger	ND	ND
Fusarium graminearum	30.037	ND
Cladosporium sp.	ND	ND
Mucor sp.	ND	ND
Alternaria alternata	ND	ND
Leptosphaeria maculans	ND	ND
8.0 × 10^{-4} ng of *S. sclerotiorum* DNA	33.068	33.662

600 [a] ND, no detection; indicates that there was no amplification of the fungal DNA or that the C_q
601 value was at least 3 cycles greater than the lowest standard (8.0 × 10^{-4} ng of *S. sclerotiorum*
602 DNA).
603

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

604



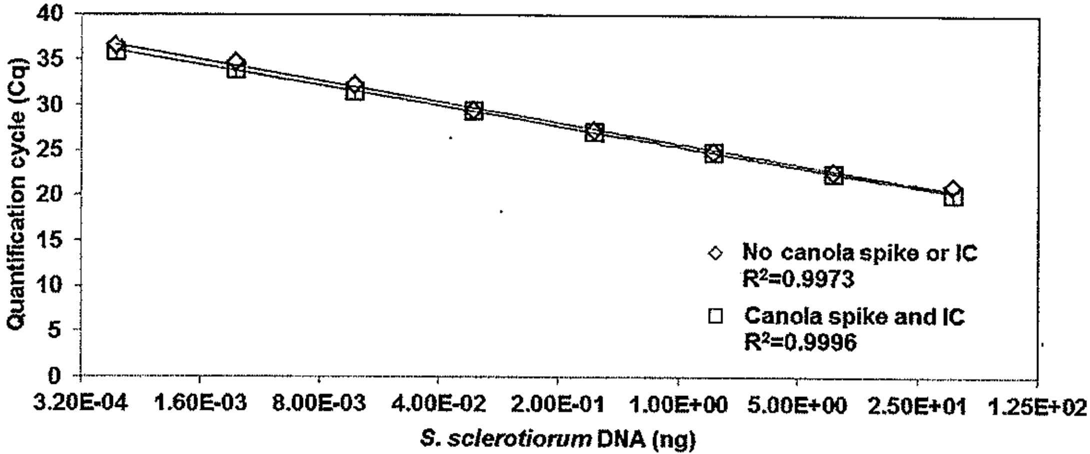
605

606 **Fig. 1.** Standard curves obtained with DNA extracted from *Sclerotinia sclerotiorum* and
607 subjected to quantitative PCR analysis with the primer set SSBZF/SSBZR and hydrolysis probe
608 SSBZP. Four replicates of the standard curve were run in separate 96-well plates. The standards
609 were prepared from a serial dilution of 8.0 ng to 8.0×10^{-4} ng *S. sclerotiorum* DNA. The four
610 replications are denoted by different symbols, which may not be clearly visible because the
611 curves overlap. The PCR efficiency (E) of the four standard curves ranged from 88.9 to 90.25
612 and the R^2 values for the individual standard curves ranged from 0.9928 to 0.9999 (average:
613 0.996).

614

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.



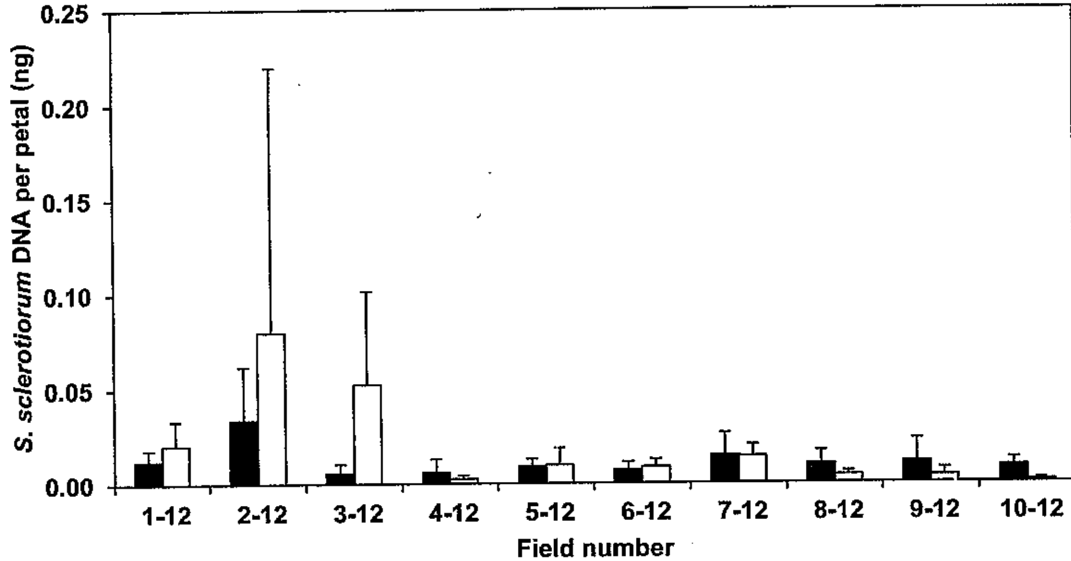
615

616 **Fig. 2.** Quantification of *Sclerotinia sclerotiorum* DNA in the presence or absence of canola
617 (*Brassica napus*) DNA and a *ToxB*-internal control. The fungal DNA was serially diluted by a
618 factor of 1:5 from 4.0×10^1 ng to 5.0×10^{-4} ng, and analyzed by qPCR with the primer set
619 SSBZF/SSBZR and hydrolysis probe SSBZP. Samples were analyzed in the absence (diamonds)
620 or presence of a *ToxB*-internal control (IC) and *Brassica napus* DNA ('Canola spike') (squares).
621 The standard deviation of the mean C_q for any point along the curve was not > 0.54, suggesting
622 that the presence of host DNA or the internal control did not affect the quantification of *S.*
623 *sclerotiorum* DNA. The standard deviation at each point of the standard curve is reflected by the
624 error bars.

625

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

626



627

628 **Fig. 3.** The quantification of *Sclerotinia sclerotiorum* DNA on canola petals collected from
629 commercial fields in the Edmonton, AB, region, as determined by qPCR analysis with the primer
630 set SSBZF/SSBZR and hydrolysis probe SSBZP. Five sites per field in each of 10 fields were
631 sampled at 20-30% bloom (black bars) and 40-50% bloom (white bars). The error bars represent
632 the standard deviation of the mean for each field and reflect the amount of variation across the
633 five sampling sites within each field.

1 **Evaluation of PCR-based quantification of *Sclerotinia sclerotiorum* infestation**
2 **levels as a predictive tool for stem rot of canola (*Brassica napus*)**

3

4 **(Draft Manuscript)**

5

6 **B.R. Ziesman[1], T.K. Turkington[2], B.D. Gossen[3], R. Kutcher[4], D. McLaren[5], K. Rashid[6],**
7 **and S.E. Strelkov[1]**

8

9 [1]Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton,
10 AB

11 [2]Lacombe Research and Development Centre, Agriculture and Agri-Food Canada, Lacombe, AB

12 [3]Saskatoon Research and Development Centre, Agriculture and Agri-Food Canada, Saskatoon,
13 SK

14 [4]Department of Plant Sciences, University of Saskatchewan, Saskatoon, SK

15 [5]Brandon Research and Development Centre, Agriculture and Agri-Food Canada, Brandon, MB

16 [6]Morden Research and Development Centre, Agriculture and Agri-Food Canada, Morden, MB

17 **Abstract**

18 Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum*, is an economically important disease of
19 canola (*Brassica napus*) in the Prairies region of western Canada. The disease is managed
20 primarily via the routine application of fungicides, often without any indication of stem rot risk.
21 The objective of this study was to evaluate the relationship between the levels of canola petal
22 infestation by *S. sclerotiorum*, as determined by quantitative (q)PCR analysis, and final stem rot
23 incidence in the field. This relationship was explored two studies. In the first study, conducted
24 over 2 years, petal infestation was compared with disease incidence in 34-35 commercial canola
25 fields distributed across Alberta, Saskatchewan and Manitoba. In the second study, these
26 parameters were compared over 3 years in 9-11 fields located in central Alberta. In the fields
27 sampled across the Prairies, no consistent relationship was observed between petal infestation
28 and stem rot incidence at harvest. However, no information was available on whether or not
29 these fields received a fungicide treatment, which may have reduced the ability to detect any
30 correlations. In contrast, sampling points in the second study were located in fungicide-free
31 check strips, and the incidence of stem rot was generally found to increase with increasing petal
32 infestation. The strength of the relationship varied across the study years, and was strongest
33 when canola petals were analyzed at full bloom and in years when disease pressure was high.

34

35 **Keywords:** Canola, disease forecasting, qPCR, risk assessment, *Sclerotinia sclerotiorum*, stem
36 rot

37 **Introduction**

38 Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, is one of the most
39 economically important diseases of canola (*Brassica napus* L.), causing yield losses high as 50%
40 (del Río et al. 2007). Ascospores produced through carpogenic germination of the overwintering
41 sclerotia are the primary inoculum initiating disease development (Abawi and Grogan 1979;
42 Bardin and Hwang 2001; Purdy 1979; Willets and Wong 1980). The ascospores come into
43 contact with canola petals onto which they adhere, germinate and penetrate the petal tissue.
44 When the petals senesce and fall onto leaf or stem surfaces, the fungus can grow out of the petals
45 and infect the living stem and leaf tissue (Jamaux et al. 1995; Purdy 1979). Infection of the
46 petals has been demonstrated to be an important stage in the Sclerotinia stem rot life cycle
47 (Jamaux et al. 1995; Morrall and Dueck 1982), and is influenced by environmental conditions
48 and the timing of ascospore release.

49 In the Prairies region of western Canada, Sclerotinia stem rot is managed primarily by the
50 routine application of fungicides. However, as a result of the influence of environmental
51 conditions on the success of infection, Sclerotinia stem rot is not present at high levels every
52 year. As a result, the application of fungicide without any indication of disease risk can result in
53 fungicides being applied when stem rot risk is low and fungicides are not required to prevent
54 yield loss. To improve the sustainability and accuracy of fungicide application, a timely and
55 reliable risk assessment tool is required to assist producers in making informed Sclerotinia stem
56 rot management decisions.

57 In Canada, several risk assessment tools have been developed for Sclerotinia stem rot of
58 canola, including weather-based risk maps, risk point tables and a petal plate test. Weather-based
59 risk maps take into account soil moisture, daily precipitation and air temperature to provide

60 regional assessments of stem rot risk (McLaren et al. 2004). The weather-based risk maps do not
61 take into account the amount of inoculum available, but rather focus on whether or not the
62 environment is favorable for disease development. Risk point tables provide essentially
63 qualitative assessments of risk, based on a variety of factors that influence the Sclerotinia stem
64 rot disease cycle (Twengstrom et al. 1998). Risk point tables assign weight to factors such as
65 crop rotation history, the amount or frequence of precipitation, the presence or absence of
66 apothecia in a field, and the incidence of Sclerotinia stem rot in the last susceptible crop, all of
67 which are all known to influence disease development in the field. Although the number of
68 apothecia present represents a quantitative estimate of inoculum presence, disease has been
69 found in fields in the absence of apothecia (Gugel 1986), indicating that this may not be a
70 reliable indication of the amount of inoculum available to initiate infection. In contrast, the petal
71 plate test developed by Morrall and Thompson (1991) provides a quantitative assessment of risk.
72 This method involves plating field collected canola petals onto potato dextrose agar (PDA),
73 incubating for 3-5 days, and assessing the percentage of petals infected with *S. sclerotiorum*
74 based on a visual inspection. Percent petal infestation takes into account an important stage in the
75 stem rot disease cycle, and has been shown to be related to Sclerotinia stem rot incidence in
76 canola (Gugel 1986; Turkington and Morrall 1993; Turkington et al. 1990). However, the delay
77 associated with the incubation period need for fungal growth has led to limited uptake of this risk
78 assessment tool by canola producers and agronomists in Canada.

79 Quantitative polymerase chain reaction (qPCR)-based methodologies can be used as a
80 method to measure the amount of *S. sclerotiorum* DNA on infected canola petals. Quantitative
81 PCR analysis can provide sensitive, robust and highly reproducible quantification without the
82 time delay associated with the traditional petal test (Smith and Osborn 2008). Several qPCR-

83 based techniques have been reported for the detection and quantification of *S. sclerotiorum*
84 (Parker et al. 2014; Rogers et al. 2008; Yin et al. 2009). Most recently, a hydrolysis probe-based
85 qPCR assay was described to measure *S. sclerotiorium* DNA in canola petals (Ziesman et al.
86 2016). This qPCR assay was found to be highly specific to *S. sclerotiorum* and did not amplify
87 DNA of a variety of closely related fungi. The assay was shown to accurately quantify the
88 amount of *S. sclerotiorum* DNA in field collected canola petals. Nevertheless, the relationship
89 between the level of petal infestation as measured by qPCR analysis, and eventual stem rot
90 incidence in the sampled canola crops was not established. An evaluation of this relationship is
91 needed if qPCR-based analysis of petal infestation is to serve as the basis of a stem rot risk
92 assessment system.

93 The aim of this study was to establish the relationship between levels of petal infestation,
94 as measured at 10-20% and at 40-50% bloom by qPCR analysis, and final stem rot incidence at
95 harvest, in order to determine whether the qPCR assay of Ziesman et al. (2016) could serve as a
96 stem rot risk assessment tool. To provide reliable estimates of stem rot risk, the results obtained
97 via qPCR analysis should be strongly related to disease incidence across a variety of fields and
98 weather conditions. Specifically, the objectives of this study were to: (1) evaluate the
99 relationship between the qPCR results and final Sclerotinia stem rot incidence in the field, 2)
100 determine if the qPCR assay can be used independently as a risk assessment tool across the
101 Prairies, and 3) assess whether or not the quantifications are correlated with percent petal
102 infestation estimates determined with the earlier petal test developed by Morrall and Thompson
103 (1991).

104 **Materials and Methods**

105 **Field selection and sample collection.** Two experiments (Exp. 1 and Exp. 2) were conducted to
106 evaluate the relationship between petal infestation level and final stem rot disease incidence.
107 Experiment 1 was conducted over two years (2011 and 2012) by monitoring petal infestation and
108 subsequent disease incidence in 35 (in 2011) or 34 (in 2012) commercial canola fields in Alberta
109 (near Edmonton and Lacombe), Manitoba (Brandon, Morden and Carman) and Saskatchewan
110 (Melfort and Saskatoon). Experiment 2 was conducted in commercial canola fields located near
111 Edmonton, Alberta, in 2011, 2012 and 2013. Nine fields were included in 2011, 10 fields in
112 2012, and 11 fields in 2013. The same central Alberta fields were monitored for Exp. 1 and Exp.
113 2 in 2011 and 2012. Experiment 1 was focussed on a Prairies-wide evaluation of the
114 relationship between petal infestation and stem rot incidence, while Exp. 2 examined this
115 relationship within a smaller regional scale.

116 With the exception of the fields near Edmonton, the fields in Exp. 1 were selected at
117 random. For the fields sampled in Manitoba and Saskatchewan, field histories were not known,
118 and no information was available as to whether or not fungicides were applied for stem rot
119 control during the growing season. In each field, there were 5 sampling sites spaced 50 m apart
120 and at least 75 m from the field edge. In Exp. 2, the sampling sites were situated in fungicide-
121 free check strips. There were five sampling sites per field arranged 50 m apart in a linear fashion.

122 Canola petals were collected at early flowering (10-20% bloom) and full flowering (40-
123 50% bloom). In 2012, the first sampling of the fields near Edmonton was delayed until 20-30%
124 bloom. Samples were collected between 1100 h and late afternoon, with the exception of the
125 fields near Edmonton in 2012 where sampling occurred early in the morning due to high

126 temperatures during the flowering period. At each sampling site, the top 20-30 cm of 20
127 randomly selected inflorescences were collected and placed into clean labelled plastic bags and
128 kept on ice in a cooler during transport back to the laboratory. In the laboratory, the samples
129 were maintained at 4°C prior to processing, which was carried out within 24 hours of sample
130 collection.

131 **Estimates of *S. sclerotiorum* infestation.** Petals were assessed for *S. sclerotiorum* infestation by
132 qPCR analysis in both Exp. 1 and Exp. 2, as well as by the traditional petal plate test in Exp. 2.
133 The qPCR analysis was carried out as described by Ziesman et al. (2016). Briefly, 20 petals
134 were selected at random from the petals stored at -80°C for each sampling site and each sampling
135 date, with no more than one petal selected from each intact flower. Petal samples were
136 homogenized using a TissueLyser II (Qiagen, Toronto, ON, Canada) and a single 5-mm stainless
137 steel bead in a 2 ml locking tube. The DNA was isolated following the protocol of Liang et al.
138 (2013), and total DNA was measured with a NanoDrop 2000c spectrophotometer (Thermo
139 Scientific, Waltham, MA, USA). An exogenous control was added to the tissue samples prior to
140 DNA isolation and carried through the full analysis (Ziesman et al. 2016).

141 Standards were prepared as a 1:10 dilution series of pure *S. sclerotiorum* genomic DNA
142 ranging from 8 ng to 8.0×10^{-4} ng. Samples were analyzed in triplicates. Triplicates with a
143 standard deviation of the Cq value > 0.5 were reanalyzed b qPCR. If a single outlier occurred in
144 a triplicate and the remaining duplicates had near equal Cq values, the outlier was removed. Any
145 sample for which no *S. sclerotiorum* DNA could be detected, or which was outside the range of
146 the standard curve, was recorded as a zero. Results are expressed on a per petal basis by first
147 accounting for the dilution, then by dividing the estimate by 20. The qPCR-based estimates of

148 petal infestation were averaged for each field on each sampling date, and the standard deviation
149 of the mean was calculated for each field.

150 The petal plate test was conducted as described by Turkington et al. (1988). Briefly,
151 petals were plated on Petri dishes containing solid potato dextrose agar amended with 25 ppm
152 ampicillin and 25 ppm streptomycin and incubated for 4-5 days in darkness at room temperature
153 (approximately 20-24°C). The percentage of petals infected with *S. sclerotiorum* was determined
154 through visual assessment, and the petal infestation estimates were averaged for each field on
155 each sampling date.

156 **Sclerotinia stem rot assessment.** Sampling sites were revisited prior to swathing, in order to
157 assess Sclerotinia stem rot incidence and severity in the canola crops. Twenty randomly selected
158 plants were evaluated at each sampling site, for a total of 100 plants per field. The plants were
159 rated for stem rot of severity on a 0-5 scale as described by Kutcher and Wolf (2006). In Exp. 1,
160 disease incidence was defined as the percentage of plants that had any symptoms of stem rot on
161 any organ (i.e., rated as 1 or greater). In Exp. 2, disease incidence was adjusted to include only
162 plants with a severity rating of 2 or higher. Thus, the adjusted disease incidence values excluded
163 infections rated as 1 (pod infections), since upper canopy (i.e., pod) infections would be expected
164 to be a minor contributor to yield loss. Statistical analysis for Exp. 2 indicated that the adjusted
165 disease incidence values improved the R^2 values slightly, but did not change the significance of
166 the regression models. As a result, the calculations for disease incidence in Exp. 1 were not
167 adjusted.

168 **Statistical analysis.** The relationship between qPCR estimates of petal infestation and final stem
169 rot levels was determined with quadratic regression using proc reg in SAS (Statistical Analysis

170 System) software. Assumptions of regression were tested and no transformations were required.
171 Scatter plots indicated a non-linear regression. This was confirmed by the lower Akiake
172 information criterion (AIC) and Bayesian information criterion (BIC) values for quadratic
173 regression models over simple linear regression models, indicating a better fit. The AIC/BIC
174 values were calculated for both linear and quadratic models using proc mixed and the residual
175 maximum likelihood (REML) method. Regression analysis was conducted on the average qPCR
176 value for each field at each of the sampling dates individually. Analysis was carried out
177 separately for the three sampling years because of differences in the respective environmental
178 conditions. The year to year variation in the strength of the statistical relationship was used to
179 assess whether or not qPCR-based estimates of petal infestation could consistently indicate stem
180 rot risk without consideration of other factors. Regression models were considered significant
181 when the slopes of the independent variables and the overall models were significant at $P = 0.05$.

182 In Exp. 1, the data were further sorted in three different ways: by province, by average
183 regional annual precipitation, and by average annual regional precipitation from April to August
184 ('summer precipitation'). The aim of this analysis was to determine which method accounted for
185 the most variation and produced the stronger regression model. Precipitation data were obtained
186 from Environment Canada. The data were separated into three main groups based on average
187 regional annual precipitation: > 500 mm (Carman and Morden), between 400-500 mm (Brandon,
188 Lacombe and Edmonton), and < 400 mm (Melfort and Saskatoon). Summer precipitation was
189 averaged for the months of April through August and was used to separate the data into two main
190 groups: > 300mm (Morden, Edmonton, Lacombe, Brandon and Carman) and < 300mm (Melfort
191 and Saskatoon).

192

193 In Exp. 2, the relationship between Sclerotinia stem rot disease incidence and percent
194 petal infestation (PPI), determined with the plate test, was assessed by simple linear regression
195 using proc reg in SAS. Regression analysis with non-transformed disease incidence did not meet
196 the assumptions of regression. Disease incidence values were Arcsine transformed as discussed
197 by Turkington and Morrall (1993), and the resulting residuals more closely met the assumptions
198 of regression. Thus, arcsine transformed disease incidence (TDI) was used for all regressions
199 with percent petal infestation (PPI) values. Simple linear regression models were confirmed to
200 have lower AIC and BIC values than the quadratic regression models, indicating a better fit for
201 the linear models. As with the qPCR results, regression analysis was conducted separately for
202 each of the three sampling years. Regression models were considered significant when the slopes
203 of the independent variables and the overall models were significant at $P = 0.05$.

204 Correlation analysis with proc reg in SAS was used in Exp. 2 to determine the
205 relationship between the qPCR-based and petal plate test estimates of petal infestation level. In
206 all three years, there was a slight deviation from normality for at least one variable. As a result,
207 Spearman Rank Correlation was used.

208 **Results**

209 **Experiment 1.** Sclerotinia stem rot disease incidence and the qPCR estimates of petal infestation
210 for individual fields were variable over the sampling years and between locations (Table 1). In
211 both 2011 and 2012, the disease incidence ranged from 0 to 92% among the sampled fields, but
212 the mean disease incidence (MDI) for all fields, presented as MDI ± standard deviation, was
213 higher in 2012 (27.2% ± 29.5%) than in 2011 (11.9% ± 17.7%) (Figs. 1-3). The mean amount of
214 *S. sclerotiorum* DNA per petal for all fields, as measured by qPCR analysis, was highest at full
215 bloom in both 2011 (0.013 ng/petal ± 0.0018 ng/petal) and 2012 (0.068 ng/petal ± 0.18 ng/petal).

216 There was variation between sampling sites, years and individual fields for disease incidence and
217 the amount of *S. sclerotiorum* DNA per petal. When the data were separated by province, none
218 of the relationships between the qPCR results and disease incidence were significant at either
219 sampling date in 2011. The coefficient of variation (CV) for these regression models ranged
220 from 94.14 to 144.05. In 2012, however, the relationship between the early bloom qPCR results
221 and disease incidence for the fields in Alberta was found to be significant, with the early bloom
222 qPCR results accounting for 59.1% of the variation in disease incidence ($R^2 = 0.591$; $P = 0.0073$;
223 CV 42.694) (Fig. 4). The regression models for the Alberta fields at full bloom and for the
224 Saskatchewan and Manitoba fields at both sampling dates were not significant, with CVs ranging
225 from 51.05 to 111.67.

226 When the data from Exp. 1 were separated by average regional annual precipitation, the
227 relationships were not significant for the locations with >400 mm precipitation in 2011, and for
228 none of the groups in 2012. The CV for these models ranged from 51.046 to 159.16. The
229 regression analysis for locations with >500 mm of annual precipitation at the late bloom
230 sampling date in 2011 could not be conducted using a quadratic model because of the large
231 number of fields with a disease incidence of 0%. For this group of locations, the simple linear
232 regression was analyzed and found not to be significant. The relationship between late bloom
233 qPCR results and disease incidence for fields with average regional annual precipitation was
234 found to be significant, with the late bloom qPCR results accounting for 57.6% of the variation
235 in disease incidence ($R^2 = 0.576$; $P = 0.0356$; $CV = 95.559$) (Fig. 5).

236 When the data were separated by average regional summer precipitation, none of the
237 regression models were significant in 2011. In 2012, the regression models for the group of
238 locations with average summer precipitation levels > 300 mm were significant at both early

239 bloom ($R^2 = 0.263$; P = 0.035; CV = 95.116) and late bloom ($R^2 = 0.244$; P = 0.0459; CV
240 96.301) (Fig. 6). The CV values for the non-significant regression models ranged from 95.56 to
241 129.022 in 2011 and from 84.295 to 99.600 in 2012.

242 **Experiment 2.** Sclerotinia stem rot incidence, PPI and the estimates of petal infestation obtained
243 by qPCR analysis were variable over the three years of the study (Table 2). Mean stem rot
244 incidence across all fields, presented as the mean ± standard deviation, was lower in 2011 (7.8%
245 ± 6.6%) than in 2012 and 2013 (64% ± 23.2% and 39.36% ± 24.8%, respectively). For both
246 sampling dates, estimates of petal infestation determined by qPCR analysis also were lowest in
247 2011 (early bloom: 0.005 ng/petal ± 0.005 ng/petal; full bloom: 0.017 ng/petal ± 0.013 ng/petal)
248 (Fig. 7). On a field level, qPCR estimates of petal infestation at full bloom were higher for all 9
249 fields in 2011, for five of 10 fields in 2012, and for 5 of 11 fields in 2013. As in Exp. 1, there
250 was a high amount of variation in the levels of petal infestation, as determined by qPCR analysis,
251 in samples from some of the fields.

252 Mean PPI, as assessed by the petal plate test of Turkington et al. (1988), was similar
253 across both sampling dates in 2011 (early bloom: 35.9% ± 22.3%; full bloom: 40.1% ± 6.84%)
254 and 2012 (early bloom: 34.3% ± 17.1%; full bloom: 41.2% ± 15.1%) (Table 2). In contrast, PPI
255 was generally higher in 2013 (early bloom: 43.85% ± 18.2%; full bloom: 51.0% ± 18.5%). A
256 comparison of petal infestation levels, as determined by qPCR analysis or the petal plate test,
257 found similar results in 2011 and 2012: both methods indicated that petal infestation levels were
258 lower at early bloom than at late bloom. In 2013, however, the different methods yielded
259 different results. Petal infestation was found to be lower at early bloom when evaluated by the
260 petal plate test, and lower at late bloom when evaluated by qPCR analysis.

261 **Relationship between qPCR-based petal infestation estimates and disease incidence.** In each
262 year of Exp. 2, three non-linear regression models were analyzed to determine the relationship
263 between qPCR estimates of petal infestation and final stem rot disease incidence (Table 3). In all
264 three years, disease incidence increased as the amount of *S. sclerotiorum* DNA per canola petal
265 increased (Fig. 8). However, the strength of the relationship varied with year and was not always
266 significant. In all years, there was a significant amount of variation in both the final disease
267 incidence and the petal infestation estimates in the fields sampled. In 2011, none of the three
268 regression models were significant at P = 0.05. The field with the highest stem rot incidence also
269 had a very low level of petal infestation (as determined by qPCR) and appeared to be an outlier.
270 If this field is removed from the analysis, the relationship between the qPCR results at full bloom
271 and disease incidence becomes significant ($R^2 = 0.9194$; P = 0.0018; CV = 27.21). In 2012,
272 there was no significant statistical relationship between the qPCR estimates and disease
273 incidence at early bloom. However, there was a significant relationship between disease
274 incidence and qPCR estimates at full bloom ($R^2 = 0.6904$; P = 0:0165; CV = 22.91).

275 In 2013, there was no significant statistical relationship at either early or full bloom when
276 all fields were included in the analysis. When a single outlier was removed, the relationship
277 between the qPCR estimates at full bloom and stem rot disease incidence became significant
278 ($R^2 = 0.6047$; P = 0.039; CV = 40.79). In 2013, there was a wider range of seeding dates (May 9-
279 May 20), with some canola fields seeded later than in 2011 and 2012. When only those fields
280 seeded on or prior to May 15 were included in the analysis, the regression became highly
281 significant at full bloom ($R^2 = 0.9204$; P = value 0.0063; CV = 20.83). The amount of variation
282 in the data set was high in all years, as indicated by the coefficients of variation, which ranged
283 from 37.22 to 89.056 for all non-significant regressions.

284 **Relationship between PPI and disease incidence.** In 2011 and 2013, there was not a significant
285 statistical linear relationship between PPI and disease incidence at either early bloom or late
286 bloom, even in 2013 when only early seeded fields were included in the analysis. In 2012, the
287 relationship was significant at full bloom ($R^2 = 0.682$; $P = 0.003$; CV = 16.189).

288 **Relationship between PPI and qPCR estimates of petal infestation.** Correlation analysis was
289 used to determine the relationship between PPI and the amount of *S. sclerotiorum* DNA per
290 canola petal as determined by qPCR analysis. In 2011, the only significant correlation was at
291 early bloom ($R^2 = 0.71$; $P = 0.03$). In 2012, the correlation was not significant at early bloom but
292 was significant at full bloom ($R^2 = 0.80$; $P = 0.0056$). In 2013, there was no significant
293 correlation at either early or full bloom.

294 **Discussion:**

295 In Canada, risk assessment tools for Sclerotinia stem rot of canola have focussed on a
296 variety of factors that are known to influence the incidence of the disease in the field. However,
297 only one of these risk assessment tools, the petal test developed by Morrall and Thompson
298 (1991), involves a quantitative estimation of the amount of inoculum present during a critical
299 period of the disease cycle. The petal test provides a measure of petal infestation, which has been
300 demonstrated to be strongly related to Sclerotinia stem rot incidence at the end of the growing
301 season. Fungicides are applied for control of stem rot during flowering. An estimation of petal
302 infestation should provide an indication of disease risk during the period over which fungicide
303 spray decisions are being made. However, the 3-5 day incubation period associated with the
304 traditional petal testing procedure causes a delay in the results, which may only become available
305 after the optimal time for fungicide application has passed (Wallenhammar et al. 2007). In

306 contrast, an evaluation of field collected canola petals by quantitative PCR analysis can provide
307 an estimate of inoculum pressure within a single day.

308 In all fields and locations included in this study, there was variation in both the amount of
309 *S. sclerotiorum* DNA detected by qPCR analysis and in the final disease incidence. There also
310 was significant year to year variation in these parameters, most likely reflecting differences in
311 environmental conditions that in turn influence carpogenic germination of the sclerotia and the
312 timing of ascospore release. Similarly, the differences observed between fields could also be a
313 result of regional differences in environmental conditions, but they may also reflect differences
314 in crop stand density, seeding date, seeding rate and crop history, all of which can influence stem
315 rot development (Turkington and Morrall 1993; Jurke and Fernando 2008; Twengstrom et al.
316 1998). In addition to field to field variation, variation across sampling sites within particular
317 fields also was observed. This variation likely reflected differences in crop stand and
318 microclimate at different points within the same field. Five sites were sampled per field, since
319 this was reported to be an accurate sampling size for evaluating the incidence of petal infestation
320 (Turkington et al. 1988). The large amount of variation observed in this study, however,
321 indicates that a larger number of sampling sites may be needed to provide a more reliable
322 estimate of petal infestation in a particular field.

323 The amount of *S. sclerotiorum* DNA quantified per canola petal varied across the
324 flowering season. These findings are consistent with previous reports that found that inoculum
325 pressure is not consistent across the flowering period or between fields (Almquist and
326 Wallenhammar 2015; Turkington and Morrall 1993). Differences in the level of infestation over
327 the growing season will influence the strength of the statistical relationship between
328 quantifications of petal infestation and stem rot incidence when evaluated across several fields.

329 Monitoring changes in petal infestation over the flowering period may serve to identify the best
330 timing for fungicide application in a particular field, but such an approach would have to be
331 balanced by cost and other practical considerations. Nonetheless, closer monitoring of infestation
332 levels on a temporal scale could prove useful in better understanding the epidemiology of
333 Sclerotinia stem rot of canola.

334 The relationship between PPI as determined by the petal plate test and petal infestation as
335 assessed by qPCR analysis was not always linear. An analysis of correlation indicated a
336 significant amount of shared variation between the two methods only at early bloom in 2011 and
337 at full bloom at 2012. Similarly, Almquist and Wallenhammar (2015) reported no correlation
338 between the results of the petal plate test and a qPCR-based *S. sclerotiorum* detection method.
339 There are several possible explanations for these differences. For example, the presence of a few
340 highly infected canola petals in a sample would be reflected in a higher concentration of *S.*
341 *sclerotiorum* DNA in the qPCR analysis, but would not be reflected in the petal plate test results
342 (Almquist and Wallenhammar 2015). Furthermore, while both the number of ascospores and the
343 amount of mycelium present in a sample can be measured by qPCR analysis, the petal plate test
344 can only indicate whether or not a petal is infected. Finally, when levels of *S. sclerotiorum*
345 infestation are low on infected petals, other fungal species (e.g. *Rhizopus* spp., *Mucor* spp.,
346 *Trichoderma* spp.) that are present on the petal tissue may outgrow colonies of *S. sclerotiorium,*
347 thus masking the presence of *S. sclerotiorum*. Given the increased sensitivity of the qPCR-based
348 method, detection and direct measurement of *S. sclerotiorum* by qPCR analysis are more likely
349 to be influenced by the environment in which those petals were produced.

