Master Degree Project

UNIVERSITY OF SKÖVDE

QUANTITATIVE DETECTION OF *SCLEROTINIA SCLEROTIORUM* AND PREDICTION OF STEM ROT RAPE SEED PLANTS DISEASE BY USING REAL TIME PCR.

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Abstract

Sclerotinia sclerotiorum fungi causes stem rot disease which is a major and economically damaging disease in *Brassica napus*. Control of the disease is difficult, since no resistant cultivars are available, and it depends mainly on the use of fungicides and different forecasting models. These models are not satisfactory since they are based on previous knowledge about crop sequences which might be lacking. Therefore, a reliable and fast method of prediction of the disease is needed in order to detect early disease stages and the fields where fungicide application is suitable. Pathogen quantification by real-time PCR, is the most rapid method, reproducible and accurate to detect the target pathogen in the plant, and to estimate the disease severity by measuring indirectly the pathogen concentration. The aim of this study was to validate previous studies of detection and quantification of *Slerotinia sclerotiorum* by real-time PCR and to compare the results obtained in oilseed rape leaves and petals (by real-time PCR). Moreover, quantification of *Sclerotinia sclerotiorum* in petals and leaves was compared to assess which is the best sample material for the detection of the fungi. Finally, results were interpreted based on the weather conditions and disease severity index during the year of recollection of the samples, 2014. Results, after comparison of percentage of detection between leaf and petal samples, showed that both samples worked fine for the detection of the fungi. Moreover, even though the fungi was detected in the plant material by real-time PCR no stem rot disease developed.

Keywords:

Stem rot disease, disease severity index, *Sclerotinia sclerotiorum*, fungi, oilseed rape, plant, quantification, real-time PCR, qPCR, detection, prediction model.

Abbreviations

BLAST: Basic Local Alignment Search Tool

CBS: Centraalbureau voor Schimmelcultures

Cq: Quantitation cycle

CTAB: Cetyl Trimethylammonium Bromide

DSI: Disease severity index

E: Amplification efficiency

EB: Elution Buffer

kb: Kilo bases

LB: Luria Bertani medium

LOD: Limit Of Detection

NTC: Non Template Control

qPCR: Quantitative Polymerase Chain Reaction

R^2: Regression Factor Value

SD: Standard Deviation Value

SOC: Super Broth Medium

TE: TrisHCl-EDTA

6-FAM: 6-carboxy-fluorescein

Popular scientific summary

"A relevant technique for avoiding economical losses"

Serious economic losses are provoked in oilseed rape cultivars worldwide by stem rot disease every year. This disease is caused by *Sclerotinia sclerotiorum* fungi and is hard to control since there are no resistant plants and the fungicides available in the market need to be applied with enough time to avoid the disease development.

Recent investigations have found an important technique that, together with weather conditions and some forecasting models, will allow to predict how the disease will developed and subsequently to decide when the application of the fungicide will be

suitable. This technique is called “real-time PCR” and it allows the detection and quantification of the fungi in a DNA samples from plant material, such as leaves and petals. This technique allows detecting the presence of the fungi and its amount in the plant at early stages of the infection. Furthermore, this method is rapid and allows studying a huge amount of samples at the same time.

Our assay aimed to use this innovative technique for assessing differences between the detection and amount of the fungi between leaves and petals from oilseed rape plants and for determining which the best sample material for the detection of the fungi is. Moreover, this study aimed to interpret these results considering the weather conditions existing in the same period and fields where the samples were collected. Finally the results obtained were compared with the disease observed a posteriori.

In conclusion, no differences were found in the results between detection and quantification of the fungi between the leaves and the petals of the oilseed rape plants studied, despite this the technique was completely able to detect and quantify the fungi. Finally, our study showed that the presence of the fungi in the plant does not imply the development of the disease because in the samples assessed the fungi was present in 69% but in the field almost none of the plants, were the samples came from, had the disease.

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Introduction

Sclerotinia sclerotiorum is a ubiquitous, nectrotrophic ascomycete pathogen with a worldwide distribution known to infect over 400 host plant species, primarly dycotyledoneus species (Clarkson *et al.*, 2003). The most important economically affected crops are bean, oilseed rape, sunflower, tobacco and a wide range of vegetables such as lettuce, cabbage, cauliflower; and tubers such as carrot and potato (Clarkson *et al*, 2003).

