Development of a Quick and Reliable Molecular Detection System for Sclerotinia Stem Rot of Canola in Western Canada

B. R. Ziesman[1], T. K Turkington[2], U. Basu[1] and S. E. Strelkov[1]

[1]Dept. Agricultural, Food and Nutritional Science, University of Alberta, 410 Agriculture/Forestry Centre, Edmonton, AB, T6G 2P5, Canada
[2]Lacombe Research Centre, Agriculture and Agri-Food Canada, 6000 C&E Trail, Lacombe, AB, T4L 1W1, Canada.

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Abstract

Sclerotinia stem rot is an important, yield-limiting disease of canola caused by the necrotrophic fungus *Sclerotinia sclerotiorum*. The development of stem rot is highly dependent on environmental conditions and inoculum levels, and as a result disease occurrence and severity are sporadic in nature. In western Canada, stem rot is primarily controlled through the routine application of fungicides, which are often applied with no indication of disease risk. In order to reduce non-economical application of fungicides, a reliable forecasting system is needed. Since the infection of canola petals in an important step in the stem rot disease cycle, a quantitative (q)PCR-based system that can give an estimate of petal infestation rates could form the basis of a reliable forecasting system to help guide fungicide spray decisions. A primer and TaqMan probe have been developed based on the sequence of a novel protein that is specific to *S. sclerotiorum*. This primer-probe system, when used in a TaqMan qPCR assay, can quantify *S. sclerotiorum* DNA with a high level of sensitivity and specificity. The development of an internal amplification control will further increase the reliability of the assay by accounting for false negatives. This assay shows great potential for use as the basis of a stem rot forecasting system for canola in western Canada.

Introduction

Sclerotinia sclerotiorum is a ubiquitous, necrotrophic plant pathogen that is known to cause disease in more than 400 plant species, including stem rot of canola (*Brassica napus*) (Willets and Wong, 1980; Boland and Hall, 1994). Sclerotinia stem rot is a sporadic and devastating disease of canola. In western Canada, stem rot is primarily managed through routine fungicide application, with fungicides often being applied when disease levels are not economically significant (Bom and Boland, 2000). In order to reduce the negative economic and environmental effects of unnecessary fungicide application, a reliable forecasting system for stem rot is needed. The severity of this disease is dependent on both inoculum levels and environmental conditions, and as a result current forecasting systems are based on these factors.

As indicated by McLaren et al. (2004), most of the forecasting systems currently available are based primarily on weather conditions and field history. Although all of these systems incorporate important aspects of the stem rot epidemiology, they are underused by growers and industry.

A petal test kit was developed by Morall and Thompson (1991) which is based on the known positive correlation between petal infestation and final disease incidence (Turkington et al. 1991, 1993). When used with weather-based information, the petal kit can be a valuable risk assessment tool. The major limitation of the petal test is that it takes 3-5 days to get results, which can represent an unacceptable delay when making fungicide spray decisions (McLaren et al. 2004). It would therefore be beneficial to develop a technique that could be used to estimate petal infestation without the extended waiting period. A quantitative (q)PCR-based detection technique to quantify the amount of *S. sclerotiorum* DNA on canola petals collected from agricultural fields could represent such an approach. This type of technology would provide growers with an indication of the amount of inoculum present in a field, and would be based on the established relationship between petal infestation and disease development in canola. The purpose of this study was to develop a qPCR assay that could be used to reliably and quickly detect and quantify *S. sclerotiorum* DNA on canola petals.

Materials and Methods

DNA extraction from fungal mycelia. Pure fungal DNA was grown on potato dextrose agar (PDA) to produce a thick mycelial mat. A plug of the pure mycelium was then transferred to potato dextrose broth (PDB) where it was incubated on a shaker for 7 days. The mycelium was then washed with distilled water, centrifuged at 4500 rpm for 5 min, flash-frozen and lyophilized. DNA was isolated from the lyophilized mycelium using the Wizard® Genomic DNA Purification Kit (Promega) as per the manufacturer's instructions, except that two phenol-chloroform-isoamyl alcohol purification steps were included after protein purification. The purified DNA was quantified with a NanoDrop® spectrophotometer (Thermo Scientific).

Design of host-specific primers and TaqMan probe. The single-copy gene SSIG_00263.1 is a secreted protein that is hypothesized to serve as a virulence factor for *S. sclerotiorum* (Liang et al. 2013), and was selected as the target for the qPCR analysis. Forward and reverse primers were designed to amplify a 59-bp amplicon in a region of the gene that has low homology to an orthologous protein in *Botrytis cinerea* de Bary (Shah et al. 2009). A TaqMan probe labelled with FAM and MGB dyes that is specific to the 59-bp amplicon was also developed.

TaqMan qPCR reaction conditions. All qPCR assays were conducted using the Roche Universal Fast Start Probe Master Mix (Rox) as per the manufacturer's recommendations. The assays were run in a 20 µl reaction volume with 100 ng of template DNA.

Primer sensitivity testing. To determine the lowest limit of reliable detection with the assay, a standard curve was generated with a dilution series of purified *S. sclerotiorum*. The limit of sensitivity was defined as the lowest amount of DNA that could be consistently quantified within the linear range of the standard curve.

Primer specificity testing. The specificity of the primers for *S. sclerotiorum* was assessed by analyzing purified DNA from 13 other fungal species using the qPCR assay. These fungal species *Botrytis cinerea, Sclerotinia minor, Sclerotinia trifoliorum, Alternaria alternate, Aspergillus niger, Trichoderma* sp., *Fusarium* sp., *Mucor* p., *Alternaria brassicae, Rhizopus* sp., *Rhizoctonia* sp., *Leptosphaeria maculans* and *Cladosporium* sp were included in the specificity testing. The species were selected based on a close taxonomic relationship to *S. sclerotiorum* or their occurrence in the same environments.

Development of an internal control. Aboukhaddour et al. (2012) cloned a 432-bp fragment of the *ToxB* gene from *Pyrenophora tritici-repentis* into the plasmid pSilent1. This plasmid was used as an internal control to prevent false negatives. Forward and reverse primers were designed based on the *ToxB* sequence that will amplify an amplicon 54-bp in length. A *ToxB*-specific TaqMan probe was also and labelled with MGB and VIC to allow for multiplexing with the host-specific primer/probe set. To eliminate the possibility of false positives, the specificity of internal control primer/probe set was also tested.

Results and Discussion

The results of the sensitivity testing indicated that the lowest detection limit of the qPCR assay was 10.2 pg of *S. sclerotiorum* DNA. Moreover, the primer/probe set was found to be highly specific to *S. sclerotiorum* and did not amplify any products from DNA of the other fungi tested. The internal control was also found to be specific to the *ToxB* target sequence with no amplification of *S. sclerotiorum* or other fungal DNA. The specificity of the internal control primers and probes suggests that there is potential to multiplex with both primer and probe sets. This would offer the advantage of quantifying the target DNA in a sample while controlling for false negatives in the same reactions.

Conclusions

The probe and primers developed for *S. sclerotiorum* in the current study are highly sensitive and specific for the fungus under the qPCR conditions evaluated. As such, this qPCR assay may serve as the basis for a reliable and rapid stem rot risk assessment tool. The inclusion of an internal control will increase confidence in the quantification and will serve to eliminate the possibility of false negatives. Additional work will focus on examining the relationship between the results of the qPCR testing of field-collected canola petals, and the eventual stem rot incidence and severity in the corresponding canola crops.

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