350 The strength of the statistical relationship between qPCR-based measurement of petal
351 infestation and final stem rot incidence in the field is critical to assessing the suitability of the

352 former for predicting the risk of disease. In Exp. 1, the strength of the relationship between the
353 qPCR results and disease incidence across the Prairies was variable across the three years of the
354 study. Moreover, when the data were analyzed separately based on the amount of annual or
355 summer precipitation in a particular region, the strength of the relationship was not increased. It
356 is important to note that, with the exception of the fields in the Edmonton region, there was no
357 information regarding fungicide application in the fields included in Exp. 1. This could have had
358 a large impact on any potential correlation. For instance, a canola field in which there was heavy
359 petal infestation may have been treated with fungicide, preventing or greatly reducing stem rot
360 development. As such, the results of Exp. 1 must be treated with caution. In Exp. 2, all sampling
361 was carried out in check-strips that were not treated with fungicide. Therefore, the results of this
362 experiment may provide a better indication of the true relationship between disease incidence
363 and petal infestation as measured by qPCR analysis. Indeed, in Exp. 2 the relationship between
364 these two parameters was stronger, with stem rot incidence generally found to be greater in fields
365 where petal infestation (*S. sclerotiorum* DNA per canola petal) also was higher. Despite this
366 stronger relationship, however, the correlations were not always significant. This highlights the
367 possible influence of other factors in stem rot development.

368 There was a wider range of seeding dates in 2013 than in 2011 or 2012, and this was
369 found to influence the statistical relationship in Exp. 2. When only the early seeded fields were
370 included in the analysis, the relationship was significant at full bloom, which was consistent with
371 the results in 2012. These findings indicate that the relationship between disease incidence and
372 the amount of *S. sclerotiorum* DNA per canola petal is strongest at full bloom and/or in years
373 when disease pressure is high. The variation across the sampling years also indicates that
374 measures of environmental conditions may need to be included in the model using multiple

375 regression analysis, in order to fully account for year to year variation in weather. More broadly,
376 comparison of the results obtained in Exp. 1 and Exp. 2 suggests that qPCR-based assessments
377 of stem rot risk are more reliable on a smaller regional scale, and that differences in environment
378 between locations should be considered when setting risk assessment thresholds.

379 The qPCR assay described in this study may serve as the basis for a risk assessment
380 system, as well as representing a useful tool for the study of the epidemiology of Sclerotinia stem
381 rot of canola. It can quantify the level of petal infestation, a key stage in the Sclerotinia stem rot
382 disease cycle, thereby providing a measure of disease risk when timely fungicide application
383 decisions need to be made. It is important to emphasize, however, that a forecasting system
384 based on qPCR quantification of petal infestation should be linked to environmental conditions,
385 as well as to cropping history, seeding date and crop canopy conditions, which may influence
386 stem rot development and the need to spray a fungicide.

387 **Acknowledgements**

388 We thank the Alberta Crop Industry Development Fund, the Alberta Canola Producers
389 Commission, the Western Grains Research Foundation, and Agriculture and Agri-Food Canada
390 and the Canola Council of Canada (via the Growing Forward program) for financial support. We
391 would also like to thank Colleen Kirkham (Agriculture and Agri-Food Canada), two WISEST
392 students (Women in Scholarship Engineering Science and Technology, University of Alberta),
393 and three summer students (University of Alberta) for their technical assistance, as well as Emile
394 deMilliano, Ryan Adams (Crop Production Services) and various farmers for their helpful
395 collaboration.

396

397 **References:**

398 Abawi, G.S., and Grogan R.G. 1979. Epidemiology of diseases caused by *Sclerotinia* species.
399 Phytopathology 69:899-904.

400 Aboukhaddour, R., Kim, Y.M., and Strelkov, S.E. 2012. RNA-mediated gene silencing of ToxB
401 in *Pyrenophora tritis-repentis*. Mol. Plant Pathol. 13:318-326.

402 Almquist, C., and Wallenhammar, A.C. 2015. Monitoring of plant and airborne inoculum of
403 *Sclerotinia sclerotiorum* in spring oilseed rape using real-time PCR. Plant Pathology 64: 109-
404 118.

405 Bardin, S.D., and Wuang, H.C. 2001. Research on biology and control of *Sclerotinia* disases in
406 Canada. Can. J. Plant Path. 23: 88-98.

407 Bolton, M.D., Bart, T., Thomma, P.H.J., and Nelson, B.D. 2006. *Sclerotinia sclerotiorum* (Lib.)
408 de Bary: biology and molecular traits of a cosmopolitan pathogen. Mol. Plant Pathol. 7:1-16.

409 del Río, L.E., Bradley, C.A., Hensen, R.A., Endres, G.J., Hanson, B.K., Mckay, K., Halvorson,
410 M., Porter, P.M., Le Gare, D.G., and Lamey, H.A. 2007. Impact of Sclerotinia stem rot on yield
411 of canola. Plant Dis. 91: 191-194.

412 Environment Canada. 2015. 1981-2010 Climate normals & averages. Found online
413 at: http://climate.weather.gc.ca/climate_normals/index_e.html.

414 Freeman, J., Ward, E., Calderon, C., and McCartney, A. 2002. A polymerase chain reaction
415 (PCR) assay for the detection of inoculum of *Sclerotinia sclerotiorum*. European Journal of Plant
416 Pathology 108:877-86.

417 Gugel. R.K. 1986. Inoculum-disease relationship in sclerotinia stem rot of rapeseed in
418 Saskatchewan. Can. J. Plant Pathol. 8(1):89-96.

419 Jamaux, I., Gelie, B. and Lamarque, C. 1995. Early stages of infection of rapeseed petals and
420 leaves by *Sclerotinia sclerotiorum* reveal by scanning electron microscopy. Plant Pathology. 44:
421 22-30.

422 Jurke, C.J. and Fernando, W.G.D. 2008. Effects of seeding rate and plant density on sclerotinia
423 stem rot incidence in canola. Arch. Phytopathol. Plant Protect. 41: 142-155.

424 Kutcher, H. R. and Wolf, T. M. 2006. Low drift fungicide application technology for sclerotinia
425 stem rot of canola. Crop Protect. 25: 640-646.

426 Lamari, L., and Strelkov, S.E. 2010. The wheat/*Pyrenophora tritici-repentis* interaction: progress
427 towards an understanding of tan spot disease. Can. J. Plant Pathol. 32:4-10.

428 Liang, Y., Yajima, W., Davis, M.R., Kav, N.N.V., and Strelkov, S.E. 2013. Disruption of a gene
429 encoding a hypothetical secreted protein for *Sclerotinia sclerotiorum* reduces its virulence on
430 canola (*Brassica napus*). Can. J. Plant Pathol. 35:46-55.

431 Ma, Z., Luo, Y., and Michailides, T.J. 2003. Nested PCR assays for detection of *manilinia*
432 *fruiticola* in stone fruit orchard and *Botryosphaeria dothidea* from pistachios in California. J.
433 Phytoptahol. 151:312-322.

434 McLaren, D.L., Conner, R.L., Platford, R.G., Lamb, J.L., Lamey, H.A. and Kutcher, H.R. 2004.
435 Predicting diseases caused by *Sclerotinia sclerotiorum* on canola and bean – a western Canadian
436 perspective. Can. J. Plant Pathol. 26: 489-497.

437 Morrall, R.A.A. and Thompson, J.R. 1991. Petal test manual for *Sclerotinia* in canola. University
438 of Saskatchewan, Saskatoon, Saskatchewan, Canada.

439 Morrall, R.A.A., and Dueck, J. 1982. Epidemiology of Sclerotinia stem rot of rapeseed in
440 Saskatchewan. Can. J. Plant Pathol. 4:161-168.

441 Nakayachiki, H., Hanada, S., Nguyen, B.Q., Kadotani, N., Tosa, Y., and Mayama, S. 2005. RNA
442 silencing as a tool for exploring gene function in ascomycete fungi. Fungal Genet. Biol. 42:275-
443 283.

444 Parker, M.L., McDonald, M.R., and Boland, G.J. 2014. Evaluation of air sampling and detection
445 methods to quantify airborne ascospores of *Sclerotinia sclerotiorum*. Plant Dis. 98:32-42.

446 Purdy, L. H. 1979. *Sclerotinia sclerotiorum* History, diseases and symptomology, host range,
447 geographic distribution and impact. Phytopathology 69: 857-880.

448 Rogers, S. L., Atkins, S.D., and West, J.S. 2008. Detection and quantification of airborne
449 inoculum of *Sclerotinia sclerotiorum* using quantitative PCR. Plant Pathol. 58: 324-331.

450 Schwartz, H.F., and Steadman, J.R. 1978. Factors affecting sclerotium populations of, and
451 apothecium production by *Sclerotinia sclerotiorum*. Phytopathology. 68:383-388.

452 Smith, C.J., and Osborn, A.M. 2008. Advantage and limitations of quantitative PCR (Q-PCR)-
453 based approaches in microbial ecology. FEMS Microbiol. Ecol. 67: 6-20.

454 Turkington, T.K., Morrall, R.A.A. and Baker, R.J. 1988. Sample size in relation to forecasting
455 Sclerotinia stem rot of canola. Can. J. Plant Pathol. 10:159-165

456 Turkington, T.K., Morrall, R.A.A. and Gugel, R.K. 1990. Use of petal infestation to forecast
457 Sclerotinia stem rot of canola: evaluation of early bloom sampling, 1985-1990. Can. J. Plant
458 Pathol. 13(1): 50-59.

459 Turkington, T.K., and Morrall, R.A.A. 1993. Use of petal infestation to forecast Sclerotinia stem
460 rot of canola: The influence of inoculum variation over the flowering period and canopy density.
461 Phytopathology 83:682-689.

462 Twengstrom, E., Sigvald, R., Svensson, C., and Yuen J. 1998. Forecasting sclerotinia stem rot in
463 spring oilseed rape. Crop. Prot. 17:405-411

464 Wallenhammar, A,-C., Redner, A., and Sjöberg, A. 2007. Development of methods improving
465 precision of risk assessment of Sclerotinia stem rot in oilseed rape. In: Fu, T., and Guan, C., eds.
466 *Proceedings of the 12th International Rapeseed Congrss, Wuhan China, March 2007*. Monmouth
467 Junction NJ, USA: Science Press. 112-115.

468 Willets, H.J. and Wong, J, A. –L. 1980. The biology of *Sclerotinia sclerotiorum*, *S. trifoliorum*
469 and *S. minor* with emphasis on specific nomenclature. Botanical Rev. 46:101-165.

470 Wu, B.M., and Werner, C.P. 2012. Infection routes for *Sclerotinia sclerotiorum* in apetalous and
471 fully petalled winter oilseed rape. Plant Pathol. 61:730-738.

472 Ziesman, B.R., Turkington, T.K., Basu, U., and Strelkov, S.E. 2016. A quantitative PCR system
473 for measuring *Sclerotinia sclerotiorum* in canola (*Brassica napus*). Plant Dis. (Accepted 13
474 December 2015). DOI: 10.1094/PDIS-05-15-0605-RE

475 **Table 1.** Stem rot disease incidence and amount of *S. sclerotiorum* DNA per canola petal, as
476 determined by qPCR analysis, in canola fields in Alberta, Saskatchewan and Manitoba
477 (Experiment 1).

Location	Stem rot incidence	2011			2012		
		Mean Disease Incidence (MDI) (%)	Mean qPCR estimates (ng of *S. sclerotiorum* DNA per petal)		MDI (%)	Mean qPCR estimates (ng of *S. sclerotiorum* DNA per petal)	
			EB	FB		EB	FB
Edmonton, AB	Average	8.67	0.005	0.017	64.8	0.012	0.02
	Minimum	1.00	0	0.003	29	0.006	0.001
	Maximum	21.00	0.013	0.038	92	0.034	0.080
Lacombe, AB	Average	11	0.001	<0.001	15.5	0.001	0.016
	Minimum	0	0	0	0	0	0.001
	Maximum	40	0.002	0.001	35	0.003	0.475
Melfort, SK	Average	28.860	0.012	0.025	1.8	0.037	0.030
	Minimum	4	0.001	0.005	0	0.002	0.001
	Maximum	92	0.017	0.047	4	0.087	0.130
Saskatoon, SK	Average	6	0.001	0.009	34.4	0.046	0.382
	Minimum	3	0	0.004	26	0.004	0.017
	Maximum	11	0.004	0.018	38	0.190	0.893
Brandon, MB	Average	0	0.002	0.012	2.4	0.007	0.004
	Minimum	0	0	0	0	0.002	0.002
	Maximum	0	0.006	0.082	11	0.016	0.006
Carman, MB	Average	10.667	0.001	0	5	0.005	0.001
	Minimum	5	0	0	1	0.004	0
	Maximum	20	0.003	0	11	0.006	0.001
Morden, MB	Average	12	0.003	0.001	14.5	0.008	0.004
	Minimum	1	0.001	0	11	0.004	0.001
	Maximum	21	0.006	0.002	18	0.011	0.007
All fields	Average	11.89	0.005	0.013	27.2	0.017	0.068
	Minimum	0	0	0	0	0	0
	Maximum	92	0.017	0.082	92	0.190	0.893

478
479

480 **Table 2.** Stem rot disease incidence and severity, and the amount of *S. sclerotiorum* DNA per

481 canola petal, as determined by qPCR analysis and the traditional petal plate test, in canola fields

482 in central Alberta (Experiment 2).

Year	Values	Mean disease incidence (%)	Mean disease severity	Mean qPCR estimates (ng of *S. sclerotiorum* DNA per petal)		Mean percent petal infestation (%) (traditional plate test)	
				Early bloom	Full bloom	Early bloom	Full bloom
2011	Average	7.78	0.340	0.005	0.017	35.9	40.11
	Minimum	1	0.020	0	0.003	13	33.5
	Maximum	20	0.79	0.013	0.038	68.5	52
2012	Average	64	2.75	0.012	0.020	34.3	41.2
	Minimum	29	1.18	0.006	0.003	21	19
	Maximum	92	3.97	0.034	0.080	74.5	63.5
2013	Average	39.64	1.54	0.081	0.051	43.9	51
	Minimum	12	0.34	0.006	0.014	18.0	25.4
	Maximum	88	3.61	0.259	0.181	73.1	79

483

484

485 **Table 3.** Significant regression models for the relationship between amount of *S. sclerotiorum*

486 DNA per canola petal, as determined by qPCR analysis, with stem rot incidence and severity at

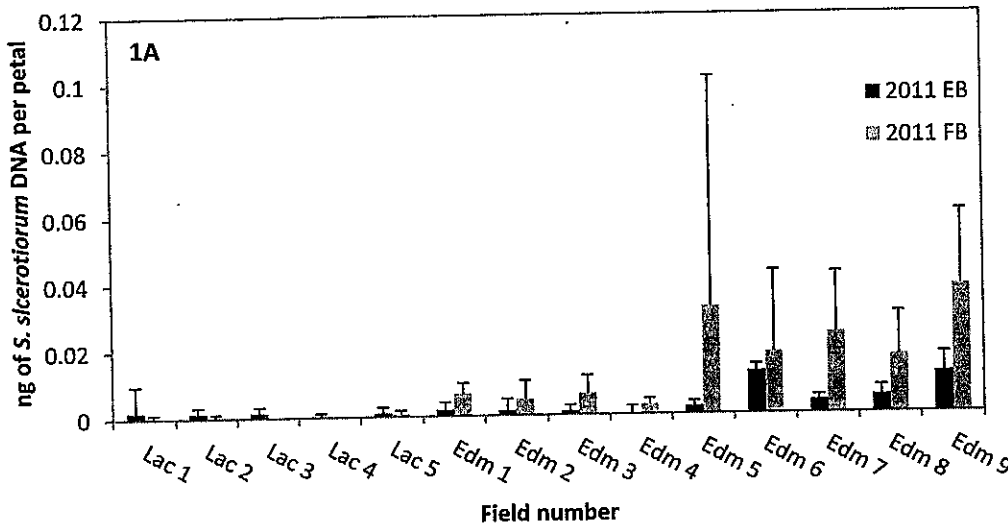
487 early bloom (EB) and full bloom (FB) in Experiment 2.

Year	Dependent Variable	Independent variables	Coefficient of determination (R^2)	Model Significance (p-value)	Coefficient of variation
2012	DI	MFB_q MFB_qsq	0.602	0.017	22.91
2012	DI	MFBPPI	0.682	0.003	16.189
2013ES	DI	MFB_q MFB_qsq	0.920	0.006	20.834

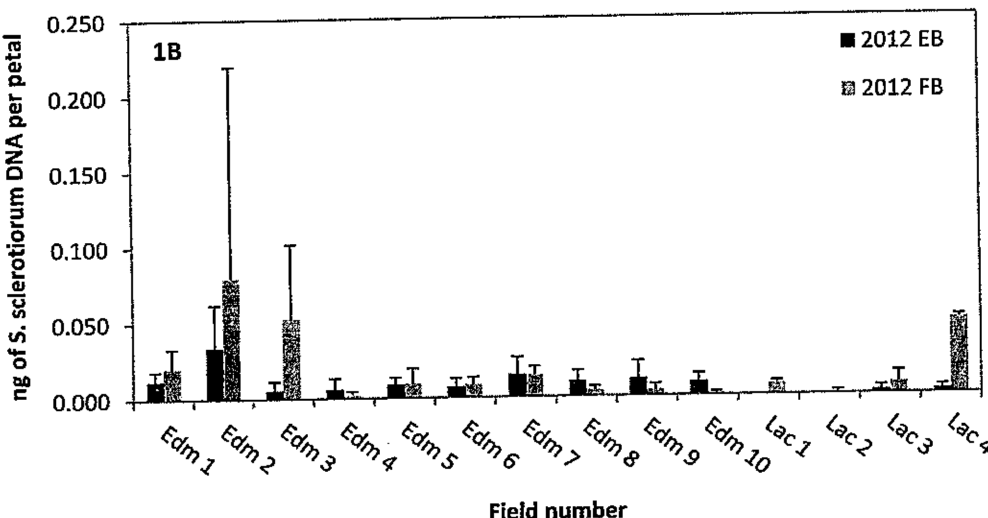
488 *2013ES = includes only the early seeded fields that were seeded on or before May 15th; MFB_q
489 = Mean qPCR estimates of petal infestation at full bloom; MFB_qsq = Mean qPCR estimate of petal
490 infestation at full bloom squared; MFBPPI = Mean percent petal infestation at full bloom.

491

492



493



494

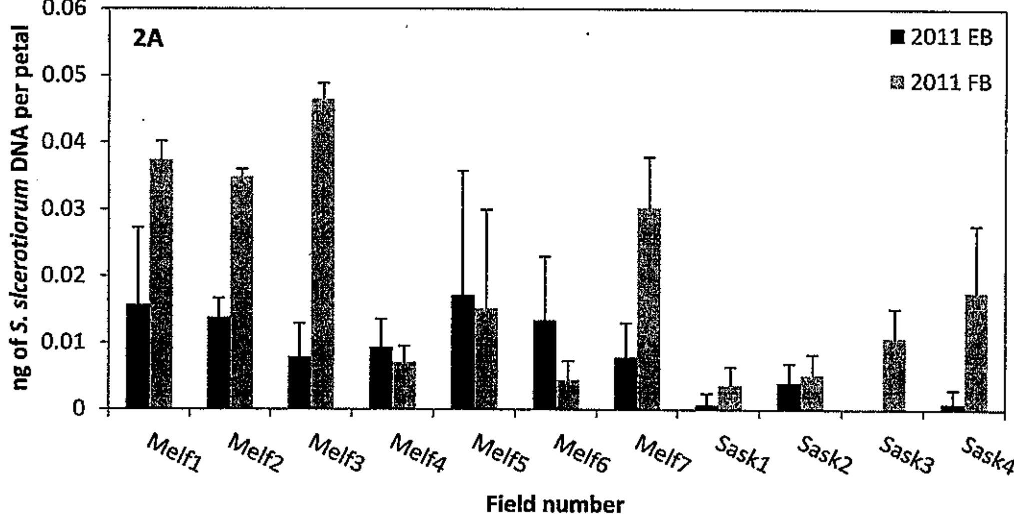
495 **Figure 1.** Amount of *S. sclerotiorum* DNA per canola petal in Alberta fields at early bloom (EB)

496 and at full bloom (FB) in Exp. 1 in (A) 2011 and (B) 2012. The error bars represent the standard

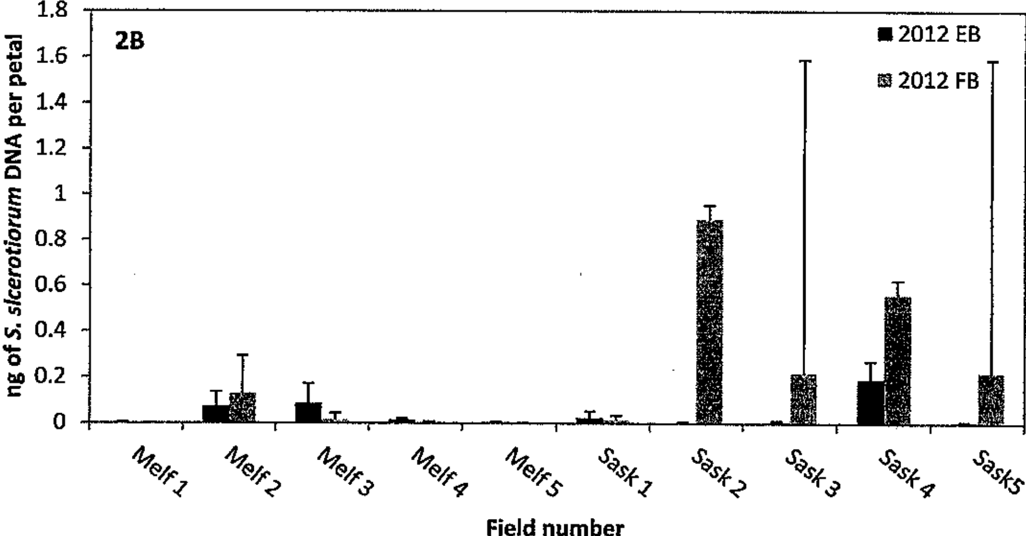
497 deviation of the mean for each field.

498

499



500



501

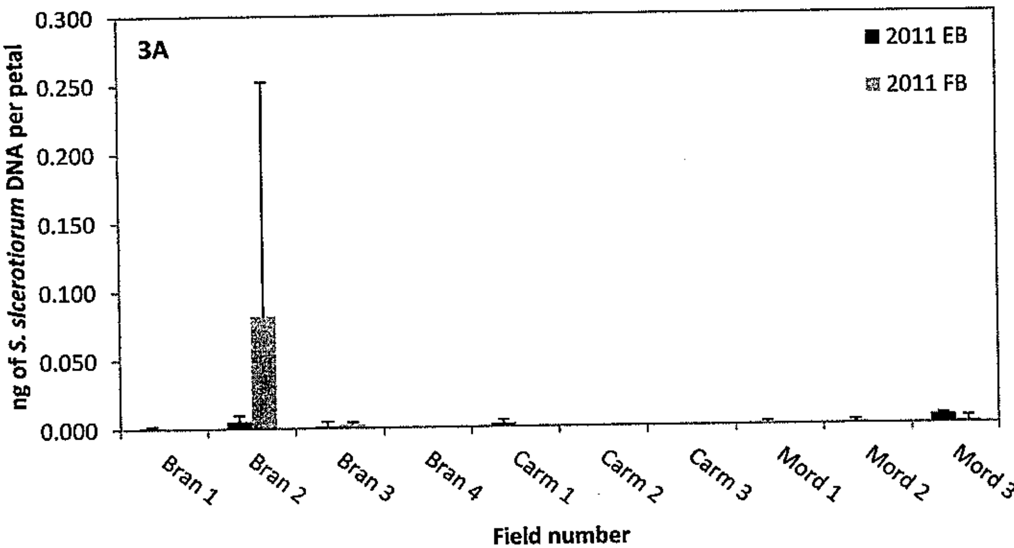
502 **Figure 2.** Amount of *S. sclerotiorum* DNA per canola petal in Saskatchewan fields at early

503 bloom (EB) and at full bloom (FB) in Exp. 1 in 2011 (A) and 2012 (B). The error bars represent

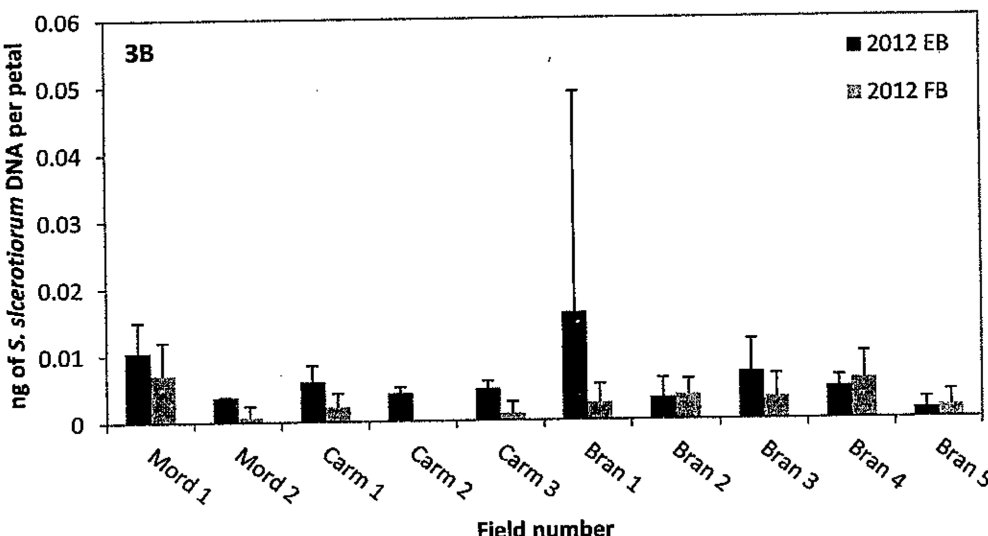
504 the standard deviation of the mean for each field.

505

506



507



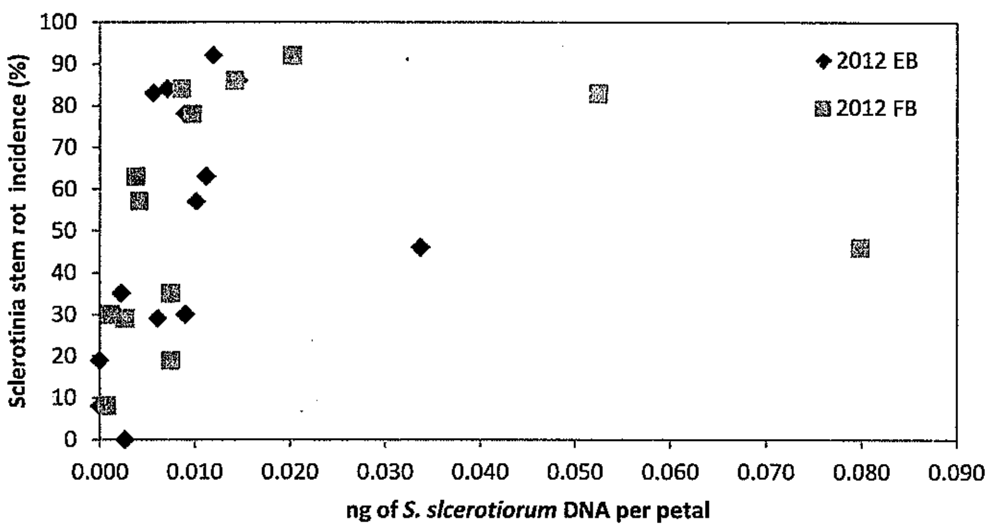
508

509 **Figure 3.** Amount of *S. sclerotiorum* DNA per canola petal in Manitoba fields at early bloom

510 (EB) and at full bloom (FB) in Exp. 1 in 2011 (A) and 2012 (B). The error bars represent the

511 standard deviation of the mean for each field.

512



513

514 **Figure 4.** Relationship between the amount of *S. sclerotiorum* DNA per canola petal as

515 determined by qPCR analysis and the incidence of Sclerotinia stem rot in the Alberta fields in

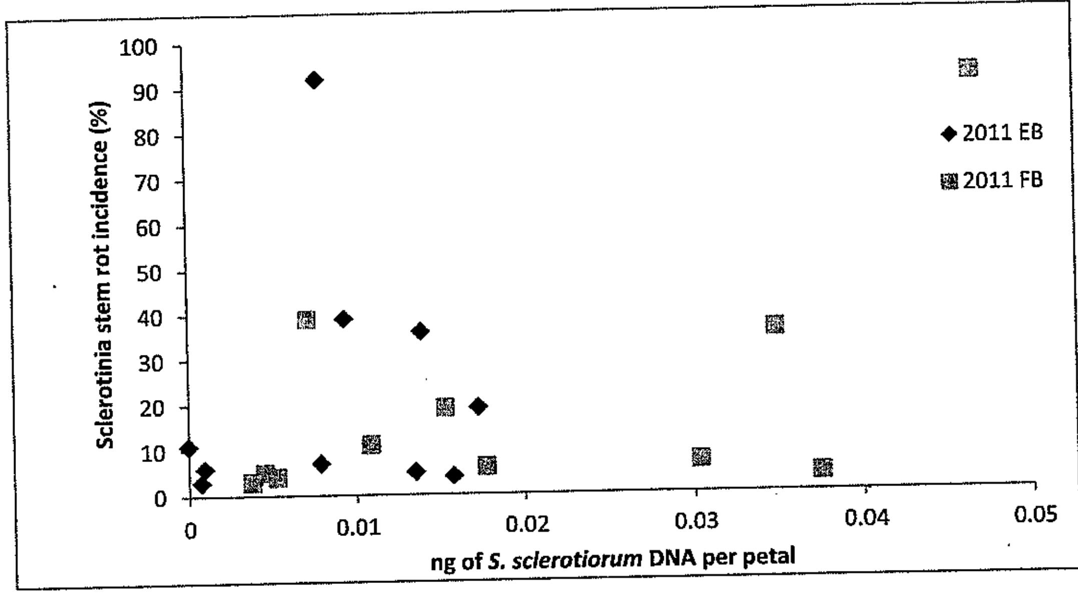
516 2012 for Exp. 1. The regression was significant at early bloom (EB) ($R^2 = 0.591$; $P = 0.007$), but

517 not at full bloom (FB) ($R^2 = 0.540$; $P = 0.106$). Relationships were not significant in the

518 Manitoba or Saskatchewan fields.

519

520



521

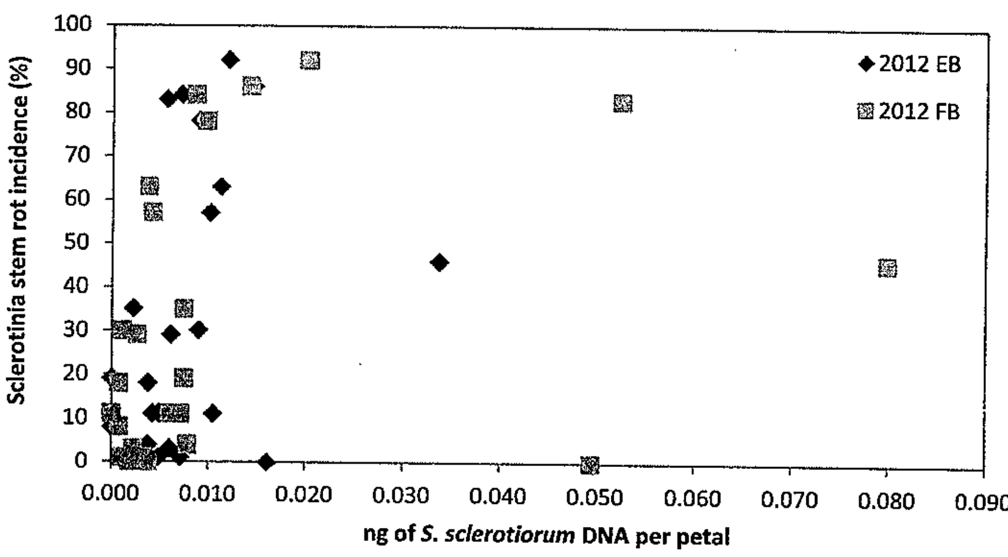
522 **Figure 5.** The relationship between the amount of *S. sclerotiorum* DNA per canola petal as

523 determined by qPCR analysis and the incidence of Sclerotinia stem rot in fields with average

524 annual precipitation < 400 mm in 2011 for Exp. 1. The regression was significant at full bloom

525 (FB) ($R^2 = 0.576$; $P = 0.032$), but not significant at early bloom (EB).

526



527

528 **Figure 6.** The relationship between the amount of *S. sclerotiorum* DNA per canola petal as

529 determined by qPCR analysis and the incidence of Sclerotinia stem rot in fields with an average

530 regional summer precipitation level of < 300 mm in 2012 for Exp. 1. The relationship was found

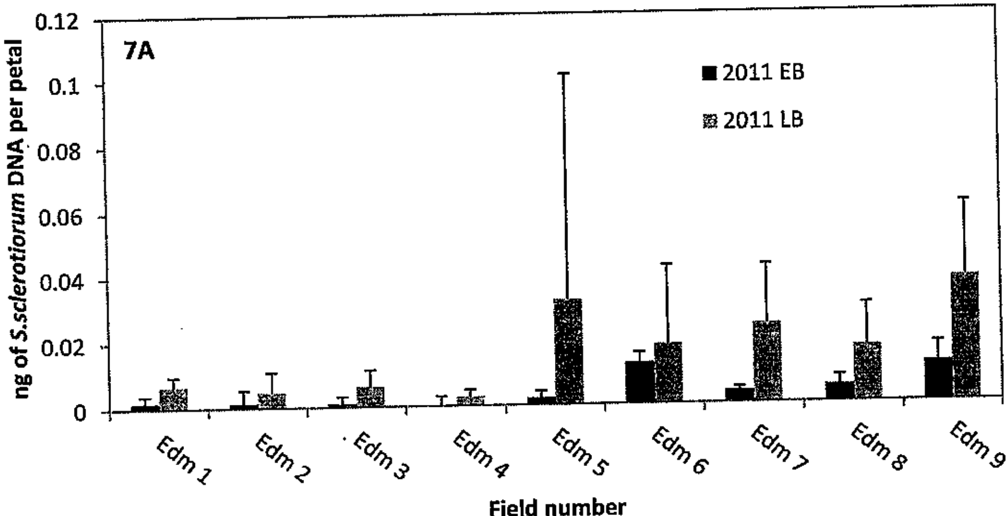
531 to be significant at both early bloom (EB) ($R^2 = 0.263$; $P = 0.035$) and full bloom (FB) ($R^2 =$

532 0.244; $P = 0.045$).