S. sclerotiorum causes stem rot rape disease which is a major and economically damaging disease in *Brassica napus* in Sweden as well as worldwide (Almquist & Wallenhammar, 2014). Disease cycle of Sclerotinia stem rot (Figure 1) is characterized by the production of "sclerotia", which are the dormant fungi long-term survival structures for overcoming adverse conditions during summer and winter. *S. sclerotiorum* may infect the plants directly, after mycelial germination; or indirectly with the production of ascospores from apothecia after carpogenic germination (Qin *et al.,* 2011). From both mechanisms, the primary infection mechanism consists in the releasing and spreading of airborne ascospores (Clarkson *et al.*, 2003; Bolton, *et al.*, 2009). Ascospores require senescent tissues or pollen as a nutrient source for the posterior development of the disease (Clarkson, *et al.,* 2003). Infection mainly starts when spores-infected petals fall and stick to the leaves; and it was shown that disease incidence of sclerotinia stem rot is related to the percentage of flower petals infected (McCartney *et al.*, 2001). Once the petal is infected it is easy for the pathogen to penetrate and access to the lower stem or roots branches, branchlets and pods and thereafter kill the whole plant (Almquist & Wallenhammar, 2014; Qin *et al.,* 2011).

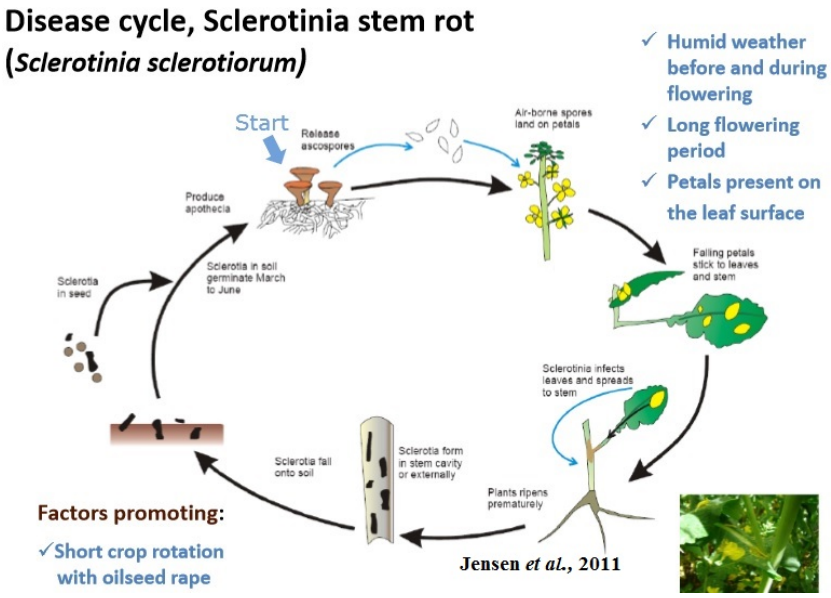

Fig.1 Disease cycle of Sclerotinia stem rot disease. The cycle starts when "sclerotia" dormant structures fall into the soil. Those structures may germinate directly or indirectly forming apothecia which release

ascospores. Ascospores infect petals and infected petals fall into the leaves. Thus, it is easy for the pathogen to penetrate into the stem and thereafter kill the whole plant.

S. sclerotiorum stem rot disease causes serious economic annual loss of 10–30% in rape seed plants yield by reducing both yield and oil content (Yang *et al.*, 2004). The disease is very hard to control since no resistant cultivars are available and this control depends mainly on the use of fungicides and different disease forecasting models (Hegedus *et al.*, 2007). The disease forecasting service in Sweden is mainly based upon a number of risk factors from the previous oilseed rape crop seasons; such as crop density and rotation, estimation of previous levels of infection and time for apothecia formation from sclerotia; and weather forecasting and amount of rainfall during early summer and flowering periods (Yuen *et al.*, 1996; Twengström *et al.*, 1998). Another prediction model from Germany is called SkleroPro, it is the first crop-loss-related forecasting model that has a minimum requirement of field-specific data and it is based on climate, crop rotation and economy (Koch *et al.*, 2007). These models are not satisfactory since there could be a lack of knowledge of previous crop sequences (Heltoft *et al.*, 2011). Therefore a reliable and fast method is needed in order to accurately detect early disease stages and the fields where fungicide application is suitable (Yin *et al.*, 2009). An efficient detection method could facilitate grower's decision on whether the disease status is correct or advanced enough to apply the fungicide; or conversely if the fungicide should not be applied because the disease has progressed too far (Almquist & Wallenhammar, 2014; Heltoft *et al.*, 2011). In order to be able to create a good disease prediction model it would be interesting to know precisely the amount of the pathogen in the field, rather than simply its presence.

Different methods of detection have been developed to detect and quantify soilborne pathogens, conventional methods and non-conventional methods. Conventional methods include visual grading, agar plating and soil bioassays. On the other side non-conventional methods include denaturing gradient gel electrophoresis, microarrays, sequencing and pyrosequencing, dot immunobinding, end-point and reverse transcriptase PCR, indirect inmunfluorescence and real- time PCR (Freeman *et al.*, 2002; West, 2008; Almquist & Wallenhammar, 2014). Conventional methods are time-consuming, not able to give a quantitative result of the infection neither to evaluate disease development before symptoms appear (Suarez *et al.*, 2005). Non-conventional methods are low time-consuming, highly specific, sensitive, rapid and reliable methods that provide an accurate assessment in pathogens that cannot be cultured and most importantly in earlier stages of the disease development (Wallenhammar *et al.*, 2012).

Among non-conventional methods, pathogen quantification by real-time PCR, is the most rapid method, reproducible, accurately, and effective to detect the target pathogen in the plant, and to estimate the disease severity by indirectly measuring the pathogen concentration (Almquist & Wallenhammar, 2014; Ziesman, 2013; Wallenhammar *et al.*, 2012; Rogers *et al.*, 2009; Suarez *et al.*, 2005). Besides, qPCR showed good potential measuring rot risk from airbone spore inoculums (Almquist & Wallenhammar, 2014).

Furthermore, real-time PCR allows detecting of the presence of obligated parasites or fungi that cannot be cultured (Wallenhammar & Arwidsson, 2001). Several important plant pathogens have been reported to be detected and quantified by real-time PCR (qPCR) from various sample material like plant, soil and air (Almquist & Wallenhammar, 2014; Lees *et al.*, 2010; Wallenhammar *et al.*, 2012, Rogers *et al*, 2009). Furthermore, qPCR data obtained in early bloom stage could be related with stem rot disease incidence and severity later in the full bloom stage (Turkington *et al.*, 2013). Therefore, qPCR data is directly related with the incidence of the disease in the same fields and season that the sample was obtained (Turkington *et al.*, 2013).

Previous studies performed by Almquist & Wallenhammar (2014) indicated that an assay to detect *S. sclerotiorum* on oilseed rape leaves, rather than on petals, could potentially improve disease risk assessment. An effective qPCR risk assessment tool will avoid an unnecessary fungicide application and consequently reduce significant expenses for the farmers; and also allow understanding how is the influence of local environment conditions on disease development and spreading.

The aim of this study is to validate the previous results obtained by Almquist & Wallenhammar (2014) by comparing the detection of *S. sclerotiorum* in naturally infested oilseed rape leaves and petals by real-time PCR, and to quantify the presence of the fungi in oilseed rape material. Different DNA extraction methods will be used and evaluated in order to develop a more robust and sensitive detection method. Moreover, quantification of *S. sclerotiorum* in petals and leaves will be compared to assess which is the best sample material for the detection of the fungi. Finally, since spreading of the spores and stem rot disease development needs certain weather conditions, such as high moisture due to cumulative rainfall periods before flowering, and drying of the petals before falling; results will be interpreted based on the weather conditions and disease index during the year of recollection of the samples, 2014.

Material and Methods

A total of 230 samples of DNA, 50 samples from leaf and 180 from petal DNA extraction were obtained by DNA extraction from four different fields, Tybble, Stora Valla, Joganbo and Frommesta. Moreover, a total of twelve samples containing genomic DNA from *Sclerotinia sclerotiorum* were extracted from the fungi.

Petals from rape seed plants were collected in different growing stages, between plants with 30% of top buds flowering (BBCH Code-63) and plants with 50-100% of top buds flowering and elderly petals starting to fall (BBCH Code-65); in the summer of 2014 from ten fields in Örebro county, Sweden.

Leaves from rape seed plants in BBCH 63-65, growing stage, were collected in the summer of 2014 from ten fields in Örebro county, Sweden.

DNA extraction

Genomic DNA extraction

Fungal strain CBS 499.50 of *Sclerotinia sclerotiorum* obtained from Centraalbureau voor Schimmelcultures was grown on potato dextrose agar plates during 72 hours at room temperature. Genomic DNA was extracted from about 30 mg of fungi by following E.Z.N.A. SP Plant DNA Miniprep kit extraction method (Omega Bio-Tek, 2014) as per the manufacturer's instructions. Prior to beginning with E.Z.N.A Kit Protocol, samples obtained from agar-plate cultures, were crushed by mortar and pestle after freezing with liquid nitrogen. DNA extracts were eluted in 50 µl of elution buffer (EB) (Omega Bio-Tek, 2014) and stored as aliquots at -20°C. Concentration, DNA purity and contamination were measured by NanoDrop 2000 Spectrophotometer (Thermo Scientific). DNA purity was assessed by measuring the absorbance at wavelength ratios of A260/A280 and A260/A230. Samples were left in -20ºC until further use.

DNA extraction from petals

DNA extraction was performed using a commercial lysis buffer, MicroLYSIS-PLUS (Microzone Ltd), with some modifications in the general protocol. A single frozen petal was pre-heated at 95°C for 5 min in 0.2 ml PCR tube. Next, 40µl MicroLYSIS-PLUS (Microzone Ltd) was added and the samples were placed in a thermal cycler (PTC-200; Bio-Rad) and treated twice using the following cycling profile: 65°C for 15 min; 96°C for 2 min; 65°C for 4 min; 96°C for 1 min; 65°C for 1 min and 96°C for 30 s. The samples were vortexed between each cycle.