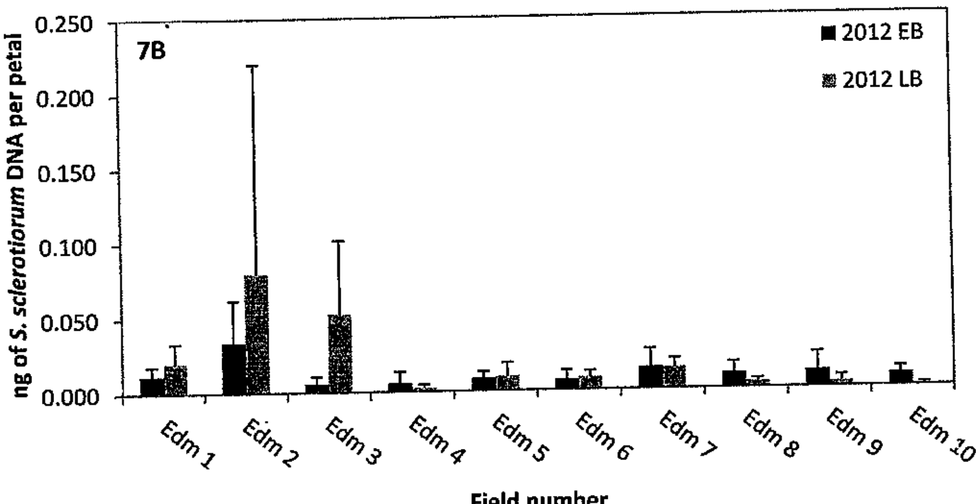
533

534

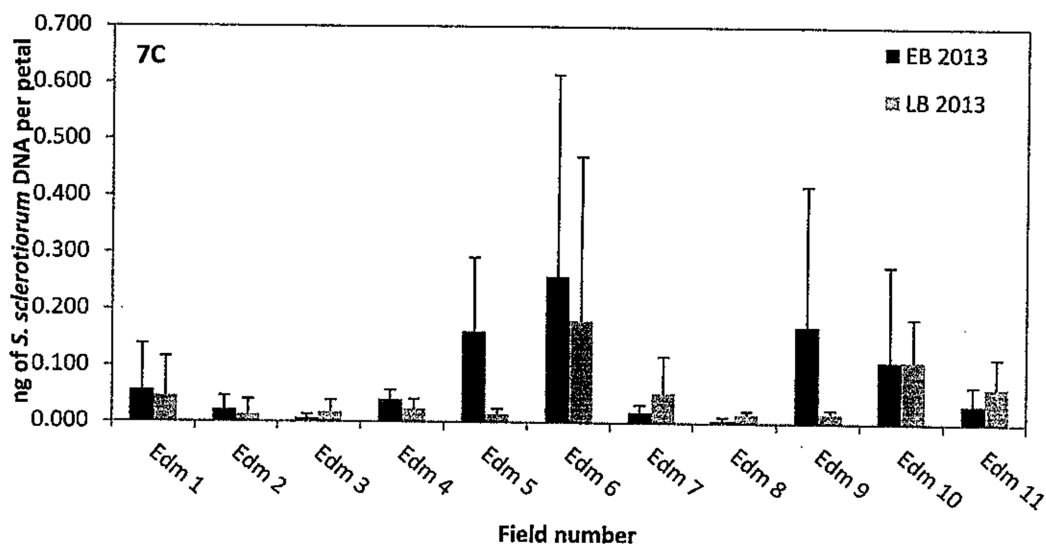
535



536



537



538

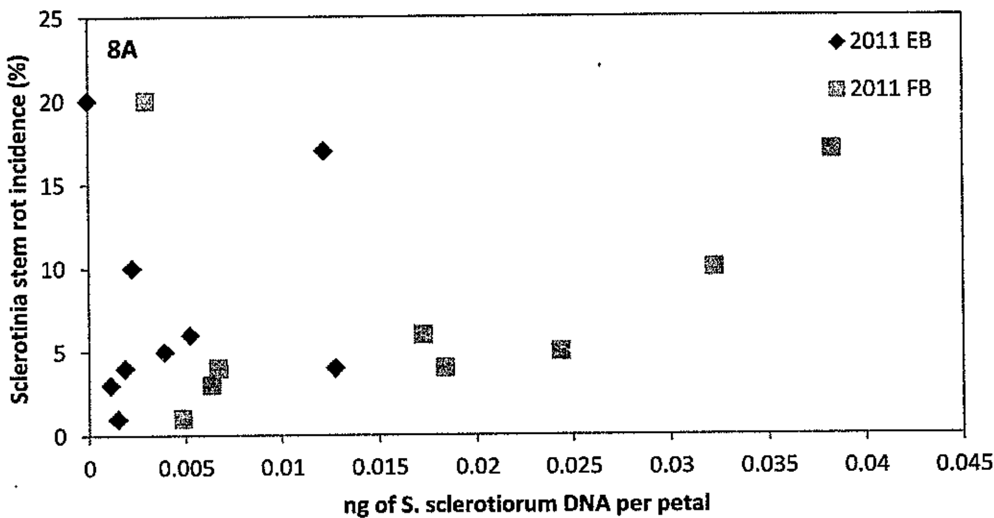
539 **Figure 7.** Amount of *S. sclerotiorum* DNA per canola petal for canola fields sampled in the

540 Edmonton region at early bloom (EB) and full bloom (FB) in (A) 2011, (B) 2012 and (C) 2013,

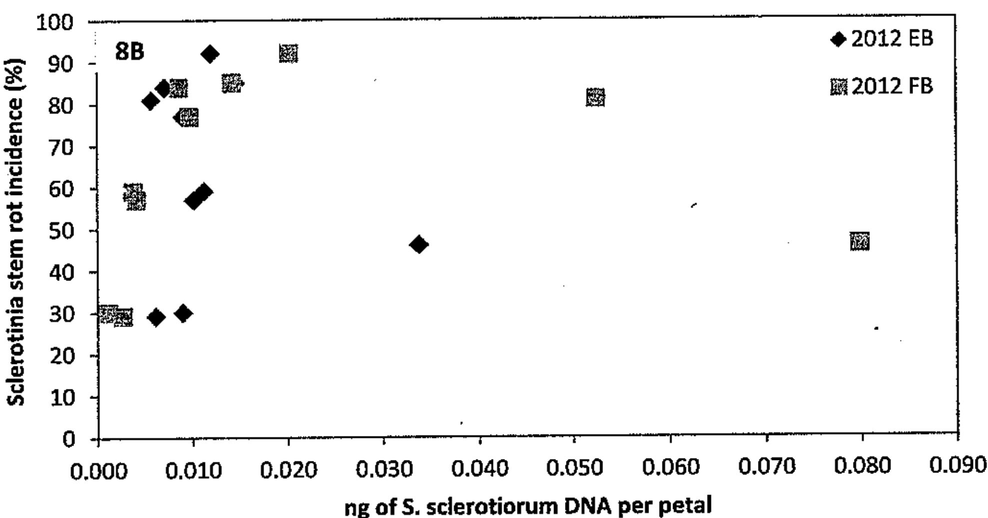
541 respectively, in Exp. 2. The error bars represent the standard deviation of the mean for each field.

542

543

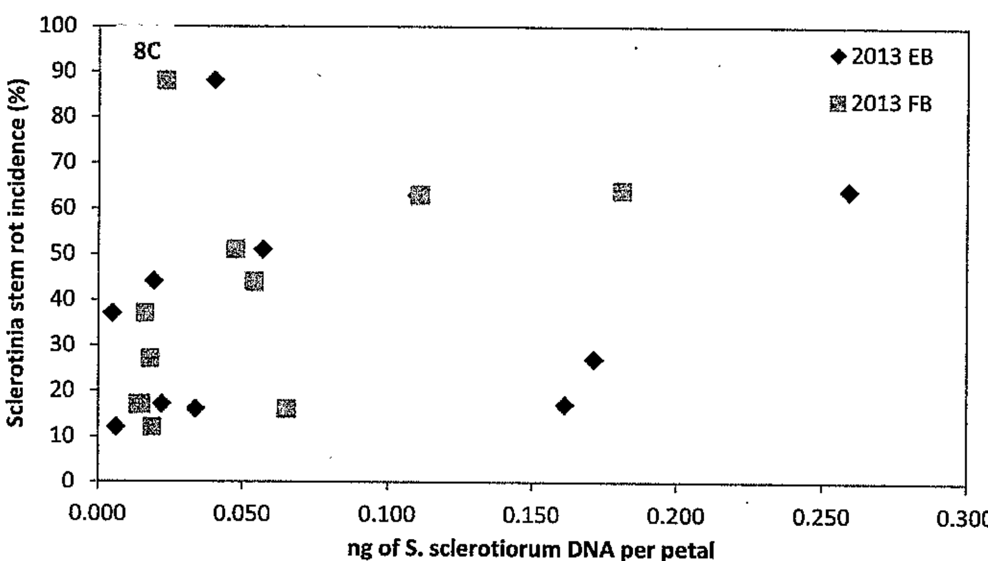


544



545

546



547 **Figure 8.** Relationship between the amount of *S. sclerotiorum* DNA per canola petal and the

548 incidence of Sclerotinia stem rot in Edmonton region fields in (A) 2011, (B) 2012 and (C) 2013,

549 respectively, in Exp. 2. When all fields were included in the regression analysis, only the full

550 bloom (FB) sampling in 2012 showed a significant relationship between the qPCR results and

551 final stem rot incidence ($R^2 = 0.602$; $P = 0.017$; CV = 22.91)

552

Date Received
For Administrative Use Only

Full Research Project Final Report

- This report must be a stand-alone report, *i.e.,* must be complete in and of itself. Scientific articles or other publications cannot be substituted for the report.
- One electronic copy and one signed original copy are to be forwarded to the lead funding agency on or before the due date as per the investment agreement.
- A detailed, signed income and expenditure statement incurred during the entire funding period of the project must be submitted along with this report. Revenues should be identified by funder, if applicable. Expenditures should be classified into the following categories: personnel; travel; capital assets; supplies; communication, dissemination and linkage; and overhead (if applicable).
- For any questions regarding the preparation and submission of this report, please contact ACIDF

Section A: Project overview

1. Project number: 2013F075R
2. Project title: Development of a rapid quantitative detection method for sclerotinia stem rot inoculum to aid disease risk assessments and fungicide spray decisions
3. Research team leader: Stephen Strelkov
4. Research team leader's organisation: University of Alberta
5. Project start date (yyyy/mm/dd): 2013/04/01
6. Project completion date (yyyy/mm/dd): 2015/12/31
7. Project final report date (yyyy/mm/dd): 2016/01/31

Section B: Non-technical summary (max 1 page)

Provide a summary of the project results which could be used by the funders for communication to industry stakeholders (*e.g.*, producers, processors, retailers, extension personnel, etc.) and/or the general public. This summary should give a brief background as to why the project was carried out, what were the principal outcomes and key messages, how these outcomes and key messages will advance the agricultural sector, how they will impact industry stakeholders and/or consumers, and what are the economic benefits for the industry.

Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum*, is an economically important disease of canola commonly managed by routine application of fungicides. Petal infestation is an important stage of the disease cycle in canola and has been the focus of previously developed Sclerotinia stem rot risk assessment methods. Quantitative (q)PCR analysis can provide a more rapid and

accurate assessment of petal infestation levels. Primers and a hydrolysis probe were designed to amplify a 70-bp region of a *S. sclerotiorum*-specific gene SS1G_00263. A hydrolysis probe-based qPCR assay was developed that had a detection limit of 8.0×10^{-4} ng of *S. sclerotiorum* DNA and only amplified *S. sclerotiorum* DNA. Evaluation of petals collected at five sampling points in each of 10 commercial canola fields on each of two sampling dates (corresponding to 20-30% bloom and 40-50% bloom) revealed infestation levels ranging from 0 to 3.3×10^{-1} ng *S. sclerotiorum* DNA per petal. The relationship between petal infestation levels and final stem rot incidence in canola was explored further in two additional studies. In the first study, conducted over 2 years, petal infestation was compared with disease incidence in 34-35 commercial canola fields distributed across Alberta, Saskatchewan and Manitoba. In the second study, these parameters were compared over 3 years in 9-11 fields located in central Alberta. In the fields sampled across the Prairies, no consistent relationship was observed between petal infestation and stem rot incidence at harvest. However, no information was available on whether or not these fields received a fungicide treatment, which may have reduced the ability to detect any correlations. In contrast, sampling points in the second study were located in fungicide-free check strips, and the incidence of stem rot was generally found to increase with increasing petal infestation. The strength of the relationship varied across the study years, and was strongest when canola petals were analyzed at full bloom and in years when disease pressure was high. The qPCR assay may serve as the basis for a risk assessment system, as well as representing a useful tool for the study of the epidemiology of Sclerotinia stem rot of canola. It can quantify the level of petal infestation, thereby providing a measure of disease risk when timely fungicide application decisions need to be made. It is important to emphasize, however, that a forecasting system based on qPCR quantification of petal infestation should be linked to environmental conditions, as well as cropping history, seeding date and crop canopy conditions, which may influence stem rot development and the need to spray a fungicide.

Section C: Project details

1. Project team (max ½ page)

Describe the contribution of each member of the R&D team to the functioning of the project. Also describe any changes to the team which occurred over the course of the project.

The project leader, Dr. Strelkov, guided the development of the quantitative (q)PCR-based methodology to detect and measure *Sclerotinia sclerotiorum* in canola tissues. The main collaborator, Dr. Turkington, contributed to the design and coordination of all of the field-related components of the research, including establishment of sampling sites, development of sample collection protocols, and sampling regimes. Barbara Ziesman is a graduate student who conducted the research as part of her studies. She developed methodologies, compiled data, and analyzed the results, receiving feedback, guidance and suggestions as needed from Drs. Strelkov and Turkington. The composition of the research team did not change over the course of the project.

2. Background (max 1 page)

Describe the project background and include the related scientific and development work that has been completed to date by your team and/or others.

Sclerotinia stem rot, caused by *S. sclerotiorum*, is one of the most important biotic stresses influencing long-term production and economic stability for canola producers. The wide host range of the stem rot pathogen, its production of wind-borne ascospores, and the sporadic nature of disease development limit the effectiveness of many management strategies (Bom and Boland 2000). Stem rot on canola is primarily managed by the routine application of fungicides, typically without any indication of disease risk. To reduce the negative economic and environmental effects associated with the application of fungicides, a reliable stem rot forecasting system is desirable. Although producers and industry have access to existing risk assessment tools, such as weather-based forecast maps, checklists and petal testing, there has been limited acceptance or widespread use of these tools (McLaren et al. 2004). Weather maps are regional and not field-specific forecasts, while checklists are qualitative in nature. Petal testing is the only available risk assessment tool providing a direct assessment of inoculum levels, but is under-used due to the time required from sample collection to availability of results. The development of a qPCR-based system to accurately measure the amount of *S. sclerotiorum* inoculum on canola petals could overcome some of these limitations, helping to provide forecasts for disease risk while taking into account environmental conditions.

There have been several previously published reports of qPCR detection systems for *S. sclerotiorum*. Rogers et al. (2009) developed a qPCR assay that could quantify *S. sclerotiorum* DNA, but was unable to differentiate low levels of *S. sclerotiorum* DNA from a high background of *B. cinerea* DNA. Yin et al. (2009) developed a qPCR assay that was able to amplify *S. sclerotiorum* DNA with a high level of sensitivity, but did not include any species closely related to *Sclerotinia* in their study, making it difficult to assess the method specificity. In addition, no Canadian isolates of the fungus were analyzed. Most recently, Almquist and Wallenhammar (2015) developed a hydrolysis probe-based qPCR assay for the detection of *S. sclerotiorum* DNA in air samples and infected leaves and petals of oilseed rape (*B. napus*) in central Sweden.

The ideal PCR-based forecasting system for deployment in Canadian canola production systems should be quantitative, highly sensitive, and selective for *S. sclerotiorum*. Most importantly, the results obtained with this forecasting system should be positively correlated with final stem rot levels in the field. A recent study (Liang et al. 2013) identified a hypothetical protein from *S. sclerotiorum* that appears to be unique to this fungus. The gene encoding this protein was selected as a target for the development of a qPCR-based assay for the detection and quantification of *S. sclerotiorum* in canola, which then was used to analyze petal infestation levels and related to final stem rot severity in commercial fields.

3. Objectives and deliverables (max 1 page)

State what the original objective(s) and expected deliverable(s) of the project were. Also describe any modifications to the objective(s) and deliverable(s) which occurred over the course of the project.

The specific objectives of the research project included:

a) Development and refinement of a rapid quantitative method for pathogen detection in canola flowers
b) Understanding of the relationship between the amount of pathogen on the petals and final stem rot levels in commercial fields

c) Assessment of correlations between pathogen detection, weather-based forecasts and final stem rot levels

The long-term goal is for this information to serve as the basis for a commercially available test for stem rot forecasting.

4. Research design and methodology (max 4 pages)
Describe and summarise the project design, methodology and methods of laboratory and statistical analysis that were actually used to carry out the project. Please provide sufficient detail to determine the experimental and statistical validity of the work and give reference to relevant literature where appropriate. For ease of evaluation, please structure this section according to the objectives cited above.

a) Development and refinement of a rapid quantitative method for pathogen detection in canola flowers

DNA isolation from field-collected canola petals. To validate the qPCR assay, canola petals were collected from 10 commercial fields in the Edmonton, AB, region in 2012. The fields were sampled once at 20-30% bloom and again at 40-50% bloom, as determined by visual assessment (Harper and Berkenkamp 1975). The incidence of Sclerotinia stem rot also was assessed at the end of the growing season for future reference (see below). Petals were collected at each of five sampling sites within each field. At each sampling site, the top 10-20 cm of the inflorescences from each of 20 plants were collected at random, placed in a plastic bag, and stored in a cooler on ice for transport back to the laboratory. DNA was isolated according to the protocol of Liang et al. (2013) from a randomly chosen subsample of 20 petals from each sampling site, and quantified with a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA) as above.

Development of *S. sclerotiorum*-specific primers. Forward and reverse primers and a hydrolysis probe were designed using Primer 3 Express (Applied Biosystems, Carlsbad, CA, USA) based on the 468-bp sequence of a single-copy gene (SS1G_00263) encoding the hypothetical secreted protein ssv263 (Liang et al. 2013) from *S. sclerotiorum*. The forward (SSBZF) and reverse (SSBZR) primers were designed to amplify a 70-bp product in a region of SS1G_00263 that exhibits the greatest difference with an orthologue in *B. cinerea.* The hydrolysis probe (SSBZP) was labeled with a non-fluorescent quencher-mini groove binder (NFQ-MGB) on the 3' end, and with the reporter dye FAM (6-carboxyfluorescein) on the 5' end. The probe and primer sequences were used to query the GenBank databases using the Basic Local Alignment Search Tool (BLAST) in order to identify any similar sequences from other organisms that could lead to false positives.

Development of the exogenous positive internal control. To identify false negatives resulting from failed DNA extraction or inhibition of the PCR, an exogenous internal control was included in the analysis of all samples. The *ToxB* gene from *Pyrenophora tritici-repentis*, causal agent of tan spot of wheat, was selected as the exogenous internal control since it is unlikely to be found in canola petals. The pSilent1 plasmid (Nakayashiki et al. 2005) containing a 432-bp fragment of *ToxB* from *P. tritici-repentis* (Aboukhaddour et al. 2012) was used as the template for the design

of the primers and hydrolysis probe. The *ToxB*-specific primers (ToxBF and ToxBR) and a probe (ToxBP) were designed using Primer 3 Express (Applied Biosystems). The *ToxB*-specific hydrolysis probe was labelled with NFQ-MGB on the 3' end and with the reporter dye VIC on the 5' end.

qPCR analysis. All qPCR analyses were conducted in a ViiA7 Real-Time PCR System (Life Technologies) using the Universal FastStart Master (Rox) mix (Roche, Indianapolis, IN, USA) in a MicroAmp® Fast Optical 96-well reaction plate (Applied Biosystems), which was sealed with MicroAmp optical adhesive film (Applied Biosystems). The reaction conditions included a hot start at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec as per the manufacturer's instructions for the master mix. Each of the primer/probe sets was analyzed separately and reactions were not multiplexed. Each sample was analyzed in triplicate for quantification of the target gene, while samples were analyzed in duplicate for assays of the internal control. If the standard deviation of the mean C_q obtained for a set of triplicates of a given sample was > 0.5, the sample was reanalyzed, and if an outlier was present, it was removed. Similarly, if the standard deviation of the mean C_q obtained for duplicates of the *ToxB* internal control was > 0.5, the sample was re-extracted.

Specificity testing. The specificity of the SS1G_00263 primer and probe set was confirmed by separately testing 100 ng of DNA from 13 fungal species and the canola host as templates in the qPCR analysis. In addition, amplification of a total of eight isolates of *B. cinerea* and seven isolates of *S. sclerotiorum* was compared. No amplification of any product, or a C_q value > 2 cycles below that of the smallest standard, were considered to be indicative of no detection. The reproducibility of the standard curve was assessed by calculating the standard deviation at each point, for 4 replications included in separate 96-well plates. Plasmids containing the *ToxB* insert were analyzed with the SS1G_00263-specific primer set to ensure that SSBZF/SSBZR did not amplify the internal control. Amplicons obtained with the SSBZF/SSBZR primer set were sequenced to confirm the identity of the product.

Sensitivity testing. To test the limit of detection (LOD) of the assay, standard curves were generated with 1:10 serial dilutions of purified *S. sclerotiorum* DNA. All standard curves tested consisted of five serial dilutions ranging from 8.0 ng to 8.0×10^{-4} ng or from 5.0 ng to 5.0×10^{-4} ng. Each standard was included in triplicate in the same 96-well plate as the samples being analyzed. The LOD was regarded as the lowest quantity of DNA that could be detected with confidence in all three of the triplicates. The efficiency and R^2 of the standard curves were determined for each replication. To determine if inclusion of the internal control and the presence of *B. napus* DNA reduced the LOD for *S. sclerotiorum*, fungal DNA was quantified in the presence or absence of the *ToxB* internal control and *B. napus* DNA. The mean C_q and standard deviation of the mean for each point in the dilution series were compared between the spiked and non-spiked samples.

Evaluation of canola petals. Total genomic DNA isolated from samples consisting of 20 canola petals each was diluted by a factor of 1:5 with nuclease free water (Life Technologies) prior to qPCR analysis with the SS1G_00263- and *ToxB*-specific primers and probes. DNA was analyzed from samples collected at five sampling sites from each of 10 commercial canola fields at each of two sampling dates as described above. Results are expressed on a per petal basis by first

accounting for the dilution, then by dividing the estimate by 20. The qPCR estimates were averaged over each field on each sampling date and the standard deviation of the mean was calculated for each field.

Assessment of the SsF and SsR primers. Isolates of *S. sclerotiorum* collected from canola fields in central Alberta were subjected to qPCR analysis with the primers SsF and SsR developed by Yin et al. (2009). Quantitative PCR assays were conducted in a Step One Plus Real-Time PCR System (Applied Biosystems). After the reaction was complete, a melting point analysis was conducted to confirm the presence of a single amplification product that had a melting temperature (T_M) consistent with the predicted T_M of 84°C. The specificity of the SsF/SsR primer set also was evaluated as described above for the other primer and probe sets.

b) Understanding of the relationship between the amount of pathogen on the petals and final stem rot levels in commercial fields

Field selection and sample collection. Two experiments (Exp. 1 and Exp. 2) were conducted to evaluate the relationship between petal infestation level and final stem rot disease incidence. Experiment 1was conducted over two years (2011 and 2012) by monitoring petal infestation and subsequent disease incidence in 35 (in 2011) or 34 (in 2012) commercial canola fields in Alberta (near Edmonton and Lacombe), Manitoba (Brandon, Morden and Carman) and Saskatchewan (Melfort and Saskatoon). Experiment 2 was conducted in commercial canola fields located near Edmonton, Alberta, in 2011, 2012 and 2013. Nine fields were included in 2011, 10 fields in 2012, and 11 fields in 2013. The same central Alberta fields were monitored for Exp. 1 and Exp. 2 in 2011 and 2012. Experiment 1 was focussed on a Prairies-wide evaluation of the relationship between petal infestation and stem rot incidence, while Exp. 2 examined this relationship within a smaller regional scale.

With the exception of the fields near Edmonton, the fields in Exp. 1 were selected at random. For the fields sampled in Manitoba and Saskatchewan, field histories were not known, and no information was available as to whether or not fungicides were applied for stem rot control during the growing season. In each field, there were 5 sampling sites spaced 50 m apart and at least 75 m from the field edge. In Exp. 2, the sampling sites were situated in fungicide-free check strips. There were five sampling sites per field arranged 50 m apart in a linear fashion.

Canola petals were collected at early flowering (10-20% bloom) and full flowering (40-50% bloom). In 2012, the first sampling of the fields near Edmonton was delayed until 20-30% bloom. Samples were collected between 1100 h and late afternoon, with the exception of the fields near Edmonton in 2012 where sampling occurred early in the morning due to high temperatures during the flowering period. At each sampling site, the top 20-30 cm of 20 randomly selected inflorescences were collected and placed into clean labelled plastic bags and kept on ice in a cooler during transport back to the laboratory. In the laboratory, the samples were maintained at 4°C prior to processing, which was carried out within 24 hours of sample collection.

Estimates of *S. sclerotiorum* infestation. Petals were assessed for *S. sclerotiorum* infestation by qPCR analysis in both Exp. 1 and Exp. 2, as well as by the traditional petal plate test in Exp. 2.

The qPCR analysis was conducted as described above. The petal plate test was conducted as described by Turkington et al. (1988). Briefly, petals were plated on Petri dishes containing solid potato dextrose agar amended with 25 ppm ampicillin and 25 ppm streptomycin and incubated for 4-5 days in darkness at room temperature (approximately 20-24°C). The percentage of petals infected with *S. sclerotiorum* was determined through visual assessment, and the petal infestation estimates were averaged for each field on each sampling date.

Sclerotinia stem rot assessment. Sampling sites were revisited prior to swathing, in order to assess Sclerotinia stem rot incidence and severity in the canola crops. Twenty randomly selected plants were evaluated at each sampling site, for a total of 100 plants per field. The plants were rated for stem rot of severity on a 0-5 scale as described by Kutcher and Wolf (2006). In Exp. 1, disease incidence was defined as the percentage of plants that had any symptoms of stem rot on any organ (i.e., rated as 1 or greater). In Exp. 2, disease incidence was adjusted to include only plants with a severity rating of 2 or higher. The adjusted disease incidence values excluded infections rated as 1 (pod infections), since upper canopy (i.e., pod) infections would be expected to be a minor contributor to yield loss. Statistical analysis for Exp. 2 indicated that the adjusted disease incidence values improved the R^2 values slightly, but did not change the significance of the regression models. As a result, the calculations for disease incidence in Exp. 1 were not adjusted.

Statistical analysis. The relationship between qPCR estimates of petal infestation and final stem rot levels was determined with quadratic regression using proc reg in SAS (Statistical Analysis System) software. Assumptions of regression were tested and no transformations were required. Scatter plots indicated a non-linear regression. This was confirmed by the lower Akiake information criterion (AIC) and Bayesian information criterion (BIC) values for quadratic regression models over simple linear regression models, indicating a better fit. The AIC/BIC values were calculated for both linear and quadratic models using proc mixed and the residual maximum likelihood (REML) method. Regression analysis was conducted on the average qPCR value for each field at each of the sampling dates individually. Analysis was carried out separately for the three sampling years because of differences in the respective environmental conditions. The year to year variation in the strength of the statistical relationship was used to assess whether or not qPCR-based estimates of petal infestation could consistently indicate stem rot risk without consideration of other factors. Regression models were considered significant when the slopes of the independent variables and the overall models were significant at $P = 0.05$.

In Exp. 2, the relationship between Sclerotinia stem rot disease incidence and percent petal infestation (PPI), determined with the plate test, was assessed by simple linear regression using proc reg in SAS. Regression analysis with non-transformed disease incidence did not meet the assumptions of regression. Disease incidence values were Arcsine transformed as discussed by Turkington and Morrall (1993), and the resulting residuals more closely met the assumptions of regression. Thus, arcsine transformed disease incidence (TDI) was used for all regressions with percent petal infestation (PPI) values. Simple linear regression models were confirmed to have lower AIC and BIC values than the quadratic regression models, indicating a better fit for the linear models. As with the qPCR results, regression analysis was conducted separately for each of the three sampling years. Regression models were considered significant when the slopes of the independent variables and the overall models were significant at $P = 0.05$.

Correlation analysis with proc reg in SAS was used in Exp. 2 to determine the relationship between the qPCR-based and petal plate test estimates of petal infestation level. In all three years, there was a slight deviation from normality for at least one variable. As a result, Spearman Rank Correlation was used.

c) Assessment of correlations between pathogen detection, precipitation and final stem rot levels

In Exp. 1, the data were further sorted in three different ways: by province, by average regional annual precipitation, and by average annual regional precipitation from April to August ('summer precipitation'). The aim of this analysis was to determine which method accounted for the most variation and produced the stronger regression model. Precipitation data were obtained from Environment Canada. The data were separated into three main groups based on average regional annual precipitation: >500 mm (Carman and Morden), between 400-500 mm (Brandon, Lacombe and Edmonton), and <400 mm (Melfort and Saskatoon). Summer precipitation was averaged for the months of April through August and was used to separate the data into two main groups: >300mm (Morden, Edmonton, Lacombe, Brandon and Carman) and <300mm (Melfort and Saskatoon).

5. Results, discussion and conclusions (max 8 pages)

Present the project results and discuss their implications. Discuss any variance between expected targets and those achieved. Highlight the innovative, unique nature of the new knowledge generated. Describe implications of this knowledge for the advancement of agricultural science. For ease of evaluation, please structure this section according to the objectives cited above.
NB: Tables, graphs, manuscripts, etc., may be included as appendices to this report.

a) Development and refinement of a rapid quantitative method for pathogen detection in canola flowers

Amplicon generated with SSBZF/SSBZR. The amplicons generated from six isolates of *S. sclerotiorum* with the SSBZF/SSBZR primer set targeting the gene SS1G_00263 were sequenced and confirmed to be of the expected 70-bp size. Moreover, the sequences were identical to each other, and a query of the GenBank database using BLASTN revealed 100% identity with accession number XM_001598127.1 from *S. sclerotiorum* (e-value = 3e-31).

Specificity testing. While the SS1G_00263-specific primers consistently amplified a 70-bp product from DNA of each of the *S. sclerotiorum* isolates evaluated, they did not generate an amplicon from any of the other 13 species tested, including the closely related *B. cinerea, S. trifoliorum*, or *S. minor*. Similarly, no amplicon was obtained from DNA of the host canola plant. Specificity testing with the *ToxB*-specific primers did not yield an amplicon from *S. sclerotiorum* or any of the non-target organisms tested.

Sensitivity testing. The lowest reliable LOD was 8.0×10^{-4} ng *S. sclerotiorum* DNA. Although the assay could detect quantities of *S. sclerotiorum* DNA as low as 5.0×10^{-4} ng, such detection could not be accomplished with a standard deviation of the mean $C_q < 0.5$. As such, the lowest

point of the standard curve was set to 8.0×10^{-4} ng DNA. To compare replicates of the standard curve, the C_q values were averaged for a set of comparisons and the standard deviation of the mean for each point on the curve was calculated. An average standard deviation of 0.19 was obtained for the mean C_q over all five points on the curve, among four replicates of the standard curve in separate 96-well plates; none of the points had a standard deviation > 0.5. For the four replicates of the standard curve generated in separate 96-well plates, the PCR efficiencies ranged from 88.90 to 90.25 and the R^2 values ranged from 0.9928 to 0.9999.

Curves generated with the SS1G_00263-specific primers in the presence or absence of the internal control and *B. napus* DNA were compared to identify any potential reduction in the quantification of *S. sclerotiorum* DNA. The C_q values were averaged for each dilution point in the two dilution series and the standard deviation of the C_q values for the replicates was calculated. Each point on the standard curves was compared individually and no single point had a standard deviation of the mean $C_q > 0.54$.

Quantification of *S. sclerotiorum* in canola petals. Total genomic DNA extracted from 20 petals from each of five sampling sites in each of 10 commercial canola fields on two different sampling dates (100 DNA samples in total) was used as a template for qPCR analysis. The petal infestation estimates for individual sampling sites ranged from 0 ng to 3.3×10^{-1} ng *S. sclerotiorum* DNA per petal. The amount of *S. sclerotiorum* DNA in each of the 5 sampling sites per field was averaged to give a single petal infestation estimate for each field on each sampling date. On the first sampling date, when the canola was at 20-30% bloom, average infestation in the 10 fields ranged from a mean (± standard deviation) of 6.0×10^{-3} ng ± 7.0×10^{-3} ng to 3.4×10^{-2} ng ± 2.8×10^{-2} ng *S. sclerotiorum* DNA per petal (C_q values = 30.5-36.1). On the second sampling date, at 40-50% bloom, average infestation in the 10 fields ranged from a mean (± standard deviation) of 1.0×10^{-3} ng ± 1.0×10^{-3} ng to 8.0×10^{-2} ng ±1.4×10^{-1} ng *S. sclerotiorum* DNA per petal (C_q values = 28.9-36.1).

In addition to the analysis with the SS1G_00263-specific primer and probe set, all samples were analyzed with the *ToxB* (internal control)-specific primer and probe set in separate, singleplex qPCR assays. The mean and standard deviation of the mean C_q value for duplicates was calculated for early bloom and late bloom sample sets to get an estimate of the amount of variation. At 20-30% bloom, the overall mean C_q (± standard deviation) was 29.6 ± 1.08, and at 40-50% bloom, the overall mean C_q (± standard deviation) was 27.6 ± 0.70.