After incubation, 40 µl TE buffer (1 mM Tris-HCl; pH 8.0; 0.01 mM EDTA) with 2 mg polyvinylpyrrolidone (Sigma) was added and the samples were vortexed. Samples were centrifuged at approximately 17 900 x g for 15 min and 50µl supernatant were transferred to new 0.2 mL PCR tubes. Precipitation of DNA was carried out by adding 125µl ice-cold 95 % ethanol and 10µl 7.5 M ammonium acetate (Sigma); and by mixing and incubating at -20°C for a minimum of 30 min. After centrifugation at 17 900 x g for 15 min, the supernatant was discarded. Finally, DNA pellet was left to air-dry before resuspension in 50 µl sterile milli-Q water. Concentration, DNA purity and contamination was measured as in genomic DNA extraction protocol. Samples were left in -20ºC until further use.

DNA extraction from leaves

DNA extraction from ten oilseed rape basal leaves from each field (five fields) was performed by using a modified E.Z.N.A. SP Plant DNA Miniprep kit (Omega Bio-Tek). First, oilseed leaves were cut into small pieces, approximately 1 cm^2, and all pieces were placed in 30 ml tubes. Then, 10 ml of CTAB (cetyl trimethylammonium bromide)

solution (ITW Reagents, PanReac AppliChem) and 30 µl of proteinase K (20 mg/ml, Omega Bio-Tek) was added and incubated at 60°C for 2 h. After incubation, samples were centrifuged at 1750 x g and 1000 µl of the supernatant was transferred to a 1.5 ml Eppendorf tube. Then 5µl of RNAse A (10 mg/ml, Omega Bio-Tek) were added and the samples were incubated at 60°C for 15 min. Thereafter samples were centrifuged at 20000 x g for 1 min and 600 µl of the supernatant was transferred to a new tube. 210 µl of SP2 Buffer (Omega Bio-Tek) were added and the mixture was incubated at 4°C for 5 min. After incubation, samples were centrifuged at 20000 x g for 10 min and 400 µl of the supernatant were transferred to a new tube, were 600 µl of SP3 Buffer (Omega Bio-Tek) was added. From this step, the protocol followed was the general E.Z.N.A. SP Plant DNA Miniprep kit extraction method (Omega Bio-Tek).The DNA from leaves was eluted in 50 µl of elution buffer (EB) (Omega Bio-Tek) preheated at 65ºC. Concentration, DNA purity and contamination were measured as the protocols above. Samples were left in -20ºC until further use.

Preparation of plasmid samples for quantification curve

PCR Assay

The targeted gene in the quantitative real-time PCR was a 278 bp fragment of the 5.8S ribosomal RNA gene of *Sclerotinia sclerotiorum* with GenBank accession number of M96382 (Z73799 and Z73800).

Genomic DNA samples from *S. sclerotiorum* were assessed by PCR assay in order to check for amplification of the target sequence. The PCR followed the cycling profile times and temperatures for AmpliTaq Gold from Applied Biosystems. Amplification was performed in a thermal cycler PTC-200 Bio-Rad system in a total volume of 50 µl. The reaction mixture contained 10 ng genomic DNA of *S. sclerotiorum,* 1X PCR Buffer II (Life Technologies), 3 mM $MgCl_2$, 0.8 mM dNTP, 0.5 mM of each primer SSFWD (5′-GCTGCTCTTCGGGGCCTTGTATGC-3′) and SSREV 5′ CAGCTTGGTATTGAGTCCATGTCA-3′) (Sigma) and 1.25 U AmpliTaq Gold DNA polymerase (Life Technologies). The thermal cycling conditions were: An initial denaturation step of 10 min at 95°C, followed by 29 cycles of 45 s at 95°C, annealing temperature gradient from 60°C to 72°C and 1 min at 72°C and finally 10 min at 72°C. After amplification, the samples were loaded onto a 1.5% Agarose electrophoresis gel together with a 100 and 50 base pair ladder (New England Biolabs) in order to ascertain that the desired length fragment (278 bp) was properly amplified.

Cloning and Transformation

The amplified PCR product was cloned by TOPO® TA Cloning® Kit for Sequencing (Life Technologies) in One Shot® TOP10 and DH5α ™ – T1 ᴿ *Escherichia coli* competent cells. First, TOPO® cloning reaction was set up by mixing together the PCR product (2 µl), TOPO® vector (1 µl), water (2 µl) and salt solution (1 µl). After mixing the reaction was incubated at room temperature for 5 minutes and placed on ice.

Transformation followed One Shot chemical transformation protocol (Life Technologies) by adding 2 µl from cloning reaction into a vial of One Shot® TOP10 *E.coli* competent cells and incubated on ice for 30 minutes. After incubation, cells were heat-shocked at 42ºC for 30 seconds without shaking and immediately transferred to ice. Then 250 µl of SOC medium was added and the tube was shaken at 240 rpm at 37ºC. Three different volumes of the previous reaction, 10 µl, 30 µl and 50 µl, were spread on LB (Luria Bertani medium) pre-warmed plates containing 50 µg/µl kanamycin (Sigma) or 50 µg/µl ampicillin (Sigma). Plates were incubated overnight at 37ºC. After incubation, colony PCR was performed in order to check that the bacterial colony contained plasmid DNA with the desired 278 bp insert from *S. sclerotiorum*. Eight colonies were analyzed by PCR as described previously. Colonies corresponding with a clear band with the desired length fragment (278 bp) were cultured in 50 ml of LB, containing either 50 µg/µl kanamycin (Sigma) or 50µg/µl ampicillin (Sigma), overnight in conical flasks at 37ºC with agitation 240 rpm. After incubation, plasmid DNA was isolated.

Plasmid Isolation and Sequencing

Plasmid DNA, from cells in the LB media flasks, was isolated by QIAprep® Miniprep Kit (QIAGEN) according to the manufacturer's instructions. Bacterial cells were harvested by centrifugation at 5400 x g for 10 min at 4ºC. Pelleted cells were resuspended in Buffer P1 and mixed thoroughly with Buffer P2 and N3. After centrifugation at 13000 rpm, 10 min, supernatants were transferred to a QIAprep spin column. After a few centrifugations and washing steps with PB and PE Buffer, DNA was eluted in 50µl of Elution Buffer (EB). Plasmid DNA concentration and purity were measured by NanoDrop 2000 Spectrophotometer (Thermo Scientific). DNA purity was assessed by measuring the absorbance at wavelength ratios of A260/A280 and A260/A230. Plasmid DNA samples were sent for sequencing (KIGene Karolinska Institutet, Stockholm) by using the M13 forward primer [-20] (5′-GTAAAACGACGGCCAG-3′, Life Technologies) and M13 reverse primer (5′-CAGGAAACAGCTATGAC-3′, Life Technologies). Sequencing was performed to confirm that *S. sclerotorioum* ribosomal RNA gene was cloned into the TOPO® vector.

Sequences obtained (Appendix B) were analyzed with BLAST and Chromas Lite by checking for the specific insert sequence by following the Map of pCR™ 4 - TOPO® vector (Appendix C), where the TOPO® cloning site is indicated (Life Technologies).

Digestion

To linearize the TOPO® cloned vector containing the target gene, 10 µg of the isolated plasmid DNA was taken for a digestion reaction with endonuclease NotI (New England Biolabs). Reaction was performed by mixing restriction enzyme NotI (100 units) and 1XNEBuffer I (New England Biolabs), plasmid DNA (10 µg) and deionized water until 100 µl of total reaction volume. Incubation digestion time was one hour at 37ºC. Inactivation of the enzyme was carried out by heating at 65ºC for 20 minutes. Cleaning of the digested samples was performed by QIA quick PCR Purification Kit (QIAGEN).

Digestion of the plasmid DNA was assessed by running digested and undigested samples into a 1% Agarose electrophoresis gel dyed with Ethidium Bromide.

Real-Time PCR Assay

The gene amplified was ~10 ng DNA fragment of a 278 bp of the ribosomal RNA gene of *S.sclerotiorum*. The primer pair used for the amplification was SSFWD (5′-GCTGCTCTTCGGGGCCTTGTATGC-3′) and SSREV (5′-CAGCTTGGTATTGAGTCCATGTCA-3′) (Freeman *et al*., 2002), purchased from Sigma.

The amplicon was detected using a hydrolysis MGB probe (5′-CGCCAGAGAATATCAA-3′) labelled with 6-carboxy-fluorescein (6-FAM) purchased from Life Technologies. Amplification and detection were performed on a 7300 Real-time PCR system (Applied Biosystems) on MicroAmp Optical 96-Well Reaction Plates (Life Technologies). All samples were run in triplicates and the reaction mixture was composed of 1X TaqMan Universal PCR Mastermix (Life Technologies), 0.2 µM of each primer, 0.2 µM of the probe and 5 µl of template DNA solution in a total volume of 25 µl. The thermal cycling conditions were an initial denaturation for 10 min at 95°C, followed by a touchdown PCR consisting of a total of 54 cycles: 14 cycles of 95°C for 15 s followed by annealing for 60 s with temperature decreasing by 0.5°C every cycle from 72–65°C; 40 cycles of 95°C for 15 s followed by annealing for 60 s at 65°C. Samples were regarded as positive if two or more replicates resulted in detection of the target sequence. Furthermore, every assay included triplicates of a negative control (5 µl of water instead of template) and positive control (genomic DNA from *S.sclerotiorum*).