Assessment of the SsF and SsR primers. The primers SsF and SsR (Yin et al. 2009) were evaluated to determine their suitability for quantifying *S. sclerotiorum* DNA in canola petals under Canadian conditions. The primers amplified a product of approximately 225-bp from all isolates of *S. sclerotiorum*, and did not amplify DNA of *S. minor*, *Aspergillus niger*, *Cladosporium* sp., *Mucor* sp., *Alternaria alternata*, or *Leptosphaeria maculans*. However, the primers were found to amplify products from DNA of *S. trifoliorum*, as well as from all isolates tested of *B. cinerea* and *B. fuckeliana*, *Rhizopus* sp., *Trichoderma* sp., *Rhizoctonia solani*, *Penicillium* sp., and *Fusarium graminearum*. The amplicons obtained from *B. cinerea*, *S. trifoliorum* and *Trichoderma* sp. all had temperature peaks between 84 and 85°C, which was within the range expected for the product from *S. sclerotiorum*. For the products amplified from DNA of *B. fuckeliana*, *Rhizopus* sp., *R. solani*, *Penicillium* sp., and *F. graminearum*, the melting

curve analysis revealed the presence of multiple temperature peaks associated with non-specific amplification. The sensitivity of the SsF/SsR primer set was similar to that of the SSBZF/SSBFR primers, with a consistent LOD of 8.0 × 10^{-4} ng DNA.

b) Understanding of the relationship between the amount of pathogen on the petals and final stem rot levels in commercial fields

Experiment 1. Sclerotinia stem rot disease incidence and the qPCR estimates of petal infestation for individual fields were variable over the sampling years and between locations. In both 2011 and 2012, the disease incidence ranged from 0 to 92% among the sampled fields, but the mean disease incidence (MDI) for all fields, presented as MDI ± standard deviation, was higher in 2012 (27.2% ± 29.5%) than in 2011 (11.9% ± 17.7%). The mean amount of *S. sclerotiorum* DNA per petal for all fields, as measured by qPCR analysis, was highest at full bloom in both 2011 (0.013 ng/petal ± 0.0018 ng/petal) and 2012 (0.068 ng/petal ± 0.18 ng/petal). There was variation between sampling sites, years and individual fields for disease incidence and the amount of *S. sclerotiorum* DNA per petal. When the data were separated by province, none of the relationships between the qPCR results and disease incidence were significant at either sampling date in 2011. The coefficient of variation (CV) for these regression models ranged from 94.14 to 144.05. In 2012, however, the relationship between the early bloom qPCR results and disease incidence for the fields in Alberta was found to be significant, with the early bloom qPCR results accounting for 59.1% of the variation in disease incidence ($R^2 = 0.591$; $P = 0.0073$; CV 42.694). The regression models for the Alberta fields at full bloom and for the Saskatchewan and Manitoba fields at both sampling dates were not significant, with CVs ranging from 51.05 to 111.67.

Experiment 2. Sclerotinia stem rot incidence, PPI and the estimates of petal infestation obtained by qPCR analysis were variable over the three years of the study. Mean stem rot incidence across all fields, presented as the mean ± standard deviation, was lower in 2011 (7.8% ± 6.6%) than in 2012 and 2013 (64% ± 23.2% and 39.36% ± 24.8%, respectively). For both sampling dates, estimates of petal infestation determined by qPCR analysis also were lowest in 2011 (early bloom: 0.005 ng/petal ± 0.005 ng/petal; full bloom: 0.017 ng/petal ± 0.013 ng/petal). On a field level, qPCR estimates of petal infestation at full bloom were higher for all 9 fields in 2011, for five of 10 fields in 2012, and for 5 of 11 fields in 2013. As in Exp. 1, there was a high amount of variation in the levels of petal infestation, as determined by qPCR analysis, in samples from some of the fields.

Mean PPI, as assessed by the petal plate test of Turkington et al. (1988), was similar across both sampling dates in 2011 (early bloom: 35.9% ± 22.3%; full bloom: 40.1% ± 6.84%) and 2012 (early bloom: 34.3% ± 17.1%; full bloom: 41.2% ± 15.1%). In contrast, PPI was generally higher in 2013 (early bloom: 43.85% ± 18.2%; full bloom: 51.0% ± 18.5%). A comparison of petal infestation levels, as determined by qPCR analysis or the petal plate test, found similar results in 2011 and 2012: both methods indicated that petal infestation levels were lower at early bloom than at late bloom. In 2013, however, the different methods yielded different results. Petal infestation was found to be lower at early bloom when evaluated by the petal plate test, and lower at late bloom when evaluated by qPCR analysis.

Relationship between qPCR-based petal infestation estimates and disease incidence. In each year of Exp. 2, three non-linear regression models were analyzed to determine the relationship between qPCR estimates of petal infestation and final stem rot disease incidence. In all three years, disease incidence increased as the amount of *S. sclerotiorum* DNA per canola petal increased. However, the strength of the relationship varied with year and was not always significant. In all years, there was a significant amount of variation in both the final disease incidence and the petal infestation estimates in the fields sampled. In 2011, none of the three regression models were significant at P = 0.05. The field with the highest stem rot incidence also had a very low level of petal infestation (as determined by qPCR) and appeared to be an outlier. If this field is removed from the analysis, the relationship between the qPCR results at full bloom and disease incidence becomes significant ($R^2 = 0.9194$; P = 0.0018; CV = 27.21). In 2012, there was no significant statistical relationship between the qPCR estimates and disease incidence at early bloom. However, there was a significant relationship between disease incidence and qPCR estimates at full bloom (P = 0.0165; $R^2 = 0.6904$; CV = 22.91).

In 2013, there was no significant statistical relationship at either early or full bloom when all fields were included in the analysis. As in 2011, there was one outlier. When this outlier was removed, the relationship between the qPCR estimates at full bloom and stem rot disease incidence became significant ($R^2 = 0.6047$; P = 0.039; CV = 40.79). In 2013, there was a wider range of seeding dates (May 9-May 20), with some canola fields seeded later than in 2011 and 2012. When only those fields seeded on or prior to May 15 were included in the analysis, the regression became highly significant at full bloom ($R^2 = 0.9204$; P = value 0.0063; CV = 20.83).

The amount of variation in the data set was high in all years, as indicated by the coefficients of variation, which ranged from 37.22 to 89.056 for all non-significant regressions.

Relationship between PPI and disease incidence. In 2011 and 2013, there was not a significant statistical linear relationship between PPI and disease incidence at either early bloom or late bloom, even in 2013 when only early seeded fields were included in the analysis. In 2012, the relationship was significant at full bloom ($R^2 = 0.682$; P = 0.003; CV = 16.189).

Relationship between PPI and qPCR estimates of petal infestation. Correlation analysis was used to determine the relationship between PPI and the amount of *S. sclerotiorum* DNA per canola petal as determined by qPCR analysis. In 2011, the only significant correlation was at early bloom ($R^2 = 0.71$; P = 0.03). In 2012, the correlation was not significant at early bloom but was significant at full bloom ($R^2 = 0.80$; P = 0.0056). In 2013, there was no significant correlation at either early or full bloom.

c) Assessment of correlations between pathogen detection, precipitation and final stem rot levels

When the data from Exp. 1 were separated by average regional annual precipitation, the relationships were not significant for the locations with >400 mm precipitation in 2011, or for none of the groups in 2012. The CV for these models ranged from 51.046 to 159.16. The regression analysis for locations with >500 mm of annual precipitation at the late bloom sampling date in 2011 could not be conducted using a quadratic model because of the large

number of fields with a disease incidence of 0%. For this group of locations, the simple linear regression was analyzed and found not to be significant. The relationship between late bloom qPCR results and disease incidence for fields with average regional annual precipitation was found to be significant, with the late bloom qPCR results accounting for 57.6% of the variation in disease incidence ($R^2 = 0.576$; P = 0.0356; CV = 95.559).

When the data were separated by average regional summer precipitation, none of the regression models were significant in 2011. In 2012, the regression models for the group of locations with average summer precipitation levels > 300 mm were significant at both early bloom ($R^2 = 0.263$; P = 0.035; CV = 95.116) and late bloom ($R^2 = 0.244$; P = 0.0459; CV 96.301). The CV values for the non-significant regression models ranged from 95.56 to 129.022 in 2011 and from 84.295 to 99.600 in 2012.

d) Discussion

This study focussed on the development of a qPCR-based assay for the quantification of *S. sclerotiorum* DNA in canola petals, to enable rapid and accurate estimates of infestation levels. The assay targets a 70-bp region of a single-copy gene encoding the hypothetical secreted protein ssv263 (Liang et al. 2013). While the target region of the gene shares 71% similarity with an orthologue in *B. cinerea*, specificity testing indicated that there was no amplification of DNA from any of six *B. cinerea* isolates tested. The qPCR assay also did not amplify DNA from any of the 13 other species evaluated in this study, including *S. minor, S. trifoliorum* and the host plant, *B. napus*. These results indicate that the assay is highly specific for *S. sclerotiorum*, and can be used to estimate pathogen biomass in canola petals.

The sensitivity tests revealed a consistent LOD of 8.0×10^{-4} ng *S. sclerotiorum* DNA. Rogers et al. (2008) reported that an ascospore of *S. sclerotiorum* is equivalent to about 3.5×10^{-4} ng of DNA, suggesting a LOD of 2.3 ascospores per petal for the current assay. When adjusted to account for the dilution of DNA from canola petals during extraction and analysis, the LOD would correspond to 1.5×10^{-3} ng *S. sclerotiorum* DNA or 4.3 ascospores per petal. An issue with the use of ascospores as the basis for a standard curve to quantify petal infestation, however, is that this approach will not reflect increases in pathogen biomass associated with spore germination. Ascospores have been shown to begin germination as early as 3 hours after release from the apothecium under favorable environmental conditions (Willets and Wong 1980). Therefore, it is likely that any assay examining the amount of *S. sclerotiorum* DNA on canola petals is measuring DNA from the ascospores as well as from hyphae developing from germinated spores. As such, quantification of *S. sclerotiorum* on petals is more meaningful and accurate when expressed as the total amount of pathogen DNA. The specificity of the assay was not affected by the presence of *B. napus* DNA or of an internal control in the samples. These are important considerations if the assay is to be used to measure infestation of petals collected in the field.

Yin et al. (2009) developed a qPCR assay to measure *S. sclerotiorum* DNA in canola petals using intercalating dye (SYBR Green) detection technology that detected between 0.0252 ng-0.111 ng pathogen DNA per mg of canola petal tissue. Evaluation of the primers developed by Yin et al. (2009) on DNA extracted from Canadian isolates of *S. sclerotiorum* and other fungal species,

using a different master mix but the same dye detection technology, indicated sensitivity similar to that of the assay described in this report. However, the specificity of the primers seemed considerably lower and amplicons were obtained from various non-target species including *S. trifoliorum, B. cinerea, F. graminearum, Rhizopus* sp., and *Penicillium* sp. In their original report, Yin et al. (2009) found no amplification of DNA other than from *S. sclerotiorum*, but only evaluated one isolate of *B. cinerea* (*B. fuckeliana*) and did not test *S. minor, S. trifoliorum*, or *Rhizopus* sp. They did test *R. solani, Penicillium* sp. and *F. graminearum*, but did not detect any product from the isolates evaluated. This lower specificity represents a challenge for application of the earlier protocol (Yin et al. 2009) in the analysis of field samples, and hence for its use as the basis for a stem rot risk assessment system in Canada. While it may be possible to identify non-specific amplification products based on a melting curve analysis, this would complicate the procedure and introduce the possibility of additional errors. Moreover, the quantification of field samples containing a range of different fungal species in addition to *S. sclerotiorum* may be difficult. The similar sensitivity, but increased specificity of the assay developed in this study with the primers SSBZF/SSBZR and probe SSBZP may reflect the use of a hydrolysis probe instead of an intercalating dye. An intercalating dye, such as SYBR green, binds to any double-stranded DNA and releases a fluorescent signal (Ririe et al. 1997). This potentially can result in fluorescent amplification of non-target double-stranded DNA that must be further differentiated through melting curve analysis (Smith and Osborn 2008). The use of a hydrolysis probe ensures that a fluorescent signal is produced only when there is an exact match between the probe and the target (Smith and Osborn 2008). This increased level of specificity reduces the likelihood of false positives when analysing diverse field samples, without compromising sensitivity. As a result, the newly developed qPCR assay may represent a more reliable method to quantify *S. sclerotiorum* DNA in canola petals.

When the hydrolysis probe-based qPCR assay was used to quantify *S. sclerotiorum* in field-collected canola petals, considerable variation was observed in the amount of petal infestation in different fields and at different crop stages. Environmental conditions in 2012 were conducive to stem rot development in the sampled fields. In five of 10 fields, infestation levels were highest at 40-50% bloom, while in the remaining five fields, the infestation levels were highest at 20-30% bloom. This is consistent with the findings of Turkington and Morrall (1993), who reported changes in petal infestation from early to late bloom and at different locations when culturing petals on growth medium. Similarly, using qPCR analysis, Almquist and Wallenhammer (2015) also observed differences in *S. sclerotiorum* incidence at different stages of flowering. The evaluation of petals by qPCR analysis over the flowering period could provide an indication of when inoculum levels are highest, and thus when the crop may benefit most from fungicide application. The variation in the amount of *S. sclerotiorum* DNA between locations may reflect differences in inoculum level or in the timing of ascospore release, crop seeding date and growth stage, canopy density, or microclimatic conditions. It also indicates the importance of assessing petal infestation and risk potential for a particular field as opposed to an assessment of risk based on regional conditions. The inclusion of an internal control helped to identify outliers resulting from possible human error or other artifacts, such as inhibition of the PCR, thereby improving the accuracy of qPCR-based estimates of petal infestation.

In all fields and locations included in this study, there was variation in both the amount of *S. sclerotiorum* DNA detected by qPCR analysis and in the final disease incidence. There also was

significant year to year variation in these parameters, most likely reflecting differences in environmental conditions that in turn influence carpogenic germination of the sclerotia and the timing of ascospore release. Similarly, the differences observed between fields could also be a result of regional differences in environmental conditions, but they may also reflect differences in crop stand density, seeding date, seeding rate and crop history, all of which can influence stem rot development (Turkington and Morrall 1993; Jurke and Fernando 2008; Twengstrom et al. 1998). In addition to field to field variation, variation across sampling sites within particular fields also was observed. This variation likely reflected differences in crop stand and microclimate at different points within the same field. Five sites were sampled per field, since this was reported to be an accurate sampling size for evaluating the incidence of petal infestation (Turkington et al. 1988). The large amount of variation observed in this study, however, indicates that a larger number of sampling sites may be needed to provide a more reliable estimate of petal infestation in a particular field.

The amount of *S. sclerotiorum* DNA quantified per canola petal varied across the flowering season. These findings are consistent with previous reports that found that inoculum pressure is not consistent across the flowering period or between fields (Almquist and Wallenhammar 2015; Turkington and Morrall 1993). Differences in the level of infestation over the growing season will influence the strength of the statistical relationship between quantifications of petal infestation and stem rot incidence when evaluated across several fields. Monitoring changes in petal infestation over the flowering period may serve to identify the best timing for fungicide application in a particular field, but such an approach would have to be balanced by cost and other practical considerations. Nonetheless, closer monitoring of infestation levels on a temporal scale could prove useful in better understanding the epidemiology of Sclerotinia stem rot of canola.

The relationship between PPI as determined by the petal plate test and petal infestation as assessed by qPCR analysis was not always linear. An analysis of correlation indicated a significant amount of shared variation between the two methods only at early bloom in 2011 and at full bloom at 2012. Similarly, Almquist and Wallenhammar (2015) reported no correlation between the results of the petal plate test and a qPCR-based *S. sclerotiorum* detection method. There are several possible explanations for these differences. For example, the presence of a few highly infected canola petals in a sample would be reflected in a higher concentration of *S. sclerotiorum* DNA in the qPCR analysis, but would not be reflected in the petal plate test results (Almquist and Wallenhammar 2015). Furthermore, while both the number of ascospores and the amount of mycelium present in a sample can be measured by qPCR analysis, the petal plate test can only indicate whether or not a petal is infected. Finally, when levels of *S. sclerotiorum* infestation are low on infected petals, other fungal species (e.g. *Rhizopus* spp., *Mucor* spp., *Trichoderma* spp.) that are present on the petal tissue may outgrow colonies of *S. sclerotiorium*, thus masking the presence of *S. sclerotiorum*. Given the increased sensitivity of the qPCR-based method, detection and direct measurement of *S. sclerotiorum* by qPCR analysis are more likely to be influenced by the environment in which those petals were produced.

The strength of the statistical relationship between qPCR-based measurement of petal infestation and final stem rot incidence in the field is critical to assessing the suitability of the former for predicting the risk of disease. In Exp. 1, the strength of the relationship between the qPCR results

and disease incidence across the Prairies was variable across the three years of the study. Moreover, when the data were analyzed separately based on the amount of annual or summer precipitation in a particular region, the strength of the relationship was not increased. It is important to note that, with the exception of the fields in the Edmonton region, there was no information regarding fungicide application in the fields included in Exp. 1. This could have had a large impact on any potential correlation. For instance, a canola field in which there was heavy petal infestation may have been treated with fungicide, preventing or greatly reducing stem rot development. As such, the results of Exp. 1 must be treated with caution. In Exp. 2, all sampling was carried out in check-strips that were not treated with fungicide. Therefore, the results of this experiment may provide a better indication of the true relationship between disease incidence and petal infestation as measured by qPCR analysis. Indeed, in Exp. 2 the relationship between these two parameters was stronger, with stem rot incidence generally found to be greater in fields where petal infestation (*S. sclerotiorum* DNA per canola petal) also was higher. Despite this stronger relationship, however, the correlations were not always significant. This highlights the possible influence of other factors in stem rot development.

There was a wider range of seeding dates in 2013 than in 2011 or 2012, and this was found to influence the statistical relationship in Exp. 2. When only the early seeded fields were included in the analysis, the relationship was significant at full bloom, which was consistent with the results in 2012. These findings indicate that the relationship between disease incidence and the amount of *S. sclerotiorum* DNA per canola petal is strongest at full bloom and/or in years when disease pressure is high. The variation across the sampling years also indicates that measures of environmental conditions may need to be included in the model using multiple regression analysis, in order to fully account for year to year variation in weather. More broadly, comparison of the results obtained in Exp. 1 and Exp. 2 suggests that qPCR-based assessments of stem rot risk are more reliable on a smaller regional scale, and that differences in environment between locations should be considered when setting risk assessment thresholds.

The qPCR assay described in this study may serve as the basis for a risk assessment system, as well as representing a useful tool for the study of the epidemiology of Sclerotinia stem rot of canola. It can quantify the level of petal infestation, a key stage in the Sclerotinia stem rot disease cycle, thereby providing a measure of disease risk when timely fungicide application decisions need to be made. It is important to emphasize, however, that a forecasting system based on qPCR quantification of petal infestation should be linked to environmental conditions, as well as cropping history, seeding date and crop canopy conditions, which may influence stem rot development and the need to spray a fungicide.

6. Literature cited

Provide complete reference information for all literature cited throughout the report.

- Aboukhaddour, R., Kim, Y. M., and Strelkov, S. E. 2012. Mol. Plant Pathol. 13:318-326.
- Almquist, C., and Wallenhammar, A.C. 2015. Plant Pathol. 64:109-118.
- Bom, M., and Boland, G.J. 2000. Can. J. Plant Sci. 80:889-898.
- Harper, F. R., and Berkenkamp, B. 1975. Can. J. Plant Sci. 55:657-658.
- Jurke, C.J., and Fernando, W.G.D. 2008. Arch. Phytopathol. Plant Protect. 41:142-155.
- Kutcher, H.R., and Wolf, T.M. 2006. Crop Protect. 25: 640-646.

- Liang, Y., Yajima, W., Davis, M.R., Kav, N.N.V. and Strelkov, S.E. 2013. Can. J. Plant Pathol. 35:46-55.
- McLaren, D.L., Conner, R.L., Platford, R.G., Lamb, J.L., Lamey, H.A., and Kutcher, H.R. 2004. Can. J. Plant Pathol. 26:489-497
- Nakayashiki, H., Hanada, S., Nguyen, B. Q., Kadotani, N., Tosa, Y., and Mayama, S. 2005. Fungal Genet. Biol. 42:275-283.
- Ririe, K. M., Rasmussen, R. P., and Wittwer, C. T. 1997. Analytical Biochem. 245:154-160.
- Rogers, S.L., Atkins, S.D. and West, J.S. 2009. Plant Pathol. 58:324-331.
- Smith, C. J., and Osborn, A. M. 2008. FEMS Microbiol. Ecol. 67:6-20.
- Turkington, T.K., Morrall, R.A.A., and Baker, R.J. 1988. Can. J. Plant Pathol. 10:159-165.
- Turkington, T.K., and Morrall, R.A.A. 1993. Phytopathol. 83: 682-689.
- Twengstrom, E., Sigvald, R., Svensson, C., and Yuen J. 1998. Crop Prot. 17:405-411.
- Willetts, H.J. and Wong, J.A.-L. 1980. Bot. Rev. 46:101-165.
- Yin, Y., Ding, L., Lui, X., Yang,J., and Ma, Z. 2009. J. Phytopathol. 157:465-469.

7. **Benefits to the industry (max 1 page; respond to sections *a)* and *b)* separately)**
   a) Describe the impact of the project results on Alberta's agriculture and food industry (results achieved and potential short-term, medium-term and long-term outcomes).

The qPCR-based assay for detection and measurement of *S. sclerotiorum* on canola petals is the first such method to be developed and validated for canola cropping systems in western Canada. The methodology is highly robust and could serve as the basis for a stem rot forecasting system. Indeed, there has been interest from several commercial testing laboratories in assessing the method, and it has been recently made publicly available. As discussed in this report, extensive analysis also was conducted to determine the relationship between petal infestation level, as measured by qPCR testing, and final stem rot incidence. While significant correlations were often detected, the relationship between inoculum level and disease incidence was found to be complex, and is influenced by parameters such as weather, crop density and seeding date. Establishment of defined action thresholds likely will require additional evaluation and multiple regression analysis. Nonetheless, the qPCR-based evaluation of canola infestation levels may provide a general indication of stem rot risk for risk-averse producers, which could be further refined with additional epidemiological data. The detection technology developed as part of this project represents a means to acquire these additional data, and also provides a tool for more in-depth study of the epidemiology of Sclerotinia stem rot. Routine use of risk assessment tools as part of an IPM approach will result in more informed fungicide-use decisions, leading to production systems with reduced input costs that address concerns regarding a safe, sustainable, and environmentally friendly food supply.

   b) Quantify the potential economic impact of the project results (*e.g.,* cost-benefit analysis, potential size of market, improvement in efficiency, etc.).

Fungicide application is viewed as one of the main strategies for stem rot management, but application during flowering is a significant investment for canola producers. Also, because of the variability of stem rot from year to year, region to region and field to field, routine

application of fungicide may not be economical. It is estimated that up to 20% of canola acres are sprayed for stem rot each year at an estimated cost of $55 million. Spraying is typically required when disease incidence approaches 20%; however, surveys suggest that only 10-20% of fields actually have at least 20% infected plants. Routine use of risk assessment tools like qPCR-based detection and quantification of *S. sclerotiorum* will help rationalize fungicide application. Even just a 10% reduction in acres sprayed will reduce the amount of fungicide applied in the prairie ecosystem by as much as 44 million grams of active ingredient annually. The potential return per dollar invested as part of this project could be as much as $80, based on annual cost savings related to lower chemical and application costs; this excludes environmental benefits.

8. Contribution to training of highly qualified personnel (max ½ page)
Specify the number of highly qualified personnel (*e.g.*, students, post-doctoral fellows, technicians, research associates, etc.) who were involved in the project.

This project served as the basis for one graduate student program. Ms. Barb Ziesman begun as a M.Sc. student, but she transferred into the Ph.D. program in order to take full advantage of the training opportunities provided by the project. During the course of her involvement, she obtained expertise in experimental design, statistical analysis, molecular biology, and plant pathology and epidemiology. In addition, the project also served as the basis for the training of two students enrolled in the Women in Science, Engineering, Scholarship and Technology (WISEST) Program, as well as three summer students (University of Alberta undergraduates) who worked in the Plant Pathology Lab for two summers.

9. Knowledge transfer/technology transfer/commercialisation (max 1 page)
Describe how the project results were communicated to the scientific community, to industry stakeholders, and to the general public. Organise according to the following categories as applicable:

a) Scientific publications (*e.g.*, scientific journals); attach copies of any publications as an appendix to this final report
    - Ziesman, B.R., Turkington, T.K., Basu, U., and Strelkov, S.E. 2016. A quantitative PCR system for measuring *Sclerotinia sclerotiorum* in canola (*Brassica napus*). Plant Dis. (Accepted 13 December 2015). DOI: 10.1094/PDIS-05-15-0605-RE
    - Ziesman, B.R., Turkington, T.K., Basu, U., deMilliano, E.J., and Strelkov, S.E. 2014. Evaluation of a quantitative (q)PCR assay as the basis for a stem rot of canola (*Sclerotinia sclerotiorum*) risk assessment tool. Phytopathology 104(S3): S3.138 (Abstr.).
    - Ziesman, B.R., Turkington, T.K., Basu, U., and Strelkov, S.E. 2014. Initial validation of a quantitative PCR-based system for detection of *Sclerotinia sclerotiorum* on canola. Can. J. Plant Pathol. 36: 132. (Abstr.).
    - Ziesman B.R., Turkington, T.K., Basu, U., and Strelkov, S.E. 2013. Development of a quantitative PCR detection system for *Sclerotinia sclerotiorum* on canola petals. Can. J. Plant Pathol. 35: 520-521. (Abstr.).
    - Ziesman, B.R., Turkington, T.K., Basu, U., and Strelkov, S.E. 2013. Development of a quantitative PCR detection technique for *Sclerotinia sclerotiorum* on canola. Can. J. Plant Pathol. 35: 131-132 (Abstr.).

b) Industry-oriented publications (*e.g.*, agribusiness trade press, popular press, etc.) attach copies of any publications as an appendix to this final report

c) Scientific presentations (*e.g.*, posters, talks, seminars, workshops, etc.)

- Ziesman B.R., Turkington, T.K., Basu, U., and Strelkov, S.E. 2013. Initial validation of a quantitative PCR-based system for detection of *Sclerotinia sclerotiorum* on canola. Annual Meeting of the Plant Pathology Society of Alberta, November 5, 2013, Brooks, Alberta (Oral Presentation).

d) Industry-oriented presentations (*e.g.*, posters, talks, seminars, workshops, etc.)

- Canola Council of Canada (CCC) and Alberta Canola Producers Commission (ACPC), PeacePalooza field day at AAFC Beaverlodge organized by CCC and ACPC, June 25, 2015. Provided an outline of key strategies and recommendations to improve the management of sclerotinia stem rot of canola and other canola diseases.
- Canola Council of Canada and Alberta Canola Producers Commission, CanolaPalooza field day at AAFC Lacombe organized by CCC and ACPC, June 23, 2015. Provided an outline of key strategies and recommendations to improve the management of sclerotinia stem rot of canola and other canola diseases.
- Turkington, T.K., Buchwaldt, L., Orchard, D., Strelkov, S., Ziesman, B., and Lange, R. 2014. Assessing risk and spray timing for sclerotinia stem rot. Daily interactive workshops, held over three days. 2014 CanoLAB training event, Bolting and Beyond Session, Olds, Alberta, February 19-21, 2014.

e) Media activities (*e.g.*, radio, television, internet, etc.)

- Berg, M. 2014. Quickly predicting Sclerotinia is closer to reality. Top Crop Manager, Nov. 2014.
- King, C. 2014. Improving sclerotinia prediction: New tools are being developed to help canola growers with spray decisions. Top Crop Manager, March 2014.

f) Any commercialisation activities or patents

N.B.: Any publications and/or presentations should acknowledge the contribution of each of the funders of the project.

Section D: Project resources

1. Statement of revenues and expenditures:

a) In a separate document certified by the organisation's accountant or other senior executive officer, provide a detailed listing of all cash revenues to the project and expenditures of project cash funds. Revenues should be identified by funder, if

applicable. Expenditures should be classified into the following categories: personnel; travel; capital assets; supplies; communication, dissemination and linkage; and overhead (if applicable).

A statement of award and expenditures will be prepared and forwarded by the Research Services Office of the University of Alberta.

b) Provide a justification of project expenditures and discuss any major variance (*i.e.*, ± 10%) from the budget approved by the funder(s).

2. Resources:
Provide a list of all external cash and in-kind resources which were contributed to the project.

Total resources contributed to the project		
Source	**Amount**	**Percentage of total project cost**
Funders	$127,000	65%
Other government sources: Cash		%
Other government sources: In-kind	$68,000	35%
Industry: Cash		%
Industry: In-kind		%
Total Project Cost	**$195,000**	**100%**

External resources (additional rows may be added if necessary)		
Government sources		
Name (only approved abbreviations please)	Amount cash	Amount in-kind
Agriculture and Agri-Food Canada		$34,000.00
University of Alberta		$34,000.00
Industry sources		
Name (only approved abbreviations please)	Amount cash	Amount in-kind

Section E: The next steps (max 2 pages)

Describe what further work if any needs to be done.

a) Is new research required to deal with issues and opportunities that the project raised or discovered but were not dealt with within the current project?

b) Is there related work that needs to be undertaken to continue advancement of the project technology or practice?

c) Did the project identify any new technology or practice that needs to be developed?

d) What suggestions do you have that increase commercial use of results by farmers and/or companies. These may be:

1. commercial uptake.
2. further research toward commercial use.
3. extension and information disbursement.

a) Additional study of the influence of environmental and other factors (such as seeding date) on the development of stem rot of canola would be valuable. The effect of these factors could be assessed through multiple regression analysis and incorporated into predictive models. Closer monitoring of petal infestation levels over the flowering period could prove useful in better understanding the epidemiology of Sclerotinia stem rot of canola, and thereby enable optimization of the timing of stem rot management practices.

b) An improved understanding of the influence of parameters other than inoculum level on stem rot development would enable refinement of qPCR-based methodologies as predictive tools. Eventually, this may allow identification of threshold levels of infestation that, when combined with specific environmental parameters, would notify growers that economically significant damage to the crop will occur unless control measures are taken.

c) The project developed a robust qPCR-based detection system for *S. sclerotiorum* in canola petals. The technology is robust and sufficient for monitoring levels of infestation, but additional epidemiological data is needed to refine understanding of the influence of factors other than inoculum load on stem rot development.

d) The methodology developed and knowledge obtained through this project is being made publicly available through the publication of peer-reviewed articles, presentation at scientific and industry meetings, and via open lines of communication with stakeholders. This will facilitate uptake of the technology by commercial and provincial diagnostic labs, as well as increased awareness among farmers and industry personnel that these new tools are available.

UNIVERSITY OF ALBERTA

Research Services Office
222 Campus Tower
8625 - 112 Street, Edmonton, AB T6G 2E1 Canada

Statement of Award & Expenditure

For the Period Ending - December 31, 2015

Name of Grantee - Project Role	Department	Reference Award Number	
Strelkov,Stephen - Principal Investigator	100300 - ALES AFNS General	2013F075R	
University Project Number	**Project/Grant Description**	**Start Date :**	**End Date :**
RES0017396	AgFC(ACIDF/ACPC/WGRF2013F075R)	April 1, 2013	December 31, 2015

	Reporting Period April 1, 2013 to December 31, 2015	
OPENING BALANCE	0.00	
AWARD		
Direct Costs	127,000.00	cr
Total Funds Available	127,000.00	cr
EXPENDITURE		
Salaries & Benefits		
Undergrad Stu Salary & Benefit		
Grad Student Salary & Benefits		
Graduate Student Salaries	41,843.28	dr
Graduate Student Benefits		
Postdoctoral Salary & Benefits		
Postdoctoral Fellows Salaries		
Postdoctoral Fellows Benefits		
Other Sal & Adj (all benefits)		
Other Salaries	48,389.02	dr
Other Benefits	4,581.88	dr
Professional & Technical Svcs		
Equipment	109.31	dr
Materials Supplies & Other Exp	30,012.06	dr
Travel	2,064.45	dr
Transfers Out		
Total Funds Expended	127,000.00	dr
Indirect Cost Expenses	0.00	
Total EXPENDITURE	127,000.00	dr
PROJECT/ GRANT BALANCE AT: December 31, 2015	0.00	

SIGNATURES

I hereby certify that the above statement is correct and that the expenditures conform to the general conditions imposed by the sponsoring agency, and were for the purpose for which the grant was made.

Project Manager – Role: Strelkov,Stephen-Principal Investigator

January 17th, 2016

Date

I certify that the expenditures summarized above were incurred wholly by and paid on behalf of the grantee, and that the vouchers are available for monitoring purposes.

Nafissa Aktary

Business Officer, Research Services Office

January 18, 2016

Date

NAFISSA AKTARY, MEd, BSc
Senior Financial Analyst
RESEARCH SERVICES OFFICE
The University of Alberta

Section F: Research Team Signatures and Employers' Approval

The team leader and an authorised representative from his/her organisation of employment MUST sign this form.

Research team members and an authorised representative from their organisation(s) of employment MUST also sign this form.

By signing as representatives of the research team leader's employing organisation and/or the research team member's(s') employing organisation(s), the undersigned hereby acknowledge submission of the information contained in this final report to the funder(s).

Team Leader's Organisation

Team Leader	
Name: **Stephen Strelkov**	**Title/Organisation:** **University of Alberta**
Signature:	**Date:** **January 30, 2015**
Team Leader's Employer's Approval	
Name: Ruurd T. Zijlstra Chair, AFNS	**Title/Organisation:**
Signature:	**Date:** FEB - 2 2016

Research Team Members (add more lines as needed)

1. Team Member	
Name: **Kelly Turkington**	**Title/Organisation:** **Agriculture and Agri-Food Canada**
Signature:	**Date:**
Team Member's Employer's Approval	
Name:	**Title/Organisation:**
Signature:	**Date:**

Section F: Research Team Signatures and Employers' Approval

The team leader and an authorised representative from his/her organisation of employment MUST sign this form.

Research team members and an authorised representative from their organisation(s) of employment MUST also sign this form.

By signing as representatives of the research team leader's employing organisation and/or the research team member's(s') employing organisation(s), the undersigned hereby acknowledge submission of the information contained in this final report to the funder(s).