Quantification

The amount of pathogen DNA was quantified using a quantification curve generated by including reactions containing different 10-fold dilution, from 0.01 fg to 1000 fg, of a linearized plasmid carrying the 278 bp target sequence from *S. sclerotiorum*.

The 10-fold dilution series of the plasmid standard was analyzed in triplicate and Cq values were plotted against the log amount of plasmid DNA to create a quantification curve.

Primer/Probe specificity

The specificity of the primers was evaluated by running a qPCR on template DNA from different fungi species that usually can be found in the soil together with *Sclerotinia sclerotiorum*. The fungi species were: *Phoma exigua var exigua*, *Fusarium avenaceum*, *Fusarium culmorum*, *Verticillum longisporum*, *Botrytis fabae*, *Fusarium solani*, *Rhizoctonia solani* and *Pythium ultimum*. The qPCR assay was performed by following real-time PCR protocol described above. All samples were run in triplicate and with a DNA amount of 3 ng.

Evaluation of amplification efficiency (E)

Amplification efficiency (E) was calculated as a percentage using the equation:

$$E = 100 \times (10^{-1/k} - 1)$$

Where k is the slope of the equation describing the slope of the curve obtained from the plot of Cq versus the logarithm of the DNA amount.

Calculation of the number of DNA copies

Number of total DNA copies in the sample was calculated following the equation:

Nº total DNA copies = [DNA Conc (g/µl) / (660 g/mol* total base pairs)* Avogadro´s number] * Volume of sample

Where Avogadro´s constant = $6.023 * 10^{23}$ and Total base pairs = Plasmid DNA base pairs + Insert DNA base pairs

Statistical analysis

Data obtained was evaluated for any statistical significant differences between petal and leaves with a student's t-test, available in the analysis ToolPak in EXCEL (Microsoft Office Standard 2010, Microsoft Corporation), and the significant level was set to a p-value < 0.05.

Results & Discussion

The aim of this study was to detect and quantify *Sclerotinia sclerotiorum* in leaf and petal isolated DNA samples from oilseed rape plants. Moreover, quantification of *Sclerotinia sclerotiorum* in petals and leaves was compared to assess which is the best sample material for the detection of the fungi.

A total of 80 samples from four different fields, Tybble, Stora Valla, Joganbo and Frommesta; were analyzed by real-time quantitative PCR in triplicates (240 in total).

DNA extraction

A total of 230 samples of DNA, 50 samples from leaf and 180 from petal DNA extraction were obtained by DNA extraction. Moreover, a total of twelve samples containing genomic DNA from *Sclerotinia sclerotiorum* were extracted from the fungi to be used as a positive control in the real-time PCR assays (including primer/probe specificity assay).

Samples were evaluated for concentration, purity, by absorbance wavelength ratio of A260/A280; and contamination, by absorbance wavelength ratio of A260/230.

In this study, DNA samples extracted from leaves and petals had A260/280 ratios above 1.8. Petal DNA concentrations were ranging from 18.7 ng/µl to 74.4 ng/µl and leaf DNA concentrations were ranging from 1 ng/µl to 18 ng/µl. However, A260/A230 ratios were lower than preferred, between 1 and 2. High quality DNA samples, with A260/280 ratios of 1.8 and A260/A230 of 2.0-2.2, are considered pure and free of different inhibitors for ensuring confidence in all subsequent assays (Nanodrop technical bulletin, Thermo Scientific; Demeke & Jenkins, 2010). PCR inhibitors can lead to lower PCR efficiency, by interfering with DNA polymerase; to false negatives or to an underestimation of the quantity of the target DNA (Olson & Morrow, 2012). A260/230 ratios below 1.8 could indicate the presence of different inhibitors such as salt, humic acid, proteins and polysaccharides; and significant absorbance at 230 nm indicates contamination by reagents commonly used in nucleic acid isolation, such as phenol and urea (Olson & Morrow, 2012).

In spite of the lower A260/230 ratios, obtained for the DNA samples in this study, the samples were used for subsequent reactions. This decision, was supported by previous studies showing no variations in amplification plots, Cq values or in the detection and amplification (Olson & Morrow, 2012; Demeke & Jenkins, 2010). A260/280 ratio is also considered better indicator of suitability for PCR whereas A260/A230 ratio is a better indicator for suitability in microarrays (D. Olson & B. Morrow, 2012; Ning, J. *et al*., 2009). Furthermore, PCR inhibition assays showed that contaminants, from samples with A260/A230 ratios below 2, did not inhibit PCR (Olson & B. Morrow, 2012). Also, A260/A230 ratios even below 0.5 did not affect real-time PCR amplification in the case of DNA extracted from soil samples (Ning, J. *et al*, 2009). Finally, in some extraction DNA methods using CTAB and Sodium dodecyl sulphate and polyvinylpyrrolidone solution, like the methods described before for petals and leaves, effect of possible inhibitors, in samples with low A260/230 value, is reduced by the dilution of the extracted DNA in the subsequent qPCR reaction (Demeke & Jenkins, 2010).