Team Leader's Organisation

Team Leader	
Name: **Stephen Strelkov**	**Title/Organisation:** **University of Alberta**
Signature: [signature]	**Date:** **January 30, 2015**
Team Leader's Employer's Approval	
Name:	**Title/Organisation:**
Signature:	**Date:**

Research Team Members (add more lines as needed)

1. Team Member	
Name: **Kelly Turkington**	**Title/Organisation:** **Agriculture and Agri-Food Canada**
Signature: [signature]	**Date:** January 30, 2016
Team Member's Employer's Approval	
Name: François Eudes, Ph.D. Director - RDT Alberta	**Title/Organisation:** François Eudes, Ph.D. Director - RDT Alberta
Signature: [signature]	**Date:** FEB 05 2016

Development of a Rapid Quantitative Detection Method for Sclerotinia Stem Rot Inoculum to Aid Disease Risk Assessments and Fungicide Spray Decisions

Project Number: 2013F075R

January 30th, 2016

APPENDIX: (1) Paper in press (*Plant Disease*) and (2) Draft manuscript being prepared for submission to a scientific journal

Research Team: Stephen Strelkov and Kelly Turkington

Graduate Student: Barbara Ziesman

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

1 **A quantitative PCR system for measuring *Sclerotinia sclerotiorum* in canola**
2 **(*Brassica napus*)**

3

4

5 **B. R. Ziesman,** Department of Agricultural, Food and Nutritional Science, University of
6 Alberta, Edmonton, AB, Canada T6G 2P5; **T. K. Turkington**, Lacombe Research Centre,
7 Agriculture and Agri-Food Canada, Lacombe, AB, Canada T4L 1W1; **U. Basu** and **S. E.**
8 **Strelkov**, Department of Agricultural, Food and Nutritional Science, University of Alberta,
9 Edmonton, AB, Canada T6G 2P5

10

11

12

13 Corresponding author: S. E. Strelkov, E-mail: stephen.strelkov@ualberta.ca

14

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

15 **Abstract**

16 Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum*, is an economically important disease of
17 canola (*Brassica napus*) commonly managed by routine application of fungicides. Petal
18 infestation has been demonstrated to be an important stage of the disease cycle in canola and has
19 been the focus of previously developed Sclerotinia stem rot risk assessment methods.
20 Quantitative (q)PCR analysis can provide a more rapid and accurate assessment of petal
21 infestation levels. Primers and a hydrolysis probe were designed to amplify a 70-bp region of a
22 *S. sclerotiorum*-specific gene SS1G_00263. A hydrolysis probe-based qPCR assay was
23 developed that had a detection limit of 8.0×10^{-4} ng of *S. sclerotiorum* DNA and only amplified
24 *S. sclerotiorum* DNA. Evaluation of petals collected at five sampling points in each of 10
25 commercial canola fields on each of two sampling dates (corresponding to 20-30% bloom and
26 40-50% bloom) revealed infestation levels ranging from 0 to 3.3×10^{-1} ng *S. sclerotiorum* DNA
27 per petal. This qPCR assay can be used to reliably quantify petal infestation and with further
28 research has the potential to serve as the basis for a Sclerotinia stem rot risk assessment tool or as
29 a means to study Sclerotinia stem rot epidemiology.

30

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

31 Sclerotinia stem rot, caused by the ascomycete fungus *Sclerotinia sclerotiorum* (Lib.) de
32 Bary, is an economically important disease of canola (*Brassica napus* L.) (Purdy 1979; Willets
33 and Wong 1980). Yield losses can be as high as 50%, with a yield reduction of 0.5% estimated
34 for every 1% increase in disease incidence (del Río et al. 2007). The fungus overwinters as
35 sclerotia, which can germinate carpogenically in the spring to produce apothecia (Abawi and
36 Grogan 1979; Bardin and Hwang 2001; Willets and Wong 1980). Mature apothecia will forcibly
37 release wind-borne ascospores (Abawi and Grogan 1979). The ascospores come into contact
38 with canola petals onto which they adhere, germinate and penetrate the petal tissue to initiate
39 infection (Jamaux et al. 1995; Purdy et al. 1979). Petal infestation has been shown to be an
40 important stage in the Sclerotinia stem rot disease cycle (Jamaux et al. 1995; Morrall and Dueck
41 1982). After senescence, the infected petals fall into the crop canopy and the fungus grows from
42 these petals, infecting the leaf and stem tissues. The first visible symptoms of disease consist of
43 browning on and around the cast petals. As the fungal infection spreads to the stems, the
44 characteristic symptoms of Sclerotinia stem rot appear, including bleached whitish-grey lesions
45 where the infected host stem tissues are very brittle, shredding and shattering when dry (Bolton
46 et al. 2006; Jamaux et al. 1995; Young and Werner 2012). Once the infection is well-established,
47 hard black survival structures known as sclerotia are produced by the fungus inside the stem. The
48 sclerotia can be dislodged during harvest and serve as inoculum in subsequent years.

49 The development of Sclerotinia stem rot of canola is influenced by environmental
50 conditions, in particular temperature and moisture. For carpogenic germination of the sclerotia
51 to occur, continuous soil moisture, near saturation, for about 10 days and moderate temperatures
52 are required (Abawi and Grogan 1979; Schwartz and Steadman 1978; Wu and Subbarao 2008).
53 Ascospore release has been found to be highest at 20-25°C, with the ascospores being able to

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

54 survive for up to 2 weeks in the environment (Abawi and Grogan 1979; Clarkson et al. 2003).
55 Infection of stem and leaf tissue by *S. sclerotiorum* is favored in the presence of free water and
56 moderate temperatures (15-25°C), while at extreme temperatures (<4°C and >30°C) fungal
57 growth is restricted (Bolton et al. 2006; Willets and Wong 1980).

58 The wide host range of *S. sclerotiorum*, along with the influence of temperature and
59 moisture conditions on apothecial development and subsequent plant infection, have limited the
60 effectiveness of cultural stem rot management practices. Crop rotations that include four years
61 away from a susceptible host were found to be ineffective for Sclerotinia stem rot management,
62 as a consequence of the survival of viable sclerotia in the soil and the introduction of ascospores
63 from external sources (Morrall and Dueck 1982). Sclerotinia stem rot tolerant canola cultivars
64 have become available recently in Canada, but when the risk of disease is high even these
65 cultivars can suffer damage and require fungicide application (Canola Council of Canada 2014;
66 Pratt 2012). As a consequence, the application of fungicides is the primary management tool for
67 Sclerotinia stem rot of canola. To be effective, fungicides need to be applied during the key
68 stage for infection, i.e., at flowering and before the appearance of symptoms in the crop.
69 Therefore, the application of fungicide often must be made without any objective indication of
70 disease risk (del Río et al. 2007; Koch et al. 2007; Turkington et al. 2011).

71 In an effort to predict the likelihood of Sclerotinia stem rot development in a given canola
72 crop, a petal test was developed whereby field-collected petals were plated onto potato dextrose
73 agar (PDA) and incubated for 3 to 5 days (Morrall and Thomson 1991). The cultured petals
74 were then examined for growth and the resulting colonies were identified. The proportion of
75 petals that yielded colonies of *S. sclerotiorum* was taken as the percent petal infestation. Risk
76 assessments were provided based on the statistical relationship between petal infestation and

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

77 final Sclerotinia stem rot development in the field, and were calculated based on percent petal
78 infestation (Gugel and Morrall 1986; Turkington et al. 1991). The percent petal infestation was
79 used to identify the Sclerotinia stem rot risk level as low, moderate, or high based on the
80 relationship between this parameter and disease incidence (Turkington et al. 1991; Turkington
81 and Morrall 1993). The 3 to 5 day incubation period that is required for accurate estimates of
82 petal infestation represents a potential disadvantage of this system, since timely spray decisions
83 need to be made during a fairly narrow window of crop development (McLaren et al. 2004;
84 Turkington et al. 1991). A molecular approach, such as quantitative polymerase chain reaction
85 (qPCR)-based analysis, may represent an alternative method to determine petal infestation levels
86 without the time delay associated with incubation of the petals. Moreover, PCR-based methods
87 can lower the risk of human error associated with the misidentification of the fungal cultures
88 growing out of the infested petals.

89 In recent years, many qPCR-based methodologies have been developed to detect and
90 quantify plant pathogens including *S. sclerotiorum* (Almquist and Wallenhammar 2015; Freeman
91 et al. 2002; Parker et al. 2014; Rogers et al. 2008; Yin et al. 2009). Freeman et al. (2002)
92 established a touchdown PCR assay to quantify *S. sclerotiorum* DNA in airborne ascospores of
93 the pathogen and inoculated canola petals. The primers SSFWD and SSREV amplified a 278-bp
94 fragment of ribosomal DNA and were found to be specific to *S. sclerotiorum,* even in the
95 presence of a 40-fold excess of *Botrytis cinerea* DNA. However, this primer set was unable to
96 distinguish between the DNA of *S. sclerotiorum* and that of close relatives such as *S. minor* and
97 *S. trifoliorum*. Nonetheless, Almquist and Wallenhammar (2015) were able to use the primers of
98 Freeman et al. (2002) to develop a hydrolysis probe-based qPCR assay for detection of *S.*
99 *sclerotiorum* DNA in air samples and infected leaves and petals of oilseed rape (*B. napus*) in

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

100 central Sweden. Rogers et al. (2008) described an intercalating dye-based qPCR assay to
101 quantify airborne ascospores of *S. sclerotiorum* around oilseed rape crops near Rothamsted,
102 United Kingdom, using primers designed to amplify a region of the mitochondrial small subunit
103 rRNA and open reading frame 1 (ORF1) of the fungus. This assay could detect *S. sclerotiorum*
104 DNA with a high level of sensitivity and specificity, even in the presence of DNA from the
105 closely related fungus *B. cinerea*, except at 5×10^{-5} ng of DNA where the sensitivity of the
106 quantification of *S. sclerotiorum* DNA was reduced (Rogers et al. 2008). Parker et al. (2014)
107 developed another intercalating dye-based qPCR assay for the quantification of airborne
108 ascospores of *S. sclerotiorum*, as part of a model for forecasting Sclerotinia rot of carrot (*Daucus*
109 *carota* ssp. *sativus*) The assay targeted the group I intron of the mitochondrial small subunit
110 (MtSSu) rRNA, and could detect 93% of the *S. sclerotiorum* isolates tested, although the
111 quantification cycle (C_q) values varied for different isolates. While this assay may be useful for
112 the detection of airborne ascospores of *S. sclerotiorum* on a regional scale, 7% of the isolates
113 tested could not be detected, indicating the potential for false negatives. The variation in C_q
114 values observed for different isolates also is a cause for concern, since it is indicative of
115 differences in the ability to quantify isolates. This likely would result in inconsistencies in the
116 quantification of the same amount of inoculum, depending upon which isolate is tested,
117 particularly if the standard curve is based on another isolate. Moreover, on carrots there is no
118 initial petal infestation stage, with infection first occurring on senesced leaves in a closed
119 canopy, and the additional risk of postharvest infection (Kora et al. 2005).

120 Most relevant to the current study, Yin et al. (2009) developed an intercalating dye-based
121 qPCR assay for the quantification of *S. sclerotiorum* DNA on canola petals in China. The
122 primers (SsF and SsR) targeted a region amplified by the microsatellite primer M13 (Ma et al.

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

123 2003). The authors reported that this assay was very specific to *S. sclerotiorum* and could
124 quantify as little as 5.0×10^{-4} ng DNA of the fungus. The specificity of the primers SsF/SsR,
125 however, was not validated by testing with DNA of pure cultures of other *Sclerotinia* species
126 such as *S. minor* or *S. trifoliorum*. Analysis of infected canola petals resulted in quantifications
127 ranging from 2.5×10^{-2} ng to 1.1×10^{-1} ng *S. sclerotiorum* DNA per mg canola petal tissue. This
128 assay may have value as the basis for a risk assessment tool in canola production systems in
129 Canada. Nevertheless, because of evidence of a high level of genetic variation in the *S.*
130 *sclerotiorum* population (Carpenter et al. 1999; Sirjusingh and Kohn 2001), specificity testing
131 would be needed to ensure that Canadian isolates of the fungus can be detected with no false
132 positives. In addition, it would be important to ensure that there is no difference in the detection
133 and quantification of different *S. sclerotiorum* isolates.

134 The objective of the current study was to develop a *S. sclerotiorum*-specific qPCR assay
135 for the quantification of Canadian isolates of *S. sclerotiorum* on canola petals using a hydrolysis
136 probe. Development of this assay was focused on petals rather than airborne inoculum because
137 of the importance of petal infestation in the disease cycle of Sclerotinia stem rot of canola. In
138 addition, estimates of petal infestation may provide a better field-specific indication of disease
139 risk, whilst detection of airborne inoculum would be best for regional risk assessments. The
140 qPCR assay described in this study also was compared with the qPCR assay developed by Yin et
141 al. (2009), in order to evaluate the potential utility of each method for quantifying *S.*
142 *sclerotiorum* DNA on canola petals.

143 Materials and Methods

144 **DNA isolation from fungal mycelium.** Pure fungal cultures of *S. sclerotiorum* and the
145 other fungal species used for specificity testing (Table 1) were grown on Difco potato dextrose

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

146 broth (Dickinson and Company, Sparks, MD, USA) that was amended with 25 ppm ampicillin
147 (Life Technologies, Carlsbad, CA, USA) and streptomycin (Sigma Chemical Company, St,
148 Louis, MO, USA) in 200 ml Erlenmeyer flasks. The cultures were grown at room temperature
149 (approximately 20-24°C) under natural light provided by a north-facing window with gentle
150 agitation for approximately 7 days, until a large mass of mycelium had formed. The supernatant
151 was decanted and the mycelium washed with sterile water, frozen at -80°C, and lyophilized in a
152 freeze-drier. Approximately 20 mg of lyophilized tissue was homogenized to a powder in a 1.5
153 ml microcentrifuge tube with a hand-held plastic pestle, and the DNA was isolated with a Wizard
154 Genomic DNA purification kit (Promega, Madison, WI, USA) as per the manufacturer's
155 instructions, but with the addition of a phenol/chloroform/isoamyl alcohol (25:24:1, v:v:v)
156 purification step. Briefly, following the protein precipitation step, three
157 phenol/chloroform/isoamyl alcohol extractions were performed. In each extraction, 600 µl of
158 phenol/chloroform/isoamyl alcohol (25:24:1, v:v:v) was added and the solution mixed and
159 centrifuged. The DNA was quantified with a NanoDrop 2000c spectrophotometer (Thermo
160 Scientific, Waltham, MA, USA), and the 260 nm/280 nm and 260 nm/230 nm absorbance ratios
161 were calculated as an indication of DNA quality prior to qPCR analysis.

162 **DNA isolation from field-collected canola petals.** To validate the qPCR assay, canola
163 petals were collected from 10 commercial fields in the Edmonton, AB, region in 2012. These 10
164 fields were farmed by three different farmers and were known to have a history of Sclerotinia
165 stem rot. The fields were sampled once at 20-30% bloom and again at 40-50% bloom, as
166 determined by visual assessment (Harper and Berkenkamp 1975). These levels of flowering
167 correspond to growth stages 62-63 and 64-65, respectively, on the BBCH scale (Webber and
168 Bleiholder 1990; Lancashire et al. 1991). Sampling dates varied slightly in each field depending

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

169 on crop stage, but in general, sampling at 20-30% bloom was carried out in the first week of
170 July, followed by sampling at 40-50% bloom 3-4 days later. The incidence of Sclerotinia stem
171 rot also was assessed at the end of the growing season for future reference. Petals were collected
172 at each of five sampling sites within each field. The sampling sites were located 100 m from the
173 edge of each field and were situated 50 m apart along a fungicide-free check strip. At each
174 sampling site, the top 10-20 cm of the inflorescences from each of 20 plants were collected at
175 random, placed in a plastic bag, and stored in a cooler on ice for transport back to the laboratory.
176 A minimum of 80 flowers were selected randomly from the sampled inflorescences and stored in
177 an ultra-low temperature freezer (-80°C) until further processing.

178 DNA was isolated according to the protocol of Liang et al. (2013) from a randomly
179 chosen subsample of 20 petals from each sampling site. Petal samples were homogenized with a
180 TissueLyser II (Qiagen, Toronto, ON, Canada) and a single 5-mm stainless steel bead in a 2-ml
181 locking tube. The TissueLyser II adapters and petal samples were frozen at -80°C prior to
182 homogenization to prevent damage to the DNA. Prior to elution, the DNA pellets were dried in
183 a Vacufuge Plus (Eppendorf, Mississauga, ON, Canada) for 7 min at 45°C. The extracted DNA
184 was eluted with 30 μl of nuclease free water (Life Technologies, Carlsbad, CA, USA) and
185 quantified with a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA)
186 as above.

187 **Development of *S. sclerotiorum*-specific primers.** Forward and reverse primers and a
188 hydrolysis probe were designed using Primer 3 Express (Applied Biosystems, Carlsbad, CA,
189 USA) based on the 468-bp sequence of a single-copy gene (SS1G_00263) encoding the
190 hypothetical secreted protein ssv263 (Liang et al. 2013) from *S. sclerotiorum* (GenBank
191 accession no. XM_001598127). This gene is highly specific to *S. sclerotiorum* and orthologous

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

192 to a protein-encoding gene BC1G_00896 (GenBank accession no. XM_001560818) from *B.*
193 *cinerea* (Liang et al. 2013; Shah et al. 2009). The forward (SSBZF) and reverse (SSBZR)
194 primers were designed to amplify a 70-bp product in a region of SS1G_00263 that exhibits the
195 greatest difference with the *B. cinerea* orthologue. The hydrolysis probe (SSBZP) was labeled
196 with a non-fluorescent quencher-mini groove binder (NFQ-MGB) on the 3' end, and with the
197 reporter dye FAM (6-carboxyfluorescein) on the 5' end (Table 1). The amplified region shares
198 71% similarity with the orthologous gene in *B. cinerea*, but includes 20-bp pair mismatches, 17
199 of which are covered by the primer and probe sequences. The probe and primer sequences were
200 used to query the GenBank databases using the Basic Local Alignment Search Tool (BLAST) in
201 order to identify any similar sequences from other organisms that could lead to false positives.
202 **Development of the exogenous positive internal control.** To identify false negatives
203 resulting from failed DNA extraction or inhibition of the PCR, an exogenous internal control was
204 included in the analysis of all samples. The *ToxB* gene from *Pyrenophora tritici-repentis*, causal
205 agent of tan spot of wheat (Lamari and Strelkov 2010), was selected as the exogenous internal
206 control since it is unlikely to be found in canola petals. The pSilent1 plasmid (Nakayashiki et al.
207 2005) containing a 432-bp fragment of *ToxB* from *P. tritici-repentis* (Aboukhaddour et al. 2012)
208 was used as the template for the design of the primers and hydrolysis probe. The *ToxB*-specific
209 primers (ToxBF and ToxBR) and a probe (ToxBP) were designed using Primer 3 Express
210 (Applied Biosystems). The *ToxB*-specific hydrolysis probe was labelled with NFQ-MGB on the
211 3' end and with the reporter dye VIC on the 5' end.
212 For use as an internal control, every canola petal sample was spiked with 2×10^6
213 plasmids containing the *ToxB* gene prior to DNA isolation. Each sample was analyzed in two
214 separate singleplex qPCR reactions, one with the *ToxB* primers and probe set and another with

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

215 the *S. sclerotiorum*-specific primers and probe. To identify outliers and potential error, the
216 standard deviation of the C_q values obtained for the internal control in a set of samples was
217 calculated. If the standard deviation was close to or < 1.0 for a set of samples, the variation in
218 the internal control and the risk of false negatives as a result of failed DNA isolation or PCR
219 inhibition were regarded as low. Any samples that were outside of this range were discarded and
220 DNA was isolated again.

221 **qPCR analysis.** The terminology used to describe the qPCR analysis will be as
222 suggested in the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR
223 Experiments) guidelines (Bustin et al. 2009). All qPCR analyses were conducted in a ViiA7
224 Real-Time PCR System (Life Technologies) using the Universal FastStart Master (Rox) mix
225 (Roche, Indianapolis, IN, USA) in a MicroAmp® Fast Optical 96-well reaction plate (Applied
226 Biosystems), which was sealed with MicroAmp optical adhesive film (Applied Biosystems,).
227 Each qPCR was conducted in a total volume of 10 µl, including 0.1 µl of each forward and
228 reverse primer (50 µM SSBZF and 50 µM SSBZR, respectively), 0.03 µl of the hydrolysis probe
229 (100 µM SSBZP), 5 µl of the 2× master mix (Rox), 0.77 µl of molecular grade water (Life
230 Technologies), and 4 µl of the template DNA or negative control. For the quantification of the
231 internal control, the reaction mixture was as above except that 0.025 µl of the 100 µM ToxBP
232 hydrolysis probe and 0.775 µl of molecular grade water (Life Technologies) were added and the
233 primers ToxBF/ToxBR were substituted for SSBZF/SSBZR. The reaction conditions included a
234 hot start at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec as per
235 the manufacturer's instructions for the master mix. Each of the primer/probe sets was analyzed
236 separately and reactions were not multiplexed. Each sample was analyzed in triplicate for
237 quantification of the SS1G_00263 target, while samples were analyzed in duplicate for assays of

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

238 the internal control. If the standard deviation of the mean C_q obtained for a set of triplicates of a
239 given sample was > 0.5, the sample was reanalyzed, and if an outlier was present, it was
240 removed. Similarly, if the standard deviation of the mean C_q obtained for duplicates of the *ToxB*
241 internal control was > 0.5, the sample was re-extracted.

242 **Specificity testing.** The specificity of the SS1G_00263 primer and probe set was
243 confirmed by separately testing 100 ng of DNA from 13 fungal species and the canola host as
244 templates in the qPCR analysis (Table 1). In addition, amplification of a total of eight isolates of
245 *B. cinerea* and seven isolates of *S. sclerotiorum* was compared. No amplification of any product,
246 or a C_q value > 2 cycles below that of the smallest standard, were considered to be indicative of
247 no detection. The reproducibility of the standard curve was assessed by calculating the standard
248 deviation at each point, for 4 replications included in separate 96-well plates. Plasmids
249 containing the *ToxB* insert were analyzed with the SS1G_00263-specific primer set to ensure that
250 SSBZF/SSBZR did not amplify the internal control.

251 **Sequencing of PCR products.** Amplicons obtained with the SSBZF/SSBZR primer set
252 were sequenced to confirm the identity of the product. Conventional PCR was used to increase
253 the amount of amplicon obtained from pure mycelial DNA of six *S. sclerotiorum* isolates
254 (UAMH 6321, UAMH 4514, UAMH 9192, SSA-11, SSB-11, SSD-11) for sequencing purposes.
255 The PCR analysis was carried out in a 25 µl reaction volume, which consisted of 15.4 µl
256 molecular grade water (Life Technologies), 2 µl of 10× PCR buffer (no magnesium chloride)
257 (Invitrogen by Life Technologies, Carlsbad, CA, USA), 1 µl 2.5mM each dNTP, 1 µl 50 mM
258 $MgCl_2$, 0.2 µl of each 50 mM SSBZF and SSBZR, 0.2 µl Platinum Taq DNA Polymerase
259 (Intvitrogen), and 5 µl (100 ng) template DNA. The reaction conditions were 94°C for 3 min,
260 followed by 40 cycles of 94°C for 30 sec, 60°C for 20 sec and 72°C for 5 min, and ended with an

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

261 incubation at 72°C for 5 min followed by a constant 4°C. Polymerase chain reaction products
262 were resolved on a 2% agarose gel run at 90V for 50 min. The amplicon band was extracted
263 using a Qiaquick Gel Extraction Kit (Qiagen) and sent to the Molecular Biology Service Unit,
264 University of Alberta, Edmonton, AB, for sequencing. Sequencing was conducted on a 3730
265 Genetic Analyzer (Applied Biosystems) with Sanger cycle sequencing using fluorescently
266 labelled dye terminators and BigDye Terminator v1.1 chemistry.

267 **Sensitivity testing.** To test the limit of detection (LOD) of the assay, standard curves
268 were generated with 1:10 serial dilutions of purified *S. sclerotiorum* DNA. All standard curves
269 tested consisted of five serial dilutions ranging from 8.0 ng to 8.0×10^{-4} ng or from 5.0 ng to 5.0
270 $\times 10^{-4}$ ng. Each standard was included in triplicate in the same 96-well plate as the samples
271 being analyzed. The LOD was regarded as the lowest quantity of DNA that could be detected
272 with confidence in all three of the triplicates. The efficiency and R^2 of the standard curves were
273 determined for each replication. The reproducibility of the standard curve was assessed by
274 calculating the standard deviation at each point, for 4 replications included in separate 96-well
275 plates. A standard deviation of < 0.5 for the average Cq values of the 4 replications was
276 considered to indicate that the assay was reliable.

277 To determine if inclusion of the internal control and the presence of *B. napus* DNA
278 reduced the LOD for *S. sclerotiorum*, fungal DNA was quantified in the presence or absence of
279 the *ToxB* internal control and *B. napus* DNA. A 1:5 dilution series ranging from 4.0×10^1 ng to
280 5.0×10^{-4} ng *S. sclerotiorum* DNA was spiked with 2 µl *B. napus* DNA and 2×10^5 copies of the
281 plasmid containing the internal control. The *B. napus* DNA represented a 1:5 dilution of total
282 genomic DNA extracted from a set of 20 canola petals that were not infected with *S.*

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

283 *sclerotiorum*. The mean C_q and standard deviation of the mean for each point in the dilution
284 series were compared between the spiked and non-spiked samples.

285 **Evaluation of canola petals.** Total genomic DNA isolated from samples consisting of
286 20 canola petals each was diluted by a factor of 1:5 with nuclease free water (Life Technologies)
287 prior to qPCR analysis with the SS1G_00263- and *ToxB*-specific primers and probes. DNA was
288 analyzed from samples collected at five sampling sites from each of 10 commercial canola fields
289 at each of two sampling dates as described above. Results are expressed on a per petal basis by
290 first accounting for the dilution, then by dividing the estimate by 20. The qPCR estimates were
291 averaged over each field on each sampling date and the standard deviation of the mean was
292 calculated for each field. Any sample from which no *S. sclerotiorum* DNA could be amplified,
293 or which was below the lowest standard outside the range of the standard curve, was recorded as
294 0 ng *S. sclerotiorum* DNA per canola petal.

295 **Assessment of the SsF and SsR primer set.** Isolates of *S. sclerotiorum* collected from
296 canola fields in central Alberta were subjected to qPCR analysis with the primers SsF (5'
297 AGTCGAGGGACGGGTACTAA 3') and SsR (5' CTTGTCCTCATTGCCGTTT 3') developed
298 by Yin et al. (2009). The primers were evaluated using Dynamite qPCR Mastermix (Molecular
299 Biology Service Unit, University of Alberta, Edmonton, Canada) instead of the SYBR Premix
300 Ex *Taq* (TaKaRa Biotechnology Co. Ltd., Dalian, China) used by Yin et al. (2009), but both of
301 these master mixes rely on the intercalating dye SYBR Green as the basis of detection. The
302 reaction conditions consisted of an initial heat denaturation at 95°C for 2 min, followed by 40
303 cycles of 95°C for 15 sec and 60°C for 60 sec, as recommended by the manufacturer. These
304 differ slightly from the conditions used by Yin et al. (2009), but are optimized for the Dynamite
305 qPCR Mastermix utilized in the current analysis. Quantitative PCR assays were conducted in a

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

306 Step One Plus Real-Time PCR System (Applied Biosystems) in a 10 µl total reaction volume,
307 containing 5 µl Dynamite qPCR Mastermix, 0.8 µl molecular grade water, 0.1µl of each of 50
308 mM SsF and SsR, and 4 µl template DNA solution. After the reaction was complete, a melting
309 point analysis was conducted to confirm the presence of a single amplification product that had a
310 melting temperature (T_M) consistent with the predicted T_M of 84°C. The specificity of the
311 SsF/SsR primer set also was evaluated as described above for the other primer and probe sets.

312 **Results**

313 **Amplicon generated with SSBZF/SSBZR.** The amplicons generated from six isolates
314 of *S. sclerotiorum* with the SSBZF/SSBZR primer set targeting the gene SS1G_00263 were
315 sequenced and confirmed to be of the expected 70-bp size. Moreover, the sequences were
316 identical to each other, and a query of the GenBank database using BLASTN revealed 100%
317 identity with accession number XM_001598127.1 from *S. sclerotiorum* (e-value = 3e-31).

318 **Specificity testing.** While the SS1G_00263-specific primers consistently amplified a 70-
319 bp product from DNA of each of the *S. sclerotiorum* isolates evaluated, they did not generate an
320 amplicon from any of the other 13 species tested, including the closely related *B. cinerea*, *S.*
321 *trifoliorum*, or *S. minor* (Table 3). Similarly, no amplicon was obtained from DNA of the host
322 canola plant. Specificity testing with the *ToxB*-specific primers did not yield an amplicon from
323 *S. sclerotiorum* or any of the non-target organisms tested.

324 **Sensitivity testing.** The lowest reliable LOD was 8.0×10^{-4} ng *S. sclerotiorum* DNA.
325 Although the assay could detect quantities of *S. sclerotiorum* DNA as small as 5.0×10^{-4} ng, such
326 detection could not be accomplished with a standard deviation of the mean $C_q < 0.5$. As such,
327 the lowest point of the standard curve was set to 8.0×10^{-4} ng DNA. To compare replicates of
328 the standard curve, the C_q values were averaged for a set of comparisons and the standard

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

329 deviation of the mean for each point on the curve was calculated. An average standard deviation
330 of 0.19 was obtained for the mean C_q over all five points on the curve, among four replicates of
331 the standard curve in separate 96-well plates; none of the points had a standard deviation > 0.5
332 (Fig. 1). For the four replicates of the standard curve generated in separate 96-well plates, the
333 PCR efficiencies ranged from 88.90 to 90.25 and the R^2 values ranged from 0.9928 to 0.9999.
334 Curves generated with the SS1G_00263-specific primers in the presence or absence of
335 the internal control and *B. napus* DNA were compared to identify any potential reduction in the
336 quantification of *S. sclerotiorum* DNA. The C_q values were averaged for each dilution point in
337 the two dilution series and the standard deviation of the C_q values for the replicates was
338 calculated. Each point on the standard curves was compared individually and no single point had
339 a standard deviation of the mean C_q > 0.54 (Fig 2).
340 **Quantification of *S. sclerotiorum* in canola petals.** Total genomic DNA extracted from
341 20 petals from each of five sampling sites in each of 10 commercial canola fields on two
342 different sampling dates (100 DNA samples in total) was used as a template for qPCR analysis.
343 The petal infestation estimates for individual sampling sites ranged from 0 ng to 3.3×10^{-1} ng *S.*
344 *sclerotiorum* DNA per petal. The amount of *S. sclerotiorum* DNA in each of the 5 sampling
345 sites per field was averaged to give a single petal infestation estimate for each field on each
346 sampling date (Fig. 3). On the first sampling date, when the canola was at 20-30% bloom,
347 average infestation in the 10 fields ranged from a mean (± standard deviation) of 6.0×10^{-3} ng ±
348 7.0×10^{-3} ng to 3.4×10^{-2} ng ± 2.8×10^{-2} ng *S. sclerotiorum* DNA per petal (C_q values = 30.5-
349 36.1). On the second sampling date, at 40-50% bloom, average infestation in the 10 fields
350 ranged from a mean (± standard deviation) of 1.0×10^{-3} ng ± 1.0×10^{-3} ng to 8.0×10^{-2} ng ±1.4
351 $\times 10^{-1}$ ng *S. sclerotiorum* DNA per petal (C_q values = 28.9-36.1).

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

352 In addition to the analysis with the SS1G_00263-specific primer and probe set, all
353 samples were analyzed with the *ToxB* (internal control)-specific primer and probe set in separate,
354 singleplex qPCR assays. The mean and standard deviation of the mean C_q value for duplicates
355 was calculated for early bloom and late bloom sample sets to get an estimate of the amount of
356 variation. At 20-30% bloom, the overall mean C_q (± standard deviation) was 29.6 ± 1.08, and at
357 40-50% bloom, the overall mean C_q (± standard deviation) was 27.6 ± 0.70.

358 **Assessment of the SsF and SsR primers.** The primers SsF and SsR (Yin et al. 2009)
359 were evaluated to determine their suitability for quantifying *S. sclerotiorum* DNA in canola
360 petals under Canadian conditions. The primers amplified a product of approximately 225-bp
361 from all isolates of *S. sclerotiorum*, and did not amplify DNA of *S. minor*, *Aspergillus niger*,
362 *Cladosporium* sp., *Mucor* sp., *Alternaria alternata*, or *Leptosphaeria maculans* (Table 3).
363 However, the primers were found to amplify products from DNA of *S. trifoliorum*, as well as
364 from all isolates tested of *B. cinerea* and *B. fuckeliana*, *Rhizopus* sp., *Trichoderma* sp.,
365 *Rhizoctonia solani*, *Penicillium* sp., and *Fusarium graminearum*. The amplicons obtained from
366 *B. cinerea*, *S. trifoliorum* and *Trichoderma* sp. all had temperature peaks between 84 and 85°C,
367 which was within the range expected for the product from *S. sclerotiorum*. For the products
368 amplified from DNA of *B. fuckeliana*, *Rhizopus* sp., *R. solani*, *Penicillium* sp., and *F.*
369 *graminearum*, the melting curve analysis revealed the presence of multiple temperature peaks
370 associated with non-specific amplification. The sensitivity of the SsF/SsR primer set was similar
371 to that of the SSBZF/SSBFR primers, with a consistent LOD of 8.0×10^{-4} ng DNA.

372 **Discussion**

373 This study focussed on the development of a qPCR-based assay for the quantification of
374 *S. sclerotiorum* DNA in canola petals, to enable rapid and accurate estimates of infestation

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

375 levels. The assay targets a 70-bp region of a single-copy gene encoding the hypothetical secreted
376 protein ssv263 (Liang et al. 2013). While the target region of the gene shares 71% similarity
377 with an orthologue in *B. cinerea*, specificity testing indicated that there was no amplification of
378 DNA from any of six *B. cinerea* isolates tested. The qPCR assay also did not amplify DNA from
379 any of the 13 other species evaluated in this study, including *S. minor, S. trifoliorum* and the host
380 plant, *B. napus*. These results indicate that the assay is highly specific for *S. sclerotiorum*, and
381 can be used to estimate pathogen biomass in canola petals.

382 The sensitivity tests revealed a consistent LOD of 8.0×10^{-4} ng *S. sclerotiorum* DNA.
383 Rogers et al. (2008) reported that an ascospore of *S. sclerotiorum* is equivalent to about $3.5 \times 10^{-}$
384 ^{4} ng of DNA, suggesting a LOD of 2.3 ascospores per petal for the current assay. When adjusted
385 to account for the dilution of DNA from canola petals during extraction and analysis, the LOD
386 would correspond to 1.5×10^{-3} ng *S. sclerotiorum* DNA or 4.3 ascospores per petal. An issue
387 with the use of ascospores as the basis for a standard curve to quantify petal infestation, however,
388 is that this approach will not reflect increases in pathogen biomass associated with spore
389 germination. Ascospores have been shown to begin germination as early as 3 hours after release
390 from the apothecium under favorable environmental conditions (Willets and Wong 1980).
391 Therefore, it is likely that any assay examining the amount of *S. sclerotiorum* DNA on canola
392 petals is measuring DNA from the ascospores as well as from hyphae developing from
393 germinated spores. As such, quantification of *S. sclerotiorum* on petals is more meaningful and
394 accurate when expressed as the total amount of pathogen DNA. The specificity of the assay was
395 not affected by the presence of *B. napus* DNA or of an internal control in the samples. These are
396 important considerations if the assay is to be used to measure infestation of petals collected in the
397 field.

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

398 Yin et al. (2009) developed a qPCR assay to measure *S. sclerotiorum* DNA in canola
399 petals using intercalating dye (SYBR Green) detection technology that detected between 0.0252
400 ng-0.111 ng pathogen DNA per mg of canola petal tissue. Evaluation of the primers developed
401 by Yin et al. (2009) on DNA extracted from Canadian isolates of *S. sclerotiorum* and other
402 fungal species, using a different master mix but the same dye detection technology, indicated
403 sensitivity similar to that of the assay described in this report. However, the specificity of the
404 primers seemed considerably lower and amplicons were obtained from various non-target
405 species including *S. trifoliorum, B. cinerea, F. graminearum, Rhizopus* sp., and *Penicillium* sp.
406 In their original report, Yin et al. (2009) found no amplification of DNA other than from *S.*
407 *sclerotiorum*, but only evaluated one isolate of *B. cinerea* (*B. fuckeliana*) and did not test *S.*
408 *minor, S. trifoliorum,* or *Rhizopus* sp. They did test *R. solani, Penicillium* sp. and *F.*
409 *graminearum*, but did not detect any product from the isolates evaluated. This lower specificity
410 represents a challenge for application of the earlier protocol (Yin et al. 2009) in the analysis of
411 field samples, and hence for its use as the basis for a Sclerotinia stem rot risk assessment system.
412 While it may be possible to identify non-specific amplification products based on a melting
413 curve analysis, this would complicate the procedure and introduce the possibility of additional
414 errors. Moreover, the quantification of field samples containing a range of different fungal
415 species in addition to *S. sclerotiorum* may be difficult. The similar sensitivity, but increased
416 specificity of the assay developed in this study with the primers SSBZF/SSBZR and probe
417 SSBZP may reflect the use of a hydrolysis probe instead of an intercalating dye. An
418 intercalating dye, such as SYBR green, binds to any double-stranded DNA and releases a
419 fluorescent signal (Ririe et al. 1997). This potentially can result in fluorescent amplification of
420 non-target double-stranded DNA that must be further differentiated through melting curve

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

421 analysis (Smith and Osborn 2008). The use of a hydrolysis probe ensures that a fluorescent
422 signal is produced only when there is an exact match between the probe and the target (Smith
423 and Osborn 2008). This increased level of specificity reduces the likelihood of false positives
424 when analysing diverse field samples, without compromising sensitivity. As a result, the newly
425 developed qPCR assay may represent a more reliable method to quantify *S. sclerotiorum* DNA in
426 canola petals.

427 When the hydrolysis probe-based qPCR assay was used to quantify *S. sclerotiorum* in
428 field-collected canola petals, considerable variation was observed in the amount of petal
429 infestation in different fields and at different crop stages. Environmental conditions in 2012
430 were conducive to stem rot development in the sampled fields. In five of 10 fields, infestation
431 levels were highest at 40-50% bloom, while in the remaining five fields, the infestation levels
432 were highest at 20-30% bloom. This is consistent with the findings of Turkington and Morrall
433 (1993), who reported changes in petal infestation from early to late bloom and at different
434 locations when culturing petals on growth medium. Similarly, using qPCR analysis, Almquist
435 and Wallenhammer (2015) also observed differences in *S. sclerotiorum* incidence at different
436 stages of flowering. The evaluation of petals by qPCR analysis over the flowering period could
437 provide an indication of when inoculum levels are highest, and thus when the crop may benefit
438 most from fungicide application. The variation in the amount of *S. sclerotiorum* DNA between
439 locations may reflect differences in inoculum level or in the timing of ascospore release, crop
440 seeding date and growth stage, canopy density, or microclimatic conditions. It also indicates the
441 importance of assessing petal infestation and risk potential for a particular field as opposed to an
442 assessment of risk based on regional conditions. The inclusion of an internal control helped to

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

443 identify outliers resulting from possible human error or other artifacts, such as inhibition of the
444 PCR, thereby improving the accuracy of qPCR-based estimates of petal infestation.

445 The qPCR assay described in this study may serve as the basis for a risk assessment
446 system, as well as representing a useful tool for the study of the epidemiology of Sclerotinia stem
447 rot of canola. It can quantify the level of petal infestation, a key stage in the Sclerotinia stem rot
448 disease cycle, thereby providing a measure of disease risk when timely fungicide application
449 decisions need to be made. To further investigate the possibility of developing a forecasting
450 system based on this technology, the relationship between the petal infestation estimates and
451 final stem rot incidence must be determined. The strength of the relationship between petal
452 infestation as assessed through qPCR analysis and final disease incidence can be investigated
453 with the use of regression analysis. It is also important to emphasize that a forecasting system
454 based on qPCR quantification of petal infestation should be linked to environmental conditions,
455 as well as cropping history, seeding date and crop canopy conditions, which may influence
456 Sclerotinia stem rot development and the need to spray a fungicide. Validation as a predictive
457 tool under field conditions is the focus of a follow-up study.

458 **Acknowledgements**

459 This work would not have been possible without the generous support of the Alberta
460 Crop Industry Development Fund, the Alberta Canola Producers Commission, the Western
461 Grains Research Foundation, and Agriculture and Agri-Food Canada and the Canola Council of
462 Canada via the Growing Forward program. We thank Dr. R. J. Howard (Alberta Agriculture and
463 Rural Development, Brooks, AB) for providing isolates of *B. cinerea*, Dr. R. Aboukhaddour
464 (University of Alberta) for providing the plasmid used to develop the internal control, and Ileana
465 Strelkov, Derek Rennie, Troy Locke and Dr. Tiesen Cao (University of Alberta) for technical

466 assistance. We also thank Emile deMilliano and Ryan Adams (Crop Production Systems, Ft.
467 Saskatchewan, AB) and several cooperating farmers for assisting with petal collection and
468 including a fungicide-free check strip in their crops.

469 **Literature Cited**

470 Abawi, G. S., and Grogan R. G. 1979. Epidemiology of diseases caused by *Sclerotinia* species.
471 Phytopathology 69:899-904.

472 Aboukhaddour, R., Kim, Y. M., and Strelkov, S. E. 2012. RNA-mediated gene silencing of *ToxB*
473 in *Pyrenophora tritici-repentis*. Mol. Plant Pathol. 13:318-326.

474 Almquist, C., and Wallenhammar, A.- C. 2015. Monitoring of plant and airborne inoculum of
475 *Sclerotinia sclerotiorum* in spring oilseed rape using real-time PCR. Plant Pathol. 64: 109-118.

476 Bardin, S. D., and Huang, H. C. 2001. Research on biology and control of *Sclerotinia* diseases in
477 Canada. Can. J. Plant Pathol. 23:88-98.

478 Boland, G. J., and Hall, R. 1994. Index of plant hosts of *Sclerotinia sclerotiorum*. Can. J. Plant
479 Pathol.16:92-108.

480 Bolton, M. D., Bart, T., Thomma, P. H. J., and Nelson, B. D. 2006. *Sclerotinia sclerotiorum*
481 (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. Mol. Plant Pathol. 7:1-
482 16.

483 Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R.,
484 Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., and Wittwer, C. T. 2009. The MIQE
485 quidelines: Minimum information for publication of quantitative real-time PCR experiments.
486 Clinical Chem. 55:611-622.

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

487 Canola Council of Canada. 2014. Canola encyclopedia: Sclerotinia stem rot. Canola Council of
488 Canada, Winnipeg, Manitoba, Canada. [Online] Available:
489 http://www.canolacouncil.org/canola-encyclopedia/diseases/sclerotinia-stem-rot/#variety-
490 selection-and-genetic-resistancetolerance. Verified: 15 October 2014.

491 Carpenter, M. A., Frampton, C., and Stewart, A. 1999. Genetic variation in New Zealand
492 populations of the plant pathogen *Sclerotinia sclerotiorum*. NZ J. Crop Hortic. Sci. 27:13-21.

493 Clarkson, J. P., Staverley, J., Phelps, K., Young, C. S., and Whipps, J. M. 2003. Ascospore
494 release and survival in *Sclerotinia* sclerotiorum. Mycol. Res. 107:213-222.

495 del Río, L. E., Bradley, C. A., Hensen, R. A., Endres, G. J., Hanson, B. K., McKay, K.,
496 Halvorson, M., Porter, P. M., Le Gare, D. G., and Lamey, H. A. 2007. Impact of sclerotinia stem
497 rot on yield of canola. Plant Dis. 91:191-194.

498 Freeman, J., Ward, E., Calderon, C., and McCartney, A. 2002. A polymerase chain reaction
499 (PCR) assay for the detection of inoculum of *Sclerotinia sclerotiorum*. Eur. J. Plant Pathol.
500 108:877-886.

501 Gugel, R. K., and Morrall, R. A. A. 1986. Inoculum disease relationships in sclerotinia stem rot
502 of rapeseed in Saskatchewan. Can. J. Plant Pathol. 8:89-96.

503 Harper, F. R., and Berkenkamp, B. 1975. Revised growth-stage key for *Brassica campestris* and
504 *B. napus*. Can. J. Plant Sci. 55:657-658.

505 Jamaux, I., Gelie, B., and Lamarque, C. 1995. Early stages of infection of rapeseed petals and
506 leaves by *Sclerotinia sclerotiorum* revealed by scanning electron microscopy. Plant Pathol.
507 44:22-30.

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

508 Koch, S., Dunker, S., Kleinhenz, B., Röhrig, M., and Von Tiedemann, A. 2007. A crop loss-
509 related forecasting model for Sclerotinia stem rot in winter oilseed rape. Phytopathology
510 97:1186-1194.

511 Kora, C., McDonald, M. R., and Boland, G. J. 2005. Epidemiology of sclerotinia rot of carrot
512 caused by *Sclerotinia sclerotiorum*. Can. J. Plant Pathol. 27:245-258.

513 Lamari, L., and Strelkov, S. E. 2010. The wheat/*Pyrenophora tritici-repentis* interaction:
514 progress towards an understanding of tan spot disease. Can. J. Plant Pathol. 32:4-10.

515 Lancashire, P. D., Bleiholder, H., Langelüddecke, P., Stauss, R., Van Den Boom, T., Weber, E.,
516 and Witzen-Berger, A. 1991. A uniform decimal code for growth stages of crops and weeds.
517 Ann. Appl. Biol. 119:561-601.

518 Liang, Y., Yajima, W., Davis, M. R., Kav, N. N. V., and Strelkov, S. E. 2013. Disruption of a
519 gene encoding a hypothetical secreted protein from *Sclerotinia sclerotiorum* reduces its virulence
520 on canola (*Brassica napus)*. Can. J. Plant Pathol. 35:46-55.

521 Ma, Z., Luo, Y., and Michailides, T. J. 2003. Nested PCR assays for detection of *Manilinia*
522 *fruticola* in stone fruit orchards and *Botryosphaeria dothidea* from pistachios in California. J.
523 Phytopathol. 151:312-322.

524 McLaren, D. L., Conner, R. L., Platford, R. G., Lamb, J. L., Lamey, H. A., and Kutcher, H. R.
525 2004. Predicting diseases caused by *Sclerotinia sclerotiorum*on canola and bean – a western
526 Canadian perspective. Can. J. Plant Pathol. 26:489-497.

527 Morrall, R. A. A., and Thomson, J. R. 1991. Petal test manual for *Sclerotinia* in canola.
528 University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

529 Morrall, R. A. A., and Dueck, J. 1982. Epidemiology of sclerotinia stem rot of rapeseed in
530 Saskatchewan. Can J. Plant Pathol. 4:161-168.

531 Nakayashiki, H., Hanada, S., Nguyen, B. Q., Kadotani, N., Tosa, Y., and Mayama, S. 2005.
532 RNA silencing as a tool for exploring gene function in ascomycete fungi. Fungal Genet. Biol.
533 42:275-283.

534 Parker, M. L., McDonald, M. R., and Boland, G. J. 2014. Evaluation of air sampling and
535 detection methods to quantify airborne ascospores of *Sclerotinia sclerotiorum*. Plant Dis. 98:32-
536 42.

537 Pratt, S. 2012. Sclerotinia resistant canola provides new option. The Western Producer, 20 July
538 2012. [Online] Available: http://www.producer.com/2012/07/sclerotinia-resistant-canola-
539 provides-new-option/. Verified: 15 October 2014.

540 Purdy, L. H. 1979. *Sclerotinia sclerotiorum* history, diseases and symptomology, host range,
541 geographic distribution and impact. Phytopathology 69:875-880.

542 Ririe, K. M., Rasmussen, R. P., and Wittwer, C. T. 1997. Product differentiation by analysis of
543 DNA melting curves during the Polymerase Chain Reaction. Analytical Biochem. 245: 154-160.

544 Rogers, S. L., Atkins, S. D., and West, J. S. 2008. Detection and quantification of airborne
545 inoculum of *Sclerotinia sclerotiorum* using quanitative PCR. Plant Pathol. 58:324-331.

546 Schwartz, H. F., and Steadman, J. R. 1978. Factors affecting sclerotium populations of, and
547 apothecium production by *Sclerotinia sclerotiorum*. Phytopathology 68:383-388.

548 Shah, P., Atwoood III, J. A., Orland, R., Mubarek, H. E., Podila, G. K., and Davis, M. R. 2009.
549 Comparative proteomic analysis of *Botrytis cinerea* secretome. J. Proteome Res. 8:1123-1130.

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

550 Sirjusingh, C., and Kohn, L. M. 2001. Characterization of microsatellites in the fungal plant
551 pathogen *Sclerotinia sclerotiorum*. Mol. Ecol. Notes 1:267-269.

552 Smith, C. J., and Osborn, A. M. 2008. Advantage and limitations of quantitative PCR (Q-PCR)-
553 based approaches in microbial ecology. FEMS Microbiol. Ecol. 67:6-20.

554 Turkington, T. K., Kutcher, H. R., McLaren, D., and Rashid, K. Y. 2011. Managing sclerotinia in
555 oilseed and pulse crops. Prairie Soils Crops 4:105-113.

556 Turkington, T. K., and Morrall, R. A. A. 1993. Use of petal infestation to forecast sclerotinia
557 stem rot of canola: the influence of inoculum variation over the flowering period and canopy
558 density. Phytopathology 83:682-689.

559 Turkington, T. K., Morrall, R. A. A., and Gugel, R. K. 1991. Use of petal infestation to forecast
560 Sclerotinia stem rot of canola: evaluation of early bloom sampling. 1995-1990. Can. J. Plant
561 Pathol. 13:50:59.

562 Weber, E., and Bleiholder, H. 1990. Erläuterungen zu den BBCH-Dezimal-Codes für die
563 Entwicklungsstadien von Mais, Raps, Faba-Bohne, Sonnenblume und Erbse - mit Abbildungen.
564 Gesunde Pflanzen 42:308-321.

565 Willets, H. J., and Wong, J. A.-L. 1980. The biology of *Sclerotinia sclerotiorum*, *S. trifoliorum*,
566 and *S. minor* with emphasis on specific nomenclature. Botanical Rev. 46:101-165.

567 Wu, B. M., and Subbarao, K. V. 2008. Effects of soil temperature, soil moisture and burial
568 depths on carpogenic germination of *Sclerotinia sclerotiorum* and *S. minor*. Phytopathology
569 98:1144-1152.

570 Yin, Y., Ding, L., Lui, X., Yang, J. and Ma, Z. 2009. Detection of *Sclerotinia sclerotiorum in*

571 *planta* by a real-time PCR assay. J. Phytopathol. 157:465-469.

572 Young, C. S., and Werner, C. P. 2012. Infection routes for *Sclerotinia sclerotiorum* in apetalous

573 and fully petalled winter oilseed rape. Plant Pathol. 61:730-738.

574

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.
Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015

575 **Table 1.** List of species used to test the specificity of primers and hydrolysis probes developed for the detection and quantification of

576 *Sclerotinia sclerotiorum* and a *ToxB* internal control.

Species	Isolate	Origin	Source	Supplier
Sclerotinia sclerotiorum[ab]	UAMH 6321	Devon, AB	Potted *Garzania* sp.	University of Alberta Microfungus Collection and Herbarium (UAMCH)
S. sclerotiorum[b]	UAMH 4514	Central Alberta	Rapeseed	UAMCH
S. sclerotiorum[b]	UAMH 9192	Maryland, USA	Bean stem	UAMCH
S. sclerotiorum[b]	SSA-11	Edmonton, AB	Canola petals	B. Ziesman, University of Alberta (U of A)
S. sclerotiorum[b]	SSB-11	Edmonton, AB	Canola petals	B. Ziesman, U of A
S. sclerotiorum	SSC-11	Edmonton, AB	Canola petals	B. Ziesman, U of A
S. sclerotiorum[b]	SSD-11	Edmonton, AB	Canola petals	B. Ziesman, U of A
S. minor	CBS 207.25	Unknown	Unknown	Centraalbureau Voor Schimmelcultures (CBS), Royal Netherlands Academy of Arts and Sciences, Uppsalalaan, NL
S. trifoliorum	CBS 122377	Netherlands	Unknown	CBS
Botrytis cinerea	DR12-5	Unknown	Potato tuber tissue	R.J. Howard, Alberta Agriculture and Rural Development (AARD), Brooks, AB
B. cinerea	414JV	Unknown	Alfalfa	R.J. Howard, AARD
B. cinerea	DAOM 192631	Winnipeg, Manitoba	*Fragaria chiloensis*	Canadian Collection of Fungal Cultures (CCFC), Ottawa, Ontario

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

B. cinerea	DAOM 189076	Charlottetown, PEI	Potato tubers	CCFC
B. cinerea	DAOM 166439	Beaverlodge, AB	*Festuca rubra*	CCFC
B. cinerea	CGC5	PEI	Winter wheat	Canadian Grain Commission, Winnipeg, MB
Botryotinia fuckeliana[c]	UAMH 16	Unknown	Bean	UAMCH
B. fuckeliana	UAMH 1784	Beaverlodge, AB	Indoor air exchange strip from *Apis mellifera* equipment cleaning warehouse	UAMCH
Rhizoctonia solani	N/A	Unknown	Unknown	J.P. Tewari, U of A
Rhizopus sp.	N/A	Unknown	Unknown	J.P. Tewari, U of A
Trichoderma sp.	N/A	Unknown	Soil	J.P. Tewari, U of A
Penicillium sp.	N/A	Edmonton, AB	Canola petals	B. Ziesman, University of Alberta (U of A)
Aspergillus niger	N/A	Unknown	Unknown	J.P. Tewari, U of A
Cladosporium sp.	N/A	Edmonton, AB	Canola petals	B. Ziesman, U of A
Mucor sp.	N/A	Edmonton, AB	Canola petals	B. Ziesman, U of A
Alternaria alternata	N/A	Unknown	Unknown	J.P. Tewari, U of A
Fusarium graminearum	G-1	Unknown	Unknown	A. Tekauz, Agriculture and Agri-Food Canada, Winnipeg, MB
Leptosphaeria maculans	N/A	Unknown	Unknown	J.P. Tewari, U of A
Brassica napus	N/A	Edmonton, AB	N/A	B. Ziesman, U of A

577 [a] *S. sclerotiorum* UAMH 6321 was originally designated as *S. minor* in the UAMCH, but later identified as *S. sclerotiorum*.
578 [b] Amplicon obtained from the isolate sequenced to confirm identity of the PCR product.
579 [c] Teleomorph of *B. cinerea*; species names are given as provided by the original supplier.

580 **Table 2.** Primers and hydrolysis probes for detection and quantification of *Sclerotinia*
581 *sclerotiorum* and a *ToxB* internal control. The primer set SSBZF/SSBZR amplifies a 70-bp
582 fragment of the gene SSIG_00263 in *S. sclerotiorum* and was used in conjunction with SSBZP in
583 a hydrolysis probe-based assay to quantify *S. sclerotiorum* DNA. The primer set ToxBF/ToxBR
584 amplifies a 70-bp fragment of the *ToxB* gene from *Pyrenophora tritici-repentis* and was used in
585 conjunction with ToxBP to quantify the exogenous internal control in a hydrolysis probe-based
586 assay.

Primer/probe name	Sequence	Size (bp)
SSBZF	5'-GCTCCAGCAGCCATGGAA-3'	18
SSBZR	5'-TGTTGAAGCAGTTGACGAGGTAGT-3'	24
SSBZP	5'-CAGCGCCTCAAGC-3'	13
ToxBF	5'-CCATGCTACTTGCTGTGGCTAT-3'	22
ToxBR	5'-CGCAGTTGGCCGAAACA-3'	17
ToxBP	5'-CTCCCTGCTGCCCC-3'	13

587
588
589
590
591
592
593
594
595
596

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

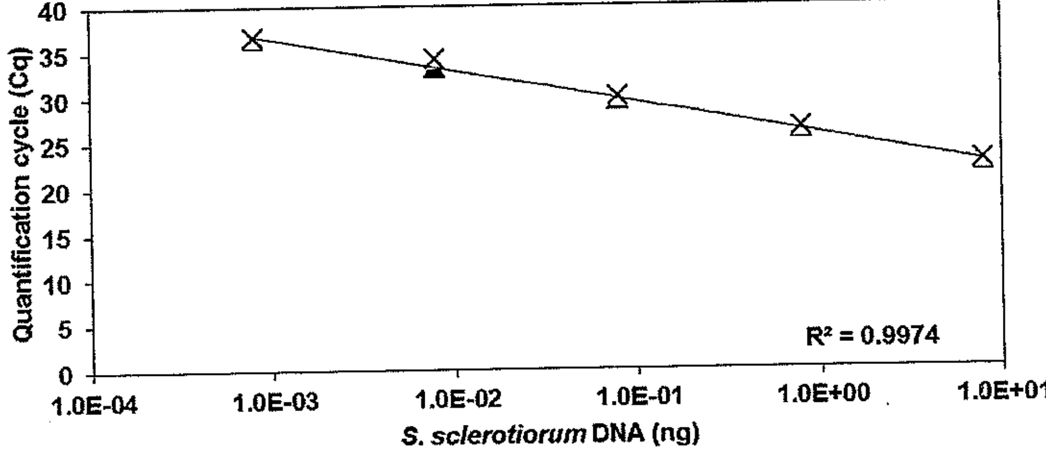
597 **Table 3.** Results of specificity testing with the SsF/SsR primer set (Yin et al. 2009) and
598 SSBZF/SSBZR primer set. The quantification cycle (C_q) value indicates the cycle number at
599 which fluorescence from amplification of a product exceeds background fluorescence.

Fungal species	C_q value (SsF/SsR)	C_q value (SSBZF/SSBZR)
Sclerotinia trifoliorum	27.673	ND[a]
Sclerotinia minor	ND	ND
Botryotinia fuckeliana UAMH 1784	33.219	ND
B. fuckeliana UAMH 16	35.017	ND
Botrytis cinerea DR12-5	34.954	ND
B. cinerea DAOM 192631	34.099	ND
B. cinerea DAOM 166439	35.017	ND
Rhizopus sp.	34.285	ND
Trichoderma sp.	30.617	ND
Rhizoctonia sp.	35.330	ND
Penicillium sp.	31.550	ND
Aspergillus niger	ND	ND
Fusarium graminearum	30.037	ND
Cladosporium sp.	ND	ND
Mucor sp.	ND	ND
Alternaria alternata	ND	ND
Leptosphaeria maculans	ND	ND
8.0×10^{-4} ng of *S. sclerotiorum* DNA	33.068	33.662

600 [a] ND, no detection; indicates that there was no amplification of the fungal DNA or that the C_q
601 value was at least 3 cycles greater than the lowest standard (8.0×10^{-4} ng of *S. sclerotiorum*
602 DNA).
603

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

604



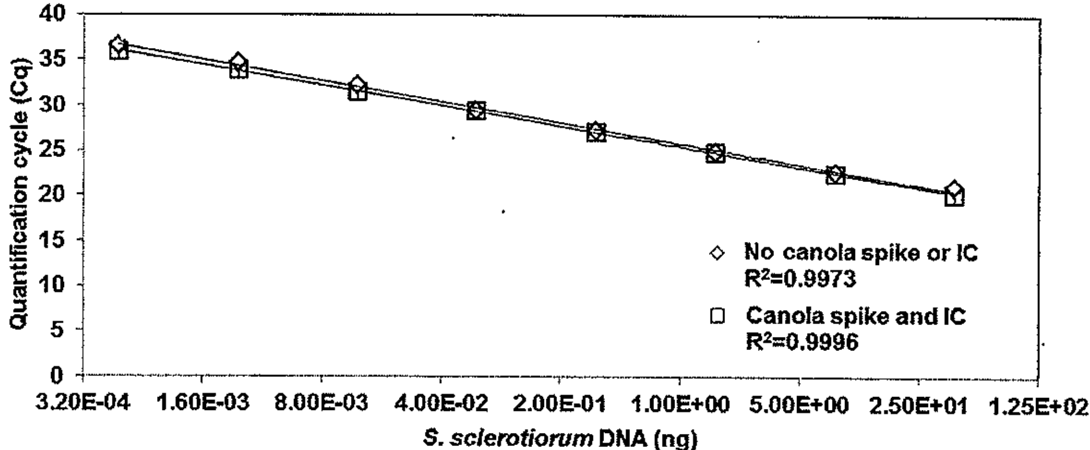
Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

605

606 **Fig. 1.** Standard curves obtained with DNA extracted from *Sclerotinia sclerotiorum* and
607 subjected to quantitative PCR analysis with the primer set SSBZF/SSBZR and hydrolysis probe
608 SSBZP. Four replicates of the standard curve were run in separate 96-well plates. The standards
609 were prepared from a serial dilution of 8.0 ng to 8.0×10^{-4} ng *S. sclerotiorum* DNA. The four
610 replications are denoted by different symbols, which may not be clearly visible because the
611 curves overlap. The PCR efficiency (E) of the four standard curves ranged from 88.9 to 90.25
612 and the R^2 values for the individual standard curves ranged from 0.9928 to 0.9999 (average:
613 0.996).

614

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.



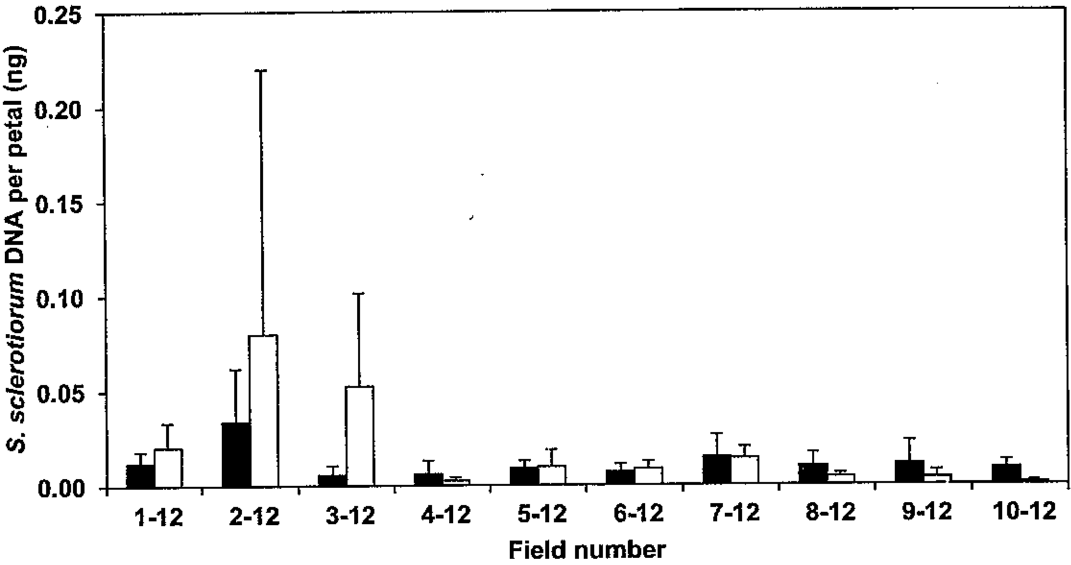
615

616 **Fig. 2.** Quantification of *Sclerotinia sclerotiorum* DNA in the presence or absence of canola
617 (*Brassica napus*) DNA and a *ToxB*-internal control. The fungal DNA was serially diluted by a
618 factor of 1:5 from 4.0×10^1 ng to 5.0×10^{-4} ng, and analyzed by qPCR with the primer set
619 SSBZF/SSBZR and hydrolysis probe SSBZP. Samples were analyzed in the absence (diamonds)
620 or presence of a *ToxB*-internal control (IC) and *Brassica napus* DNA ('Canola spike') (squares).
621 The standard deviation of the mean C_q for any point along the curve was not > 0.54, suggesting
622 that the presence of host DNA or the internal control did not affect the quantification of *S.*
623 *sclerotiorum* DNA. The standard deviation at each point of the standard curve is reflected by the
624 error bars.

625

Plant Disease "First Look" paper • http://dx.doi.org/10.1094/PDIS-05-15-0605-RE • posted 12/19/2015
This paper has been peer reviewed and accepted for publication but has not yet been copyedited or proofread. The final published version may differ.

626



627

628 **Fig. 3.** The quantification of *Sclerotinia sclerotiorum* DNA on canola petals collected from
629 commercial fields in the Edmonton, AB, region, as determined by qPCR analysis with the primer
630 set SSBZF/SSBZR and hydrolysis probe SSBZP. Five sites per field in each of 10 fields were
631 sampled at 20-30% bloom (black bars) and 40-50% bloom (white bars). The error bars represent
632 the standard deviation of the mean for each field and reflect the amount of variation across the
633 five sampling sites within each field.

1 **Evaluation of PCR-based quantification of *Sclerotinia sclerotiorum* infestation**
2 **levels as a predictive tool for stem rot of canola (*Brassica napus*)**

3

4 **(Draft Manuscript)**

5

6 **B.R. Ziesman[1], T.K. Turkington[2], B.D. Gossen[3], R. Kutcher[4], D. McLaren[5], K. Rashid[6],**
7 **and S.E. Strelkov[1]**

8

9 [1]Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton,
10 AB

11 [2]Lacombe Research and Development Centre, Agriculture and Agri-Food Canada, Lacombe, AB

12 [3]Saskatoon Research and Development Centre, Agriculture and Agri-Food Canada, Saskatoon,
13 SK

14 [4]Department of Plant Sciences, University of Saskatchewan, Saskatoon, SK

15 [5]Brandon Research and Development Centre, Agriculture and Agri-Food Canada, Brandon, MB

16 [6]Morden Research and Development Centre, Agriculture and Agri-Food Canada, Morden, MB

17 **Abstract**

18 Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum*, is an economically important disease of
19 canola (*Brassica napus*) in the Prairies region of western Canada. The disease is managed
20 primarily via the routine application of fungicides, often without any indication of stem rot risk.
21 The objective of this study was to evaluate the relationship between the levels of canola petal
22 infestation by *S. sclerotiorum*, as determined by quantitative (q)PCR analysis, and final stem rot
23 incidence in the field. This relationship was explored two studies. In the first study, conducted
24 over 2 years, petal infestation was compared with disease incidence in 34-35 commercial canola
25 fields distributed across Alberta, Saskatchewan and Manitoba. In the second study, these
26 parameters were compared over 3 years in 9-11 fields located in central Alberta. In the fields
27 sampled across the Prairies, no consistent relationship was observed between petal infestation
28 and stem rot incidence at harvest. However, no information was available on whether or not
29 these fields received a fungicide treatment, which may have reduced the ability to detect any
30 correlations. In contrast, sampling points in the second study were located in fungicide-free
31 check strips, and the incidence of stem rot was generally found to increase with increasing petal
32 infestation. The strength of the relationship varied across the study years, and was strongest
33 when canola petals were analyzed at full bloom and in years when disease pressure was high.

34

35 **Keywords:** Canola, disease forecasting, qPCR, risk assessment, *Sclerotinia sclerotiorum*, stem
36 rot

37 **Introduction**

38 Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, is one of the most
39 economically important diseases of canola (*Brassica napus* L.), causing yield losses high as 50%
40 (del Río et al. 2007). Ascospores produced through carpogenic germination of the overwintering
41 sclerotia are the primary inoculum initiating disease development (Abawi and Grogan 1979;
42 Bardin and Hwang 2001; Purdy 1979; Willets and Wong 1980). The ascospores come into
43 contact with canola petals onto which they adhere, germinate and penetrate the petal tissue.
44 When the petals senesce and fall onto leaf or stem surfaces, the fungus can grow out of the petals
45 and infect the living stem and leaf tissue (Jamaux et al. 1995; Purdy 1979). Infection of the
46 petals has been demonstrated to be an important stage in the Sclerotinia stem rot life cycle
47 (Jamaux et al. 1995; Morrall and Dueck 1982), and is influenced by environmental conditions
48 and the timing of ascospore release.