Preparation of plasmid samples for quantification curve

After PCR amplification, assessing for the target sequence from *Slerotinia sclerotiorum*, and electrophoresis, clear bands with the approximated length as the desired target sequence of *Sclerotinia sclerotiorum* were observed (data not shown). This led to the conclusion that target DNA sequence was properly amplified. Cloning and transformation was successful since results from colony PCR electrophoresis showed clear bands of the approximated length as the desired sequence of 278 bp from *Sclerotinia sclerotiorum*, in the eight colonies analyzed (data not shown). After incubation of colonies, plasmid DNA isolation was performed and DNA extracts, used for quantitative curve realization, followed strictly the required ratios of purity and contamination, A260/A280 ratios of 1.8 and A260/A230 ratios of 2.0-2.2 respectively. Plasmid DNA samples were sent for sequencing in order to confirm that the desired

sequence was cloned into the vector. Sequences obtained from sequencing was analyzed with BLAST and Chromas Lite and showed that all the samples of isolated plasmid DNA contained the vector (Appendix B) with the target sequence inserted in the cloning site (Appendix C). This target sequence matches the sequence of *Sclerotinia sclerotiorum* found in GenBank with accession number of M96382 (Z73799 and Z73800).

Plasmid Digestion

In order to linearize the TOPO® cloned vector, containing the target sequence, for posterior dilutions and quantification curve realization; digestion was carried out in two samples of isolated plasmid DNA. Results of plasmid digestion (Figure 2), with NotI, after electrophoresis showed a clear band of 4-4.5 kb approximately, that corresponds with pCR™ 4 - TOPO® vector length plus the insert with a total length of 4234 bp (Appendix B), while undigested samples showed more bands of different sizes corresponding with the plausible forms of undigested plasmid: supercoiled, nicked and linear.

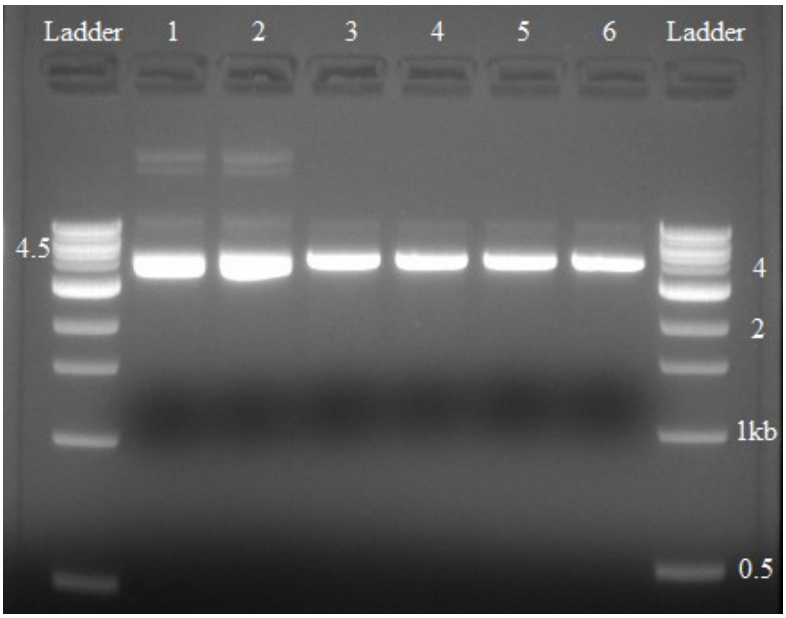

Fig.2 Electrophoretic results of cloned DNA digestion with NotI endonuclease enzyme. Lanes in both sides: Ladder 1kb DNA ladder (New England Biolabs). Lane number 1 and 2: Undigested samples of plasmid DNA. Lane number 3, 4, 5 and 6: Digested plasmid DNA samples with NotI endonuclease restriction enzyme.

Quantitative Real Time PCR Assay

Problems during qPCR assessment

During the performance of real-time PCR there were problems with DNA contamination. Amplification was detected in negative controls (NTC) and also irregularities in Cq values were observed in the quantification curve. These irregularities

in Cq values between different plasmid DNA dilutions led to a very low R^2 value. Irregularities were found in the higher dilutions of the DNA template, 4.3 copies/µl and 0.43 copies/µl, where very low Cq values, plotted against the logarithm of the total DNA copy number, showed a curved tendency instead of a linear tendency (Figure 3). The same differences were obtained in several different qPCR experiments.