49 In the Prairies region of western Canada, Sclerotinia stem rot is managed primarily by the
50 routine application of fungicides. However, as a result of the influence of environmental
51 conditions on the success of infection, Sclerotinia stem rot is not present at high levels every
52 year. As a result, the application of fungicide without any indication of disease risk can result in
53 fungicides being applied when stem rot risk is low and fungicides are not required to prevent
54 yield loss. To improve the sustainability and accuracy of fungicide application, a timely and
55 reliable risk assessment tool is required to assist producers in making informed Sclerotinia stem
56 rot management decisions.

57 In Canada, several risk assessment tools have been developed for Sclerotinia stem rot of
58 canola, including weather-based risk maps, risk point tables and a petal plate test. Weather-based
59 risk maps take into account soil moisture, daily precipitation and air temperature to provide

60 regional assessments of stem rot risk (McLaren et al. 2004). The weather-based risk maps do not
61 take into account the amount of inoculum available, but rather focus on whether or not the
62 environment is favorable for disease development. Risk point tables provide essentially
63 qualitative assessments of risk, based on a variety of factors that influence the Sclerotinia stem
64 rot disease cycle (Twengstrom et al. 1998). Risk point tables assign weight to factors such as
65 crop rotation history, the amount or frequence of precipitation, the presence or absence of
66 apothecia in a field, and the incidence of Sclerotinia stem rot in the last susceptible crop, all of
67 which are all known to influence disease development in the field. Although the number of
68 apothecia present represents a quantitative estimate of inoculum presence, disease has been
69 found in fields in the absence of apothecia (Gugel 1986), indicating that this may not be a
70 reliable indication of the amount of inoculum available to initiate infection. In contrast, the petal
71 plate test developed by Morrall and Thompson (1991) provides a quantitative assessment of risk.
72 This method involves plating field collected canola petals onto potato dextrose agar (PDA),
73 incubating for 3-5 days, and assessing the percentage of petals infected with *S. sclerotiorum*
74 based on a visual inspection. Percent petal infestation takes into account an important stage in the
75 stem rot disease cycle, and has been shown to be related to Sclerotinia stem rot incidence in
76 canola (Gugel 1986; Turkington and Morrall 1993; Turkington et al. 1990). However, the delay
77 associated with the incubation period need for fungal growth has led to limited uptake of this risk
78 assessment tool by canola producers and agronomists in Canada.

79 Quantitative polymerase chain reaction (qPCR)-based methodologies can be used as a
80 method to measure the amount of *S. sclerotiorum* DNA on infected canola petals. Quantitative
81 PCR analysis can provide sensitive, robust and highly reproducible quantification without the
82 time delay associated with the traditional petal test (Smith and Osborn 2008). Several qPCR-

83 based techniques have been reported for the detection and quantification of *S. sclerotiorum*
84 (Parker et al. 2014; Rogers et al. 2008; Yin et al. 2009). Most recently, a hydrolysis probe-based
85 qPCR assay was described to measure *S. sclerotiorium* DNA in canola petals (Ziesman et al.
86 2016). This qPCR assay was found to be highly specific to *S. sclerotiorum* and did not amplify
87 DNA of a variety of closely related fungi. The assay was shown to accurately quantify the
88 amount of *S. sclerotiorum* DNA in field collected canola petals. Nevertheless, the relationship
89 between the level of petal infestation as measured by qPCR analysis, and eventual stem rot
90 incidence in the sampled canola crops was not established. An evaluation of this relationship is
91 needed if qPCR-based analysis of petal infestation is to serve as the basis of a stem rot risk
92 assessment system.

93 The aim of this study was to establish the relationship between levels of petal infestation,
94 as measured at 10-20% and at 40-50% bloom by qPCR analysis, and final stem rot incidence at
95 harvest, in order to determine whether the qPCR assay of Ziesman et al. (2016) could serve as a
96 stem rot risk assessment tool. To provide reliable estimates of stem rot risk, the results obtained
97 via qPCR analysis should be strongly related to disease incidence across a variety of fields and
98 weather conditions. Specifically, the objectives of this study were to: (1) evaluate the
99 relationship between the qPCR results and final Sclerotinia stem rot incidence in the field, 2)
100 determine if the qPCR assay can be used independently as a risk assessment tool across the
101 Prairies, and 3) assess whether or not the quantifications are correlated with percent petal
102 infestation estimates determined with the earlier petal test developed by Morrall and Thompson
103 (1991).

104 **Materials and Methods**

105 **Field selection and sample collection.** Two experiments (Exp. 1 and Exp. 2) were conducted to
106 evaluate the relationship between petal infestation level and final stem rot disease incidence.
107 Experiment 1 was conducted over two years (2011 and 2012) by monitoring petal infestation and
108 subsequent disease incidence in 35 (in 2011) or 34 (in 2012) commercial canola fields in Alberta
109 (near Edmonton and Lacombe), Manitoba (Brandon, Morden and Carman) and Saskatchewan
110 (Melfort and Saskatoon). Experiment 2 was conducted in commercial canola fields located near
111 Edmonton, Alberta, in 2011, 2012 and 2013. Nine fields were included in 2011, 10 fields in
112 2012, and 11 fields in 2013. The same central Alberta fields were monitored for Exp. 1 and Exp.
113 2 in 2011 and 2012. Experiment 1 was focussed on a Prairies-wide evaluation of the
114 relationship between petal infestation and stem rot incidence, while Exp. 2 examined this
115 relationship within a smaller regional scale.

116 With the exception of the fields near Edmonton, the fields in Exp. 1 were selected at
117 random. For the fields sampled in Manitoba and Saskatchewan, field histories were not known,
118 and no information was available as to whether or not fungicides were applied for stem rot
119 control during the growing season. In each field, there were 5 sampling sites spaced 50 m apart
120 and at least 75 m from the field edge. In Exp. 2, the sampling sites were situated in fungicide-
121 free check strips. There were five sampling sites per field arranged 50 m apart in a linear fashion.

122 Canola petals were collected at early flowering (10-20% bloom) and full flowering (40-
123 50% bloom). In 2012, the first sampling of the fields near Edmonton was delayed until 20-30%
124 bloom. Samples were collected between 1100 h and late afternoon, with the exception of the
125 fields near Edmonton in 2012 where sampling occurred early in the morning due to high

126 temperatures during the flowering period. At each sampling site, the top 20-30 cm of 20
127 randomly selected inflorescences were collected and placed into clean labelled plastic bags and
128 kept on ice in a cooler during transport back to the laboratory. In the laboratory, the samples
129 were maintained at 4°C prior to processing, which was carried out within 24 hours of sample
130 collection.

131 **Estimates of *S. sclerotiorum* infestation.** Petals were assessed for *S. sclerotiorum* infestation by
132 qPCR analysis in both Exp. 1 and Exp. 2, as well as by the traditional petal plate test in Exp. 2.
133 The qPCR analysis was carried out as described by Ziesman et al. (2016). Briefly, 20 petals
134 were selected at random from the petals stored at -80°C for each sampling site and each sampling
135 date, with no more than one petal selected from each intact flower. Petal samples were
136 homogenized using a TissueLyser II (Qiagen, Toronto, ON, Canada) and a single 5-mm stainless
137 steel bead in a 2 ml locking tube. The DNA was isolated following the protocol of Liang et al.
138 (2013), and total DNA was measured with a NanoDrop 2000c spectrophotometer (Thermo
139 Scientific, Waltham, MA, USA). An exogenous control was added to the tissue samples prior to
140 DNA isolation and carried through the full analysis (Ziesman et al. 2016).

141 Standards were prepared as a 1:10 dilution series of pure *S. sclerotiorum* genomic DNA
142 ranging from 8 ng to 8.0×10^{-4} ng. Samples were analyzed in triplicates. Triplicates with a
143 standard deviation of the Cq value > 0.5 were reanalyzed b qPCR. If a single outlier occurred in
144 a triplicate and the remaining duplicates had near equal Cq values, the outlier was removed. Any
145 sample for which no *S. sclerotiorum* DNA could be detected, or which was outside the range of
146 the standard curve, was recorded as a zero. Results are expressed on a per petal basis by first
147 accounting for the dilution, then by dividing the estimate by 20. The qPCR-based estimates of

148 petal infestation were averaged for each field on each sampling date, and the standard deviation
149 of the mean was calculated for each field.

150 The petal plate test was conducted as described by Turkington et al. (1988). Briefly,
151 petals were plated on Petri dishes containing solid potato dextrose agar amended with 25 ppm
152 ampicillin and 25 ppm streptomycin and incubated for 4-5 days in darkness at room temperature
153 (approximately 20-24°C). The percentage of petals infected with *S. sclerotiorum* was determined
154 through visual assessment, and the petal infestation estimates were averaged for each field on
155 each sampling date.

156 **Sclerotinia stem rot assessment.** Sampling sites were revisited prior to swathing, in order to
157 assess Sclerotinia stem rot incidence and severity in the canola crops. Twenty randomly selected
158 plants were evaluated at each sampling site, for a total of 100 plants per field. The plants were
159 rated for stem rot of severity on a 0-5 scale as described by Kutcher and Wolf (2006). In Exp. 1,
160 disease incidence was defined as the percentage of plants that had any symptoms of stem rot on
161 any organ (i.e., rated as 1 or greater). In Exp. 2, disease incidence was adjusted to include only
162 plants with a severity rating of 2 or higher. Thus, the adjusted disease incidence values excluded
163 infections rated as 1 (pod infections), since upper canopy (i.e., pod) infections would be expected
164 to be a minor contributor to yield loss. Statistical analysis for Exp. 2 indicated that the adjusted
165 disease incidence values improved the R^2 values slightly, but did not change the significance of
166 the regression models. As a result, the calculations for disease incidence in Exp. 1 were not
167 adjusted.

168 **Statistical analysis.** The relationship between qPCR estimates of petal infestation and final stem
169 rot levels was determined with quadratic regression using proc reg in SAS (Statistical Analysis

170 System) software. Assumptions of regression were tested and no transformations were required.
171 Scatter plots indicated a non-linear regression. This was confirmed by the lower Akiake
172 information criterion (AIC) and Bayesian information criterion (BIC) values for quadratic
173 regression models over simple linear regression models, indicating a better fit. The AIC/BIC
174 values were calculated for both linear and quadratic models using proc mixed and the residual
175 maximum likelihood (REML) method. Regression analysis was conducted on the average qPCR
176 value for each field at each of the sampling dates individually. Analysis was carried out
177 separately for the three sampling years because of differences in the respective environmental
178 conditions. The year to year variation in the strength of the statistical relationship was used to
179 assess whether or not qPCR-based estimates of petal infestation could consistently indicate stem
180 rot risk without consideration of other factors. Regression models were considered significant
181 when the slopes of the independent variables and the overall models were significant at $P = 0.05$.

182 In Exp. 1, the data were further sorted in three different ways: by province, by average
183 regional annual precipitation, and by average annual regional precipitation from April to August
184 ('summer precipitation'). The aim of this analysis was to determine which method accounted for
185 the most variation and produced the stronger regression model. Precipitation data were obtained
186 from Environment Canada. The data were separated into three main groups based on average
187 regional annual precipitation: > 500 mm (Carman and Morden), between 400-500 mm (Brandon,
188 Lacombe and Edmonton), and < 400 mm (Melfort and Saskatoon). Summer precipitation was
189 averaged for the months of April through August and was used to separate the data into two main
190 groups: > 300mm (Morden, Edmonton, Lacombe, Brandon and Carman) and < 300mm (Melfort
191 and Saskatoon).

192

193 In Exp. 2, the relationship between Sclerotinia stem rot disease incidence and percent
194 petal infestation (PPI), determined with the plate test, was assessed by simple linear regression
195 using proc reg in SAS. Regression analysis with non-transformed disease incidence did not meet
196 the assumptions of regression. Disease incidence values were Arcsine transformed as discussed
197 by Turkington and Morrall (1993), and the resulting residuals more closely met the assumptions
198 of regression. Thus, arcsine transformed disease incidence (TDI) was used for all regressions
199 with percent petal infestation (PPI) values. Simple linear regression models were confirmed to
200 have lower AIC and BIC values than the quadratic regression models, indicating a better fit for
201 the linear models. As with the qPCR results, regression analysis was conducted separately for
202 each of the three sampling years. Regression models were considered significant when the slopes
203 of the independent variables and the overall models were significant at $P = 0.05$.

204 Correlation analysis with proc reg in SAS was used in Exp. 2 to determine the
205 relationship between the qPCR-based and petal plate test estimates of petal infestation level. In
206 all three years, there was a slight deviation from normality for at least one variable. As a result,
207 Spearman Rank Correlation was used.

208 **Results**

209 **Experiment 1.** Sclerotinia stem rot disease incidence and the qPCR estimates of petal infestation
210 for individual fields were variable over the sampling years and between locations (Table 1). In
211 both 2011 and 2012, the disease incidence ranged from 0 to 92% among the sampled fields, but
212 the mean disease incidence (MDI) for all fields, presented as MDI ± standard deviation, was
213 higher in 2012 (27.2% ± 29.5%) than in 2011 (11.9% ± 17.7%) (Figs. 1-3). The mean amount of
214 *S. sclerotiorum* DNA per petal for all fields, as measured by qPCR analysis, was highest at full
215 bloom in both 2011 (0.013 ng/petal ± 0.0018 ng/petal) and 2012 (0.068 ng/petal ± 0.18 ng/petal).

216 There was variation between sampling sites, years and individual fields for disease incidence and
217 the amount of *S. sclerotiorum* DNA per petal. When the data were separated by province, none
218 of the relationships between the qPCR results and disease incidence were significant at either
219 sampling date in 2011. The coefficient of variation (CV) for these regression models ranged
220 from 94.14 to 144.05. In 2012, however, the relationship between the early bloom qPCR results
221 and disease incidence for the fields in Alberta was found to be significant, with the early bloom
222 qPCR results accounting for 59.1% of the variation in disease incidence ($R^2 = 0.591$; $P = 0.0073$;
223 CV 42.694) (Fig. 4). The regression models for the Alberta fields at full bloom and for the
224 Saskatchewan and Manitoba fields at both sampling dates were not significant, with CVs ranging
225 from 51.05 to 111.67.

226 When the data from Exp. 1 were separated by average regional annual precipitation, the
227 relationships were not significant for the locations with >400 mm precipitation in 2011, and for
228 none of the groups in 2012. The CV for these models ranged from 51.046 to 159.16. The
229 regression analysis for locations with >500 mm of annual precipitation at the late bloom
230 sampling date in 2011 could not be conducted using a quadratic model because of the large
231 number of fields with a disease incidence of 0%. For this group of locations, the simple linear
232 regression was analyzed and found not to be significant. The relationship between late bloom
233 qPCR results and disease incidence for fields with average regional annual precipitation was
234 found to be significant, with the late bloom qPCR results accounting for 57.6% of the variation
235 in disease incidence ($R^2 = 0.576$; $P = 0.0356$; $CV = 95.559$) (Fig. 5).

236 When the data were separated by average regional summer precipitation, none of the
237 regression models were significant in 2011. In 2012, the regression models for the group of
238 locations with average summer precipitation levels > 300 mm were significant at both early

239 bloom ($R^2 = 0.263$; $P = 0.035$; CV = 95.116) and late bloom ($R^2 = 0.244$; $P = 0.0459$; CV
240 96.301) (Fig. 6). The CV values for the non-significant regression models ranged from 95.56 to
241 129.022 in 2011 and from 84.295 to 99.600 in 2012.

242 **Experiment 2.** Sclerotinia stem rot incidence, PPI and the estimates of petal infestation obtained
243 by qPCR analysis were variable over the three years of the study (Table 2). Mean stem rot
244 incidence across all fields, presented as the mean ± standard deviation, was lower in 2011 (7.8%
245 ± 6.6%) than in 2012 and 2013 (64% ± 23.2% and 39.36% ± 24.8%, respectively). For both
246 sampling dates, estimates of petal infestation determined by qPCR analysis also were lowest in
247 2011 (early bloom: 0.005 ng/petal ± 0.005 ng/petal; full bloom: 0.017 ng/petal ± 0.013 ng/petal)
248 (Fig. 7). On a field level, qPCR estimates of petal infestation at full bloom were higher for all 9
249 fields in 2011, for five of 10 fields in 2012, and for 5 of 11 fields in 2013. As in Exp. 1, there
250 was a high amount of variation in the levels of petal infestation, as determined by qPCR analysis,
251 in samples from some of the fields.

252 Mean PPI, as assessed by the petal plate test of Turkington et al. (1988), was similar
253 across both sampling dates in 2011 (early bloom: 35.9% ± 22.3%; full bloom: 40.1% ± 6.84%)
254 and 2012 (early bloom: 34.3% ± 17.1%; full bloom: 41.2% ± 15.1%) (Table 2). In contrast, PPI
255 was generally higher in 2013 (early bloom: 43.85% ± 18.2%; full bloom: 51.0% ± 18.5%). A
256 comparison of petal infestation levels, as determined by qPCR analysis or the petal plate test,
257 found similar results in 2011 and 2012: both methods indicated that petal infestation levels were
258 lower at early bloom than at late bloom. In 2013, however, the different methods yielded
259 different results. Petal infestation was found to be lower at early bloom when evaluated by the
260 petal plate test, and lower at late bloom when evaluated by qPCR analysis.

261 **Relationship between qPCR-based petal infestation estimates and disease incidence.** In each
262 year of Exp. 2, three non-linear regression models were analyzed to determine the relationship
263 between qPCR estimates of petal infestation and final stem rot disease incidence (Table 3). In all
264 three years, disease incidence increased as the amount of *S. sclerotiorum* DNA per canola petal
265 increased (Fig. 8). However, the strength of the relationship varied with year and was not always
266 significant. In all years, there was a significant amount of variation in both the final disease
267 incidence and the petal infestation estimates in the fields sampled. In 2011, none of the three
268 regression models were significant at P = 0.05. The field with the highest stem rot incidence also
269 had a very low level of petal infestation (as determined by qPCR) and appeared to be an outlier.
270 If this field is removed from the analysis, the relationship between the qPCR results at full bloom
271 and disease incidence becomes significant ($R^2 = 0.9194$; P = 0.0018; CV = 27.21). In 2012,
272 there was no significant statistical relationship between the qPCR estimates and disease
273 incidence at early bloom. However, there was a significant relationship between disease
274 incidence and qPCR estimates at full bloom ($R^2 = 0.6904$; P = 0:0165; CV = 22.91).

275 In 2013, there was no significant statistical relationship at either early or full bloom when
276 all fields were included in the analysis. When a single outlier was removed, the relationship
277 between the qPCR estimates at full bloom and stem rot disease incidence became significant
278 ($R^2 = 0.6047$; P = 0.039; CV = 40.79). In 2013, there was a wider range of seeding dates (May 9-
279 May 20), with some canola fields seeded later than in 2011 and 2012. When only those fields
280 seeded on or prior to May 15 were included in the analysis, the regression became highly
281 significant at full bloom ($R^2 = 0.9204$; P = value 0.0063; CV = 20.83). The amount of variation
282 in the data set was high in all years, as indicated by the coefficients of variation, which ranged
283 from 37.22 to 89.056 for all non-significant regressions.

284 **Relationship between PPI and disease incidence.** In 2011 and 2013, there was not a significant
285 statistical linear relationship between PPI and disease incidence at either early bloom or late
286 bloom, even in 2013 when only early seeded fields were included in the analysis. In 2012, the
287 relationship was significant at full bloom ($R^2 = 0.682$; $P = 0.003$; $CV = 16.189$).

288 **Relationship between PPI and qPCR estimates of petal infestation.** Correlation analysis was
289 used to determine the relationship between PPI and the amount of *S. sclerotiorum* DNA per
290 canola petal as determined by qPCR analysis. In 2011, the only significant correlation was at
291 early bloom ($R^2 = 0.71$; $P = 0.03$). In 2012, the correlation was not significant at early bloom but
292 was significant at full bloom ($R^2 = 0.80$; $P = 0.0056$). In 2013, there was no significant
293 correlation at either early or full bloom.

294 **Discussion:**

295 In Canada, risk assessment tools for Sclerotinia stem rot of canola have focussed on a
296 variety of factors that are known to influence the incidence of the disease in the field. However,
297 only one of these risk assessment tools, the petal test developed by Morrall and Thompson
298 (1991), involves a quantitative estimation of the amount of inoculum present during a critical
299 period of the disease cycle. The petal test provides a measure of petal infestation, which has been
300 demonstrated to be strongly related to Sclerotinia stem rot incidence at the end of the growing
301 season. Fungicides are applied for control of stem rot during flowering. An estimation of petal
302 infestation should provide an indication of disease risk during the period over which fungicide
303 spray decisions are being made. However, the 3-5 day incubation period associated with the
304 traditional petal testing procedure causes a delay in the results, which may only become available
305 after the optimal time for fungicide application has passed (Wallenhammar et al. 2007). In

306 contrast, an evaluation of field collected canola petals by quantitative PCR analysis can provide
307 an estimate of inoculum pressure within a single day.

308 In all fields and locations included in this study, there was variation in both the amount of
309 *S. sclerotiorum* DNA detected by qPCR analysis and in the final disease incidence. There also
310 was significant year to year variation in these parameters, most likely reflecting differences in
311 environmental conditions that in turn influence carpogenic germination of the sclerotia and the
312 timing of ascospore release. Similarly, the differences observed between fields could also be a
313 result of regional differences in environmental conditions, but they may also reflect differences
314 in crop stand density, seeding date, seeding rate and crop history, all of which can influence stem
315 rot development (Turkington and Morrall 1993; Jurke and Fernando 2008; Twengstrom et al.
316 1998). In addition to field to field variation, variation across sampling sites within particular
317 fields also was observed. This variation likely reflected differences in crop stand and
318 microclimate at different points within the same field. Five sites were sampled per field, since
319 this was reported to be an accurate sampling size for evaluating the incidence of petal infestation
320 (Turkington et al. 1988). The large amount of variation observed in this study, however,
321 indicates that a larger number of sampling sites may be needed to provide a more reliable
322 estimate of petal infestation in a particular field.

323 The amount of *S. sclerotiorum* DNA quantified per canola petal varied across the
324 flowering season. These findings are consistent with previous reports that found that inoculum
325 pressure is not consistent across the flowering period or between fields (Almquist and
326 Wallenhammar 2015; Turkington and Morrall 1993). Differences in the level of infestation over
327 the growing season will influence the strength of the statistical relationship between
328 quantifications of petal infestation and stem rot incidence when evaluated across several fields.

329 Monitoring changes in petal infestation over the flowering period may serve to identify the best
330 timing for fungicide application in a particular field, but such an approach would have to be
331 balanced by cost and other practical considerations. Nonetheless, closer monitoring of infestation
332 levels on a temporal scale could prove useful in better understanding the epidemiology of
333 Sclerotinia stem rot of canola.

334 The relationship between PPI as determined by the petal plate test and petal infestation as
335 assessed by qPCR analysis was not always linear. An analysis of correlation indicated a
336 significant amount of shared variation between the two methods only at early bloom in 2011 and
337 at full bloom at 2012. Similarly, Almquist and Wallenhammar (2015) reported no correlation
338 between the results of the petal plate test and a qPCR-based *S. sclerotiorum* detection method.
339 There are several possible explanations for these differences. For example, the presence of a few
340 highly infected canola petals in a sample would be reflected in a higher concentration of *S.*
341 *sclerotiorum* DNA in the qPCR analysis, but would not be reflected in the petal plate test results
342 (Almquist and Wallenhammar 2015). Furthermore, while both the number of ascospores and the
343 amount of mycelium present in a sample can be measured by qPCR analysis, the petal plate test
344 can only indicate whether or not a petal is infected. Finally, when levels of *S. sclerotiorum*
345 infestation are low on infected petals, other fungal species (e.g. *Rhizopus* spp., *Mucor* spp.,
346 *Trichoderma* spp.) that are present on the petal tissue may outgrow colonies of *S. sclerotiorium*,
347 thus masking the presence of *S. sclerotiorum*. Given the increased sensitivity of the qPCR-based
348 method, detection and direct measurement of *S. sclerotiorum* by qPCR analysis are more likely
349 to be influenced by the environment in which those petals were produced.

350 The strength of the statistical relationship between qPCR-based measurement of petal
351 infestation and final stem rot incidence in the field is critical to assessing the suitability of the

352 former for predicting the risk of disease. In Exp. 1, the strength of the relationship between the
353 qPCR results and disease incidence across the Prairies was variable across the three years of the
354 study. Moreover, when the data were analyzed separately based on the amount of annual or
355 summer precipitation in a particular region, the strength of the relationship was not increased. It
356 is important to note that, with the exception of the fields in the Edmonton region, there was no
357 information regarding fungicide application in the fields included in Exp. 1. This could have had
358 a large impact on any potential correlation. For instance, a canola field in which there was heavy
359 petal infestation may have been treated with fungicide, preventing or greatly reducing stem rot
360 development. As such, the results of Exp. 1 must be treated with caution. In Exp. 2, all sampling
361 was carried out in check-strips that were not treated with fungicide. Therefore, the results of this
362 experiment may provide a better indication of the true relationship between disease incidence
363 and petal infestation as measured by qPCR analysis. Indeed, in Exp. 2 the relationship between
364 these two parameters was stronger, with stem rot incidence generally found to be greater in fields
365 where petal infestation (*S. sclerotiorum* DNA per canola petal) also was higher. Despite this
366 stronger relationship, however, the correlations were not always significant. This highlights the
367 possible influence of other factors in stem rot development.

368 There was a wider range of seeding dates in 2013 than in 2011 or 2012, and this was
369 found to influence the statistical relationship in Exp. 2. When only the early seeded fields were
370 included in the analysis, the relationship was significant at full bloom, which was consistent with
371 the results in 2012. These findings indicate that the relationship between disease incidence and
372 the amount of *S. sclerotiorum* DNA per canola petal is strongest at full bloom and/or in years
373 when disease pressure is high. The variation across the sampling years also indicates that
374 measures of environmental conditions may need to be included in the model using multiple

375 regression analysis, in order to fully account for year to year variation in weather. More broadly,
376 comparison of the results obtained in Exp. 1 and Exp. 2 suggests that qPCR-based assessments
377 of stem rot risk are more reliable on a smaller regional scale, and that differences in environment
378 between locations should be considered when setting risk assessment thresholds.

379 The qPCR assay described in this study may serve as the basis for a risk assessment
380 system, as well as representing a useful tool for the study of the epidemiology of Sclerotinia stem
381 rot of canola. It can quantify the level of petal infestation, a key stage in the Sclerotinia stem rot
382 disease cycle, thereby providing a measure of disease risk when timely fungicide application
383 decisions need to be made. It is important to emphasize, however, that a forecasting system
384 based on qPCR quantification of petal infestation should be linked to environmental conditions,
385 as well as to cropping history, seeding date and crop canopy conditions, which may influence
386 stem rot development and the need to spray a fungicide.

387 **Acknowledgements**

388 We thank the Alberta Crop Industry Development Fund, the Alberta Canola Producers
389 Commission, the Western Grains Research Foundation, and Agriculture and Agri-Food Canada
390 and the Canola Council of Canada (via the Growing Forward program) for financial support. We
391 would also like to thank Colleen Kirkham (Agriculture and Agri-Food Canada), two WISEST
392 students (Women in Scholarship Engineering Science and Technology, University of Alberta),
393 and three summer students (University of Alberta) for their technical assistance, as well as Emile
394 deMilliano, Ryan Adams (Crop Production Services) and various farmers for their helpful
395 collaboration.

396

397 **References:**

398 Abawi, G.S., and Grogan R.G. 1979. Epidemiology of diseases caused by *Sclerotinia* species.
399 Phytopathology 69:899-904.

400 Aboukhaddour, R., Kim, Y.M., and Strelkov, S.E. 2012. RNA-mediated gene silencing of ToxB
401 in *Pyrenophora tritis-repentis*. Mol. Plant Pathol. 13:318-326.

402 Almquist, C., and Wallenhammar, A.C. 2015. Monitoring of plant and airborne inoculum of
403 *Sclerotinia sclerotiorum* in spring oilseed rape using real-time PCR. Plant Pathology 64: 109-
404 118.

405 Bardin, S.D., and Wuang, H.C. 2001. Research on biology and control of *Sclerotinia* disases in
406 Canada. Can. J. Plant Path. 23: 88-98.

407 Bolton, M.D., Bart, T., Thomma, P.H.J., and Nelson, B.D. 2006. *Sclerotinia sclerotiorum* (Lib.)
408 de Bary: biology and molecular traits of a cosmopolitan pathogen. Mol. Plant Pathol. 7:1-16.

409 del Río, L.E., Bradley, C.A., Hensen, R.A., Endres, G.J., Hanson, B.K., Mckay, K., Halvorson,
410 M., Porter, P.M., Le Gare, D.G., and Lamey, H.A. 2007. Impact of Sclerotinia stem rot on yield
411 of canola. Plant Dis. 91: 191-194.

412 Environment Canada. 2015. 1981-2010 Climate normals & averages. Found online
413 at: http://climate.weather.gc.ca/climate_normals/index_e.html.

414 Freeman, J., Ward, E., Calderon, C., and McCartney, A. 2002. A polymerase chain reaction
415 (PCR) assay for the detection of inoculum of *Sclerotinia sclerotiorum*. European Journal of Plant
416 Pathology 108:877-86.

417 Gugel. R.K. 1986. Inoculum-disease relationship in sclerotinia stem rot of rapeseed in
418 Saskatchewan. Can. J. Plant Pathol. 8(1):89-96.

419 Jamaux, I., Gelie, B. and Lamarque, C. 1995. Early stages of infection of rapeseed petals and
420 leaves by *Sclerotinia sclerotiorum* reveal by scanning electron microscopy. Plant Pathology. 44:
421 22-30.

422 Jurke, C.J. and Fernando, W.G.D. 2008. Effects of seeding rate and plant density on sclerotinia
423 stem rot incidence in canola. Arch. Phytopathol. Plant Protect. 41: 142-155.

424 Kutcher, H. R. and Wolf, T. M. 2006. Low drift fungicide application technology for sclerotinia
425 stem rot of canola. Crop Protect. 25: 640-646.

426 Lamari, L., and Strelkov, S.E. 2010. The wheat/*Pyrenophora tritici-repentis* interaction: progress
427 towards an understanding of tan spot disease. Can. J. Plant Pathol. 32:4-10.

428 Liang, Y., Yajima, W., Davis, M.R., Kav, N.N.V., and Strelkov, S.E. 2013. Disruption of a gene
429 encoding a hypothetical secreted protein for *Sclerotinia sclerotiorum* reduces its virulence on
430 canola (*Brassica napus*). Can. J. Plant Pathol. 35:46-55.

431 Ma, Z., Luo, Y., and Michailides, T.J. 2003. Nested PCR assays for detection of *manilinia*
432 *fruiticola* in stone fruit orchard and *Botryosphaeria dothidea* from pistachios in California. J.
433 Phytoptahol. 151:312-322.

434 McLaren, D.L., Conner, R.L., Platford, R.G., Lamb, J.L., Lamey, H.A. and Kutcher, H.R. 2004.
435 Predicting diseases caused by *Sclerotinia sclerotiorum* on canola and bean – a western Canadian
436 perspective. Can. J. Plant Pathol. 26: 489-497.

437 Morrall, R.A.A. and Thompson, J.R. 1991. Petal test manual for *Sclerotinia* in canola. University
438 of Saskatchewan, Saskatoon, Saskatchewan, Canada.

439 Morrall, R.A.A., and Dueck, J. 1982. Epidemiology of Sclerotinia stem rot of rapeseed in
440 Saskatchewan. Can. J. Plant Pathol. 4:161-168.

441 Nakayachiki, H., Hanada, S., Nguyen, B.Q., Kadotani, N., Tosa, Y., and Mayama, S. 2005. RNA
442 silencing as a tool for exploring gene function in ascomycete fungi. Fungal Genet. Biol. 42:275-
443 283.

444 Parker, M.L., McDonald, M.R., and Boland, G.J. 2014. Evaluation of air sampling and detection
445 methods to quantify airborne ascospores of *Sclerotinia sclerotiorum*. Plant Dis. 98:32-42.

446 Purdy, L. H. 1979. *Sclerotinia sclerotiorum* History, diseases and symptomology, host range,
447 geographic distribution and impact. Phytopathology 69: 857-880.

448 Rogers, S. L., Atkins, S.D., and West, J.S. 2008. Detection and quantification of airborne
449 inoculum of *Sclerotinia sclerotiorum* using quantitative PCR. Plant Pathol. 58: 324-331.

450 Schwartz, H.F., and Steadman, J.R. 1978. Factors affecting sclerotium populations of, and
451 apothecium production by *Sclerotinia sclerotiorum*. Phytopathology. 68:383-388.

452 Smith, C.J., and Osborn, A.M. 2008. Advantage and limitations of quantitative PCR (Q-PCR)-
453 based approaches in microbial ecology. FEMS Microbiol. Ecol. 67: 6-20.

454 Turkington, T.K., Morrall, R.A.A. and Baker, R.J. 1988. Sample size in relation to forecasting
455 Sclerotinia stem rot of canola. Can. J. Plant Pathol. 10:159-165

456 Turkington, T.K., Morrall, R.A.A. and Gugel, R.K. 1990. Use of petal infestation to forecast
457 Sclerotinia stem rot of canola: evaluation of early bloom sampling, 1985-1990. Can. J. Plant
458 Pathol. 13(1): 50-59.

459 Turkington, T.K., and Morrall, R.A.A. 1993. Use of petal infestation to forecast Sclerotinia stem
460 rot of canola: The influence of inoculum variation over the flowering period and canopy density.
461 Phytopathology 83:682-689.

462 Twengstrom, E., Sigvald, R., Svensson, C., and Yuen J. 1998. Forecasting sclerotinia stem rot in
463 spring oilseed rape. Crop. Prot. 17:405-411

464 Wallenhammar, A,-C., Redner, A., and Sjöberg, A. 2007. Development of methods improving
465 precision of risk assessment of Sclerotinia stem rot in oilseed rape. In: Fu, T., and Guan, C., eds.
466 *Proceedings of the 12th International Rapeseed Congrss, Wuhan China, March 2007.* Monmouth
467 Junction NJ, USA: Science Press. 112-115.

468 Willets, H.J. and Wong, J, A. –L. 1980. The biology of *Sclerotinia sclerotiorum, S. trifoliorum*
469 and *S. minor* with emphasis on specific nomenclature. Botanical Rev. 46:101-165.

470 Wu, B.M., and Werner, C.P. 2012. Infection routes for *Sclerotinia sclerotiorum* in apetalous and
471 fully petalled winter oilseed rape. Plant Pathol. 61:730-738.

472 Ziesman, B.R., Turkington, T.K., Basu, U., and Strelkov, S.E. 2016. A quantitative PCR system
473 for measuring *Sclerotinia sclerotiorum* in canola (*Brassica napus*). Plant Dis. (Accepted 13
474 December 2015). DOI: 10.1094/PDIS-05-15-0605-RE

475 **Table 1.** Stem rot disease incidence and amount of *S. sclerotiorum* DNA per canola petal, as

476 determined by qPCR analysis, in canola fields in Alberta, Saskatchewan and Manitoba

477 (Experiment 1).

Location	Stem rot incidence	2011			2012		
		Mean Disease Incidence (MDI) (%)	Mean qPCR estimates (ng of *S. sclerotiorum* DNA per petal)		MDI (%)	Mean qPCR estimates (ng of *S. sclerotiorum* DNA per petal)	
			EB	FB		EB	FB
Edmonton, AB	Average	8.67	0.005	0.017	64.8	0.012	0.02
	Minimum	1.00	0	0.003	29	0.006	0.001
	Maximum	21.00	0.013	0.038	92	0.034	0.080
Lacombe, AB	Average	11	0.001	<0.001	15.5	0.001	0.016
	Minimum	0	0	0	0	0	0.001
	Maximum	40	0.002	0.001	35	0.003	0.475
Melfort, SK	Average	28.860	0.012	0.025	1.8	0.037	0.030
	Minimum	4	0.001	0.005	0	0.002	0.001
	Maximum	92	0.017	0.047	4	0.087	0.130
Saskatoon, SK	Average	6	0.001	0.009	34.4	0.046	0.382
	Minimum	3	0	0.004	26	0.004	0.017
	Maximum	11	0.004	0.018	38	0.190	0.893
Brandon, MB	Average	0	0.002	0.012	2.4	0.007	0.004
	Minimum	0	0	0	0	0.002	0.002
	Maximum	0	0.006	0.082	11	0.016	0.006
Carman, MB	Average	10.667	0.001	0	5	0.005	0.001
	Minimum	5	0	0	1	0.004	0
	Maximum	20	0.003	0	11	0.006	0.001
Morden, MB	Average	12	0.003	0.001	14.5	0.008	0.004
	Minimum	1	0.001	0	11	0.004	0.001
	Maximum	21	0.006	0.002	18	0.011	0.007
All fields	Average	11.89	0.005	0.013	27.2	0.017	0.068
	Minimum	0	0	0	0	0	0
	Maximum	92	0.017	0.082	92	0.190	0.893

478

479

480 **Table 2.** Stem rot disease incidence and severity, and the amount of *S. sclerotiorum* DNA per

481 canola petal, as determined by qPCR analysis and the traditional petal plate test, in canola fields

482 in central Alberta (Experiment 2).

Year	Values	Mean disease incidence (%)	Mean disease severity	Mean qPCR estimates (ng of *S. sclerotiorum* DNA per petal)		Mean percent petal infestation (%) (traditional plate test)	
				Early bloom	Full bloom	Early bloom	Full bloom
2011	Average	7.78	0.340	0.005	0.017	35.9	40.11
	Minimum	1	0.020	0	0.003	13	33.5
	Maximum	20	0.79	0.013	0.038	68.5	52
2012	Average	64	2.75	0.012	0.020	34.3	41.2
	Minimum	29	1.18	0.006	0.003	21	19
	Maximum	92	3.97	0.034	0.080	74.5	63.5
2013	Average	39.64	1.54	0.081	0.051	43.9	51
	Minimum	12	0.34	0.006	0.014	18.0	25.4
	Maximum	88	3.61	0.259	0.181	73.1	79

483

484

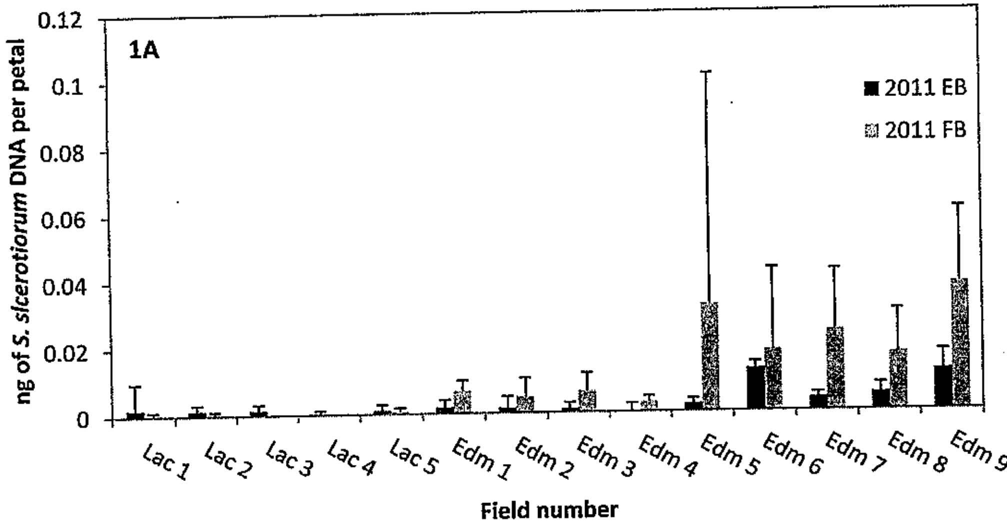
485 **Table 3.** Significant regression models for the relationship between amount of *S. sclerotiorum*
486 DNA per canola petal, as determined by qPCR analysis, with stem rot incidence and severity at
487 early bloom (EB) and full bloom (FB) in Experiment 2.

Year	Dependent Variable	Independent variables	Coefficient of determination (R^2)	Model Significance (p-value)	Coefficient of variation
2012	DI	MFB_q MFB_qsq	0.602	0.017	22.91
2012	DI	MFBPPI	0.682	0.003	16.189
2013ES	DI	MFB_q MFB_qsq	0.920	0.006	20.834

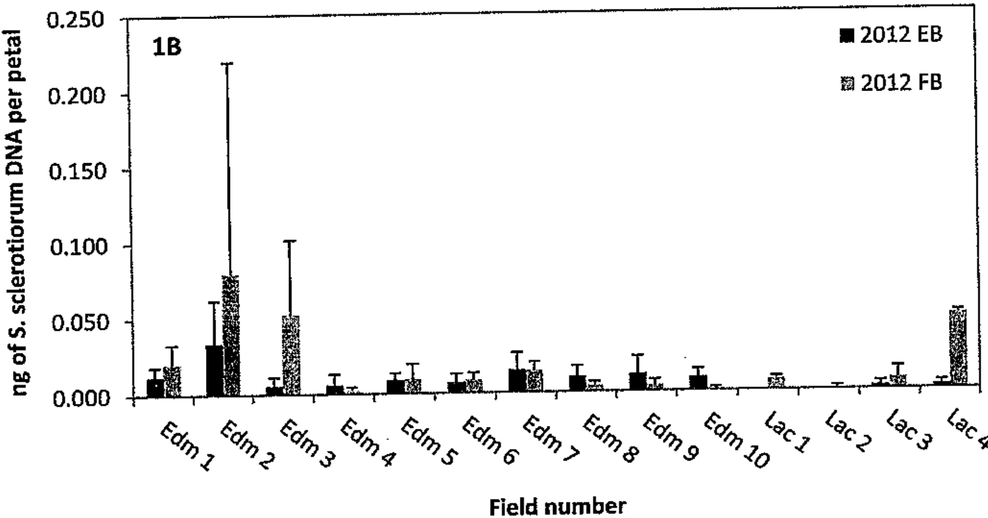
488 *2013ES = includes only the early seeded fields that were seeded on or before May 15th; MFB_q
489 = Mean qPCR estimates of petal infestation at full bloom; MFB_qsq = Mean qPCR estimate of petal
490 infestation at full bloom squared; MFBPPI = Mean percent petal infestation at full bloom.

491

492



493



494

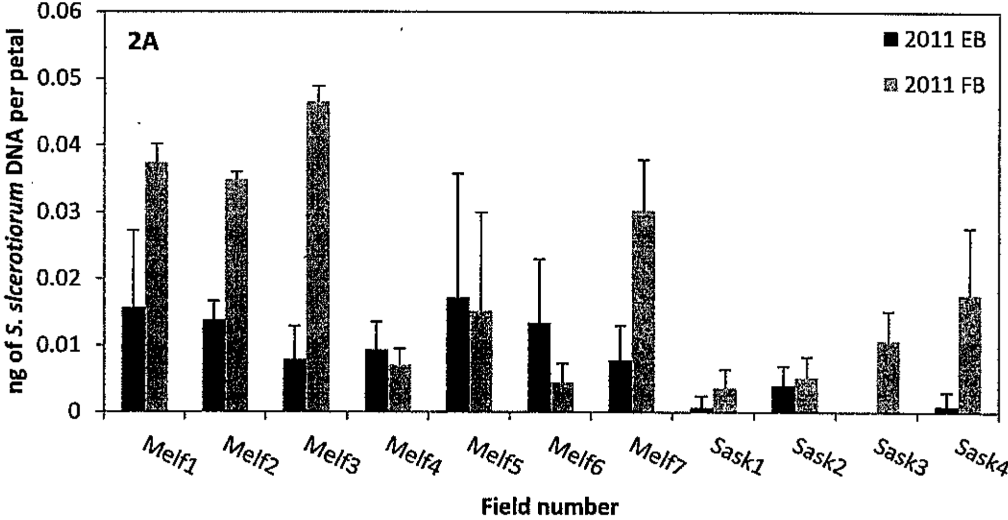
495 **Figure 1.** Amount of *S. sclerotiorum* DNA per canola petal in Alberta fields at early bloom (EB)

496 and at full bloom (FB) in Exp. 1 in (A) 2011 and (B) 2012. The error bars represent the standard

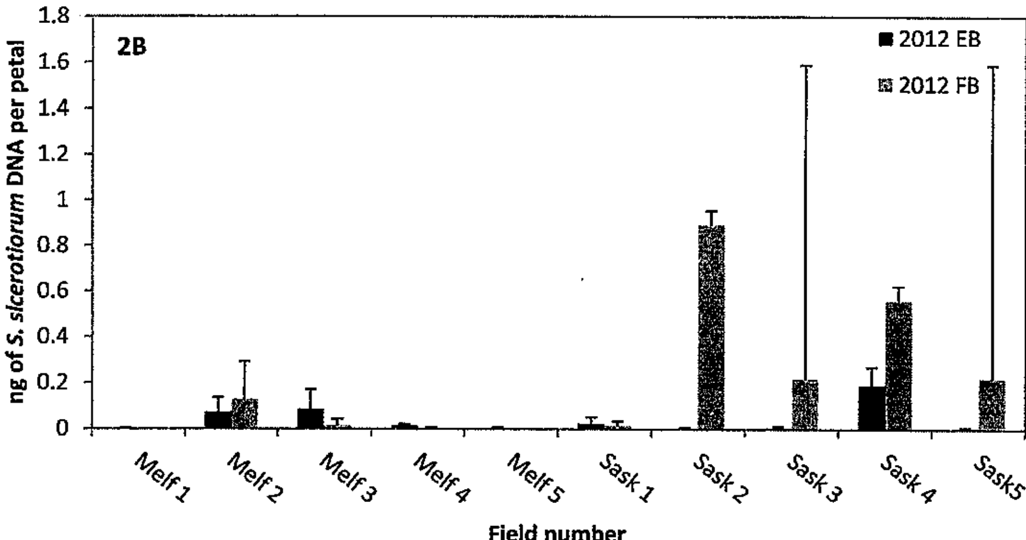
497 deviation of the mean for each field.

498

499



500



501

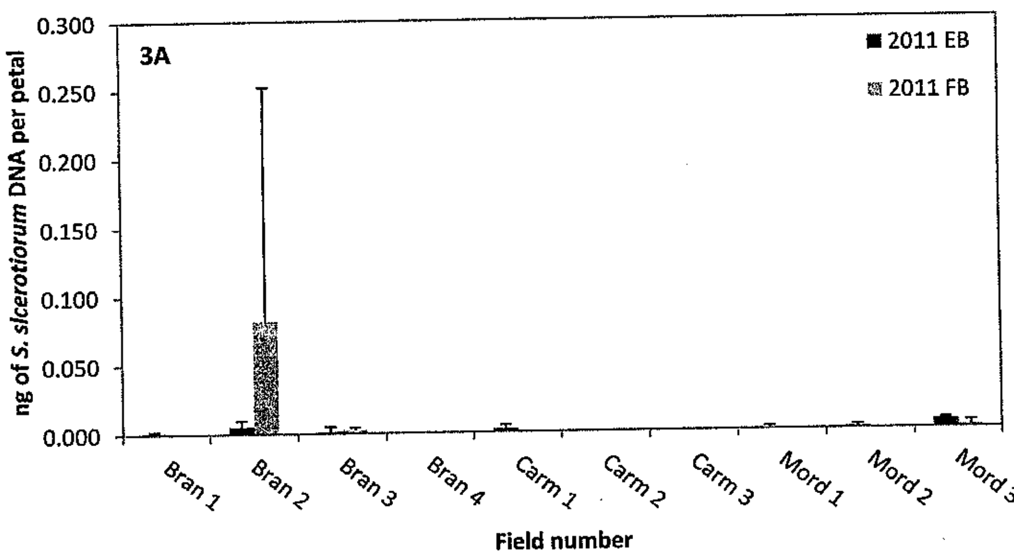
502 **Figure 2.** Amount of *S. sclerotiorum* DNA per canola petal in Saskatchewan fields at early

503 bloom (EB) and at full bloom (FB) in Exp. 1 in 2011 (A) and 2012 (B). The error bars represent

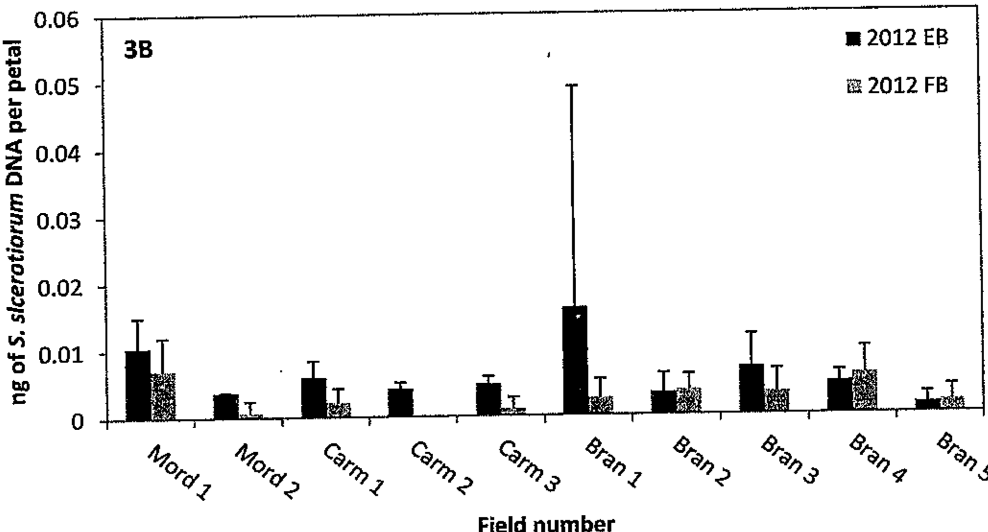
504 the standard deviation of the mean for each field.

505

506



507



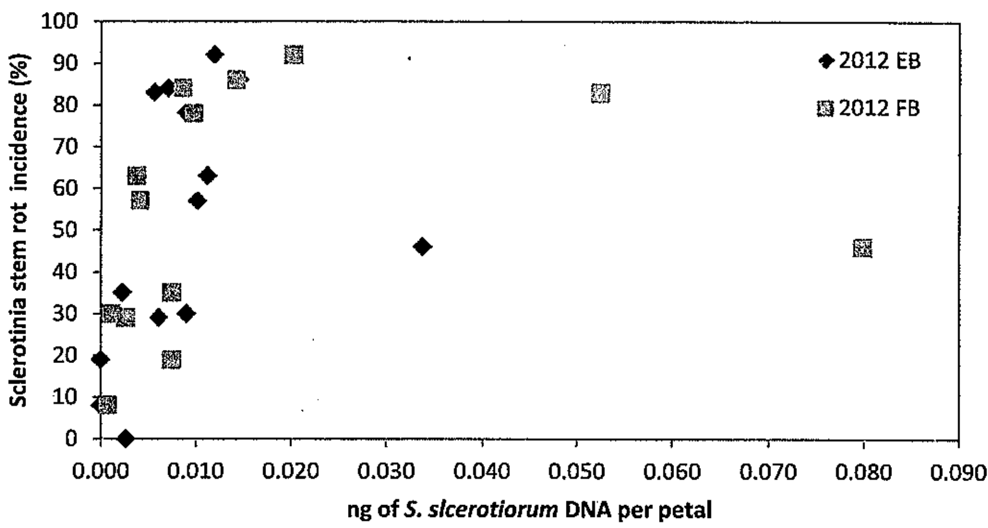
508

509 **Figure 3.** Amount of *S. sclerotiorum* DNA per canola petal in Manitoba fields at early bloom

510 (EB) and at full bloom (FB) in Exp. 1 in 2011 (A) and 2012 (B). The error bars represent the

511 standard deviation of the mean for each field.

512



513

514 **Figure 4.** Relationship between the amount of *S. sclerotiorum* DNA per canola petal as

515 determined by qPCR analysis and the incidence of Sclerotinia stem rot in the Alberta fields in

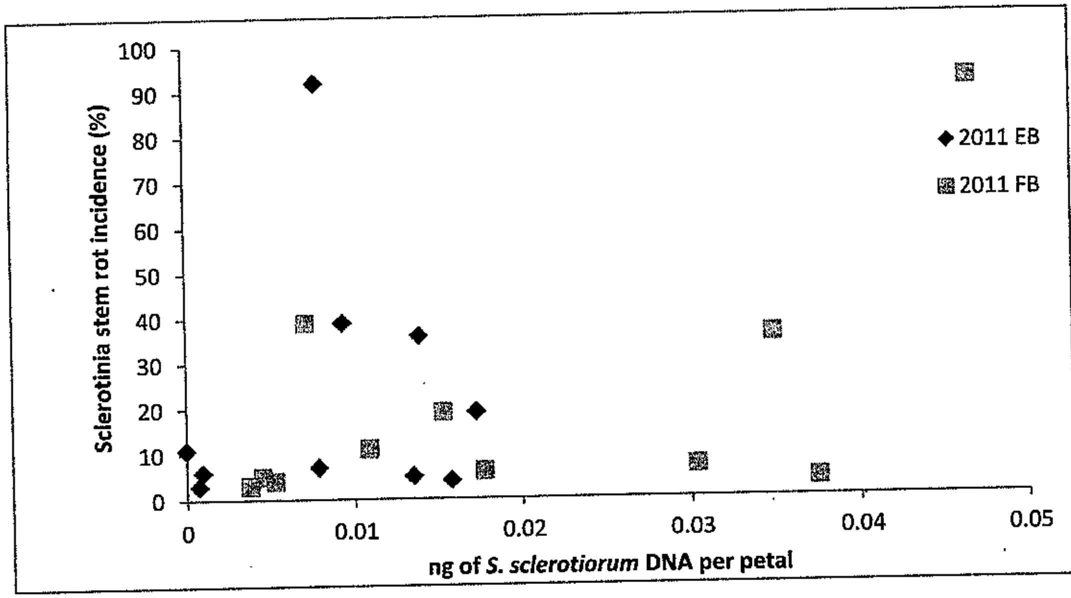
516 2012 for Exp. 1. The regression was significant at early bloom (EB) ($R^2 = 0.591$; $P = 0.007$), but

517 not at full bloom (FB) ($R^2 = 0.540$; $P = 0.106$). Relationships were not significant in the

518 Manitoba or Saskatchewan fields.

519

520



521

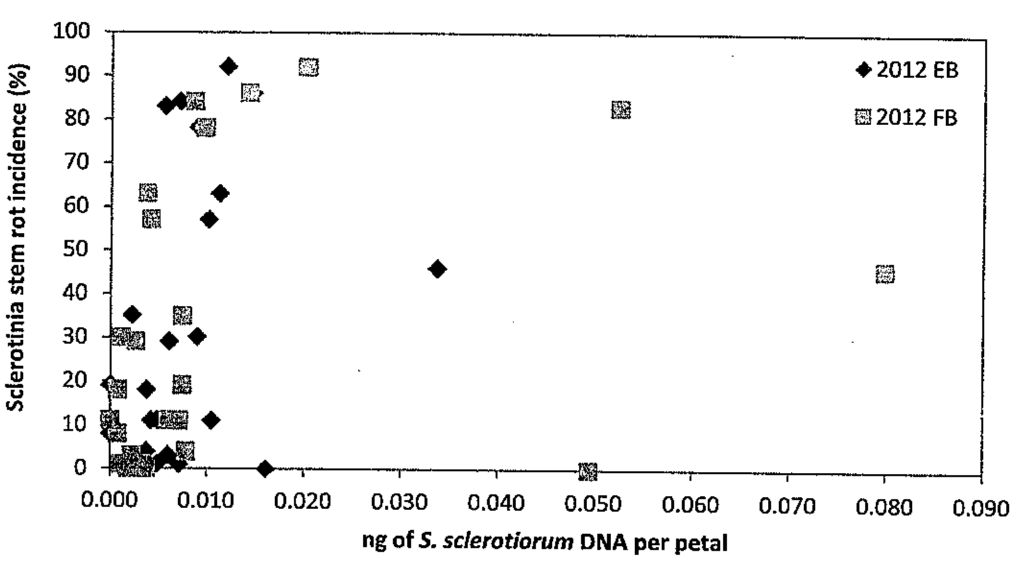
522 **Figure 5.** The relationship between the amount of *S. sclerotiorum* DNA per canola petal as

523 determined by qPCR analysis and the incidence of Sclerotinia stem rot in fields with average

524 annual precipitation < 400 mm in 2011 for Exp. 1. The regression was significant at full bloom

525 (FB) ($R^2 = 0.576$; P = 0.032), but not significant at early bloom (EB).

526



527

528 **Figure 6.** The relationship between the amount of *S. sclerotiorum* DNA per canola petal as

529 determined by qPCR analysis and the incidence of Sclerotinia stem rot in fields with an average

530 regional summer precipitation level of < 300 mm in 2012 for Exp. 1. The relationship was found

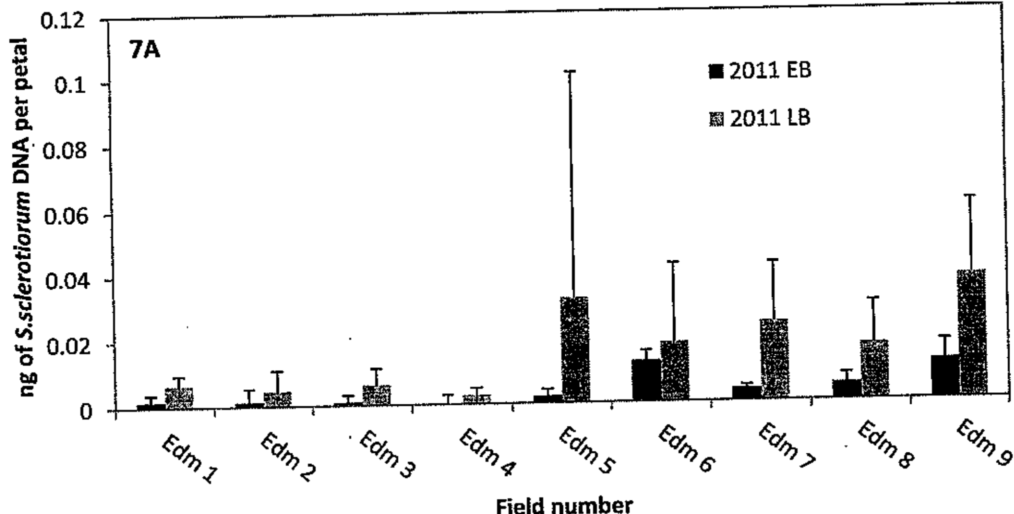
531 to be significant at both early bloom (EB) ($R^2 = 0.263$; P = 0.035) and full bloom (FB) ($R^2 =$

532 0.244; P = 0.045).

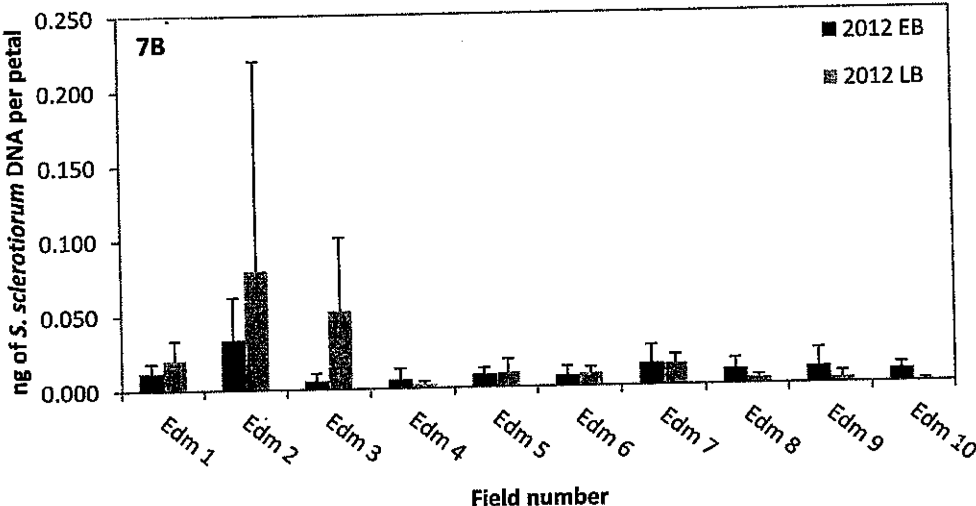
533

534

535

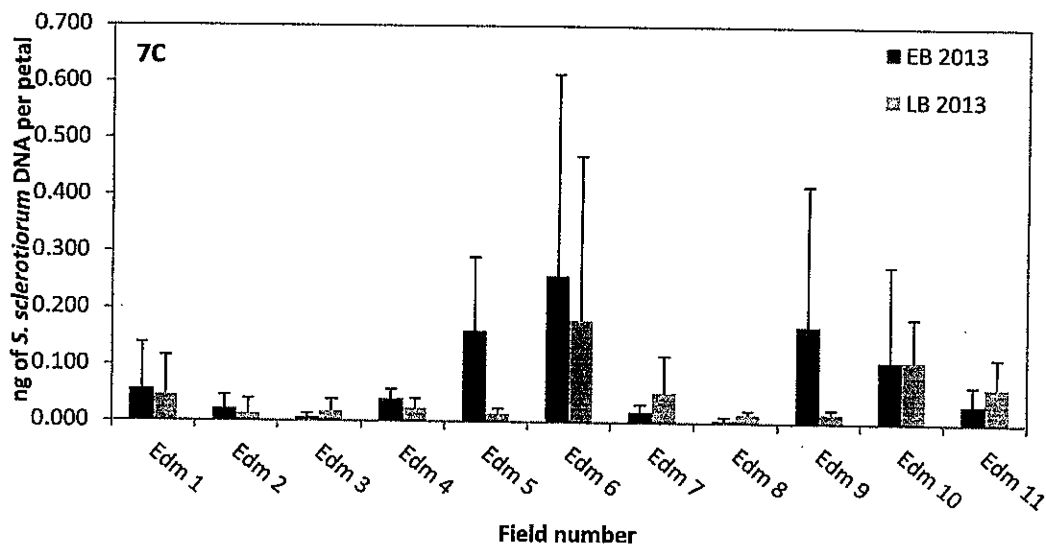


536



537

538



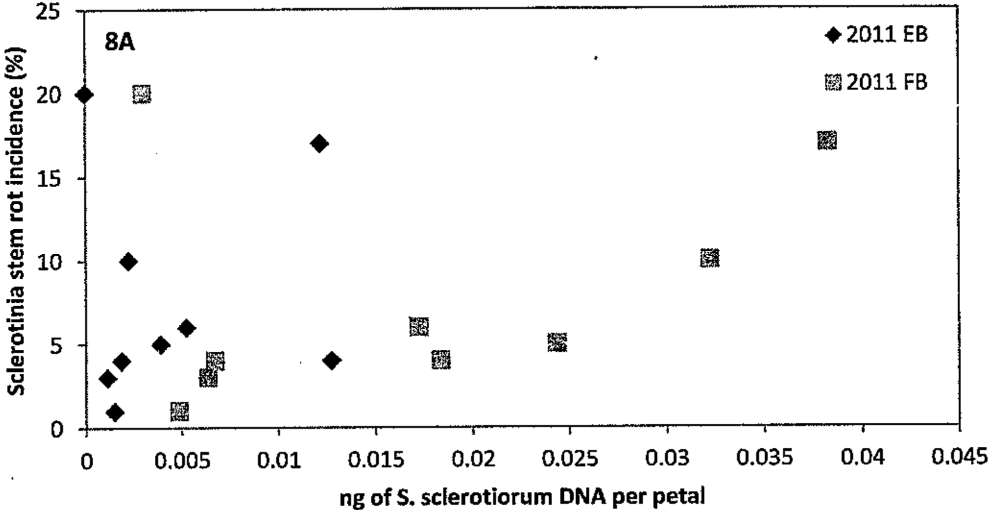
539 **Figure 7.** Amount of *S. sclerotiorum* DNA per canola petal for canola fields sampled in the

540 Edmonton region at early bloom (EB) and full bloom (FB) in (A) 2011, (B) 2012 and (C) 2013,

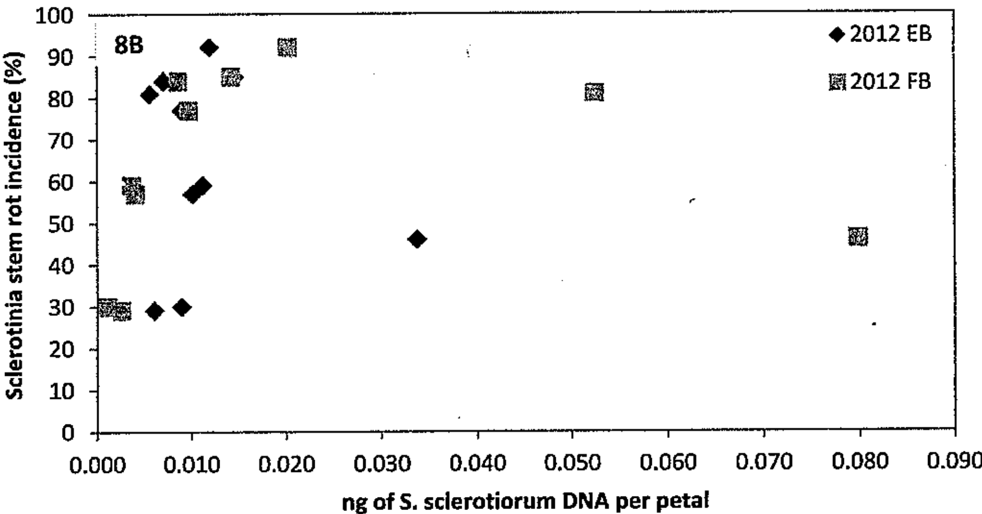
541 respectively, in Exp. 2. The error bars represent the standard deviation of the mean for each field.

542

543

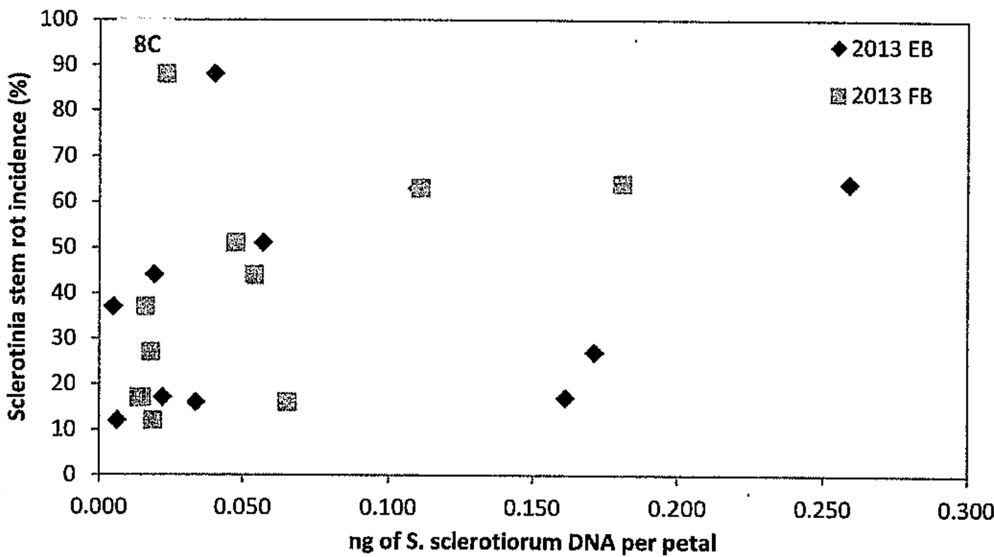


544



545

546



547 **Figure 8.** Relationship between the amount of *S. sclerotiorum* DNA per canola petal and the
548 incidence of Sclerotinia stem rot in Edmonton region fields in (A) 2011, (B) 2012 and (C) 2013,
549 respectively, in Exp. 2. When all fields were included in the regression analysis, only the full
550 bloom (FB) sampling in 2012 showed a significant relationship between the qPCR results and
551 final stem rot incidence ($R^2 = 0.602$; $P = 0.017$; CV = 22.91)

552