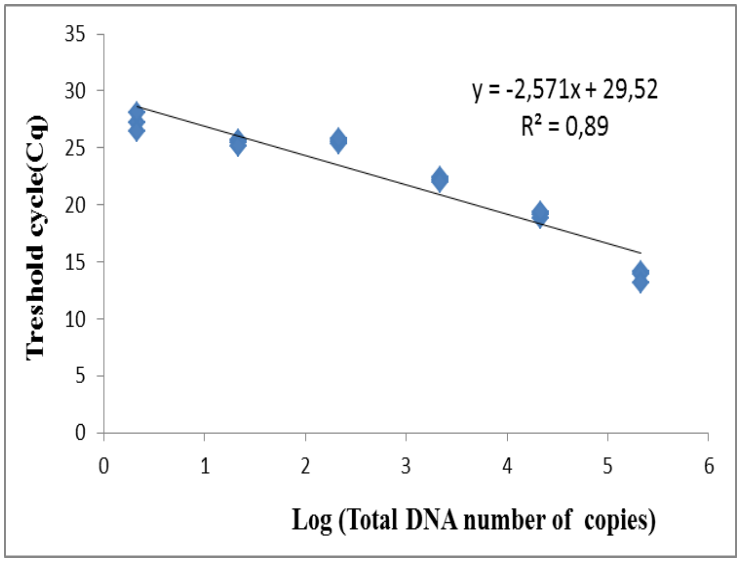

Figure 3: Quantification curve from 10-fold plasmid DNA serial dilutions, with *Sclerotinia sclerotiorum* target, generated for the quantification of the target DNA in petal and leaf samples. Linear regression was generated by plotting threshold cycle values (Cq), obtained in qPCR assay, and the logarithm of total DNA copies in each plasmid DNA dilution. Correlation values (R2) was 0.89. Graphic shows a curve tendency due to irregularities in higher template dilutions (lower values of logarithm of total DNA copy number in the graphics) owing to contamination problems. NTC values in the same assay showed amplification. Results are shown in triplicates.

Cq values among NTC replicates showed no variation, this led to the conclusion that one or more reagents were contaminated. Reagents were changed several times, but still contamination of the NTC and standard curve samples was observed. An electrophoresis of the contaminated NTC samples comparing with petal samples coming from one of the contaminated qPCR experiments, was performed in order to see if the non-desirable detected amplification was truly the amplified target DNA from *Sclerotinia sclerotiorum*. As can be observed in Figure 4, all the lanes showed a clear band between 250 and 300 base pair that matches with the expected target DNA sequence of 278bp from *Sclerotinia sclerotiorum*. This led to the conclusion that the source of DNA contamination was *Sclerotinia sclerotiorum*.

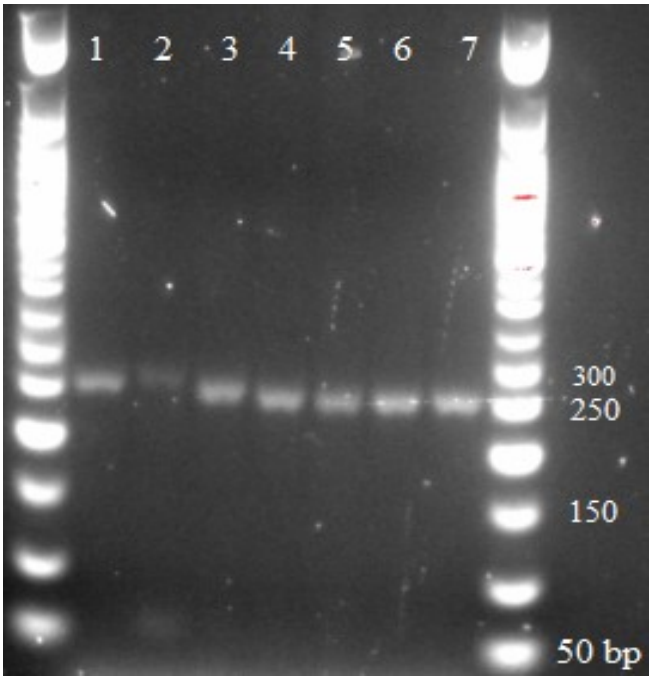

Fig.4 Electrophoretic results of different products from qPCR. Lanes in both sides: 50bp DNA ladder (New England Biolabs), lanes 1-3: Negative control (NTC), lanes 4-5: Frommesta petal samples and lanes 6-7: Plasmid DNA samples from quantification curve (100fg and 10fg).

As one of the most sensitive methods for detecting nucleic acids, real-time PCR has a strong sensibility and can be affected by very low levels of contamination from its own product. This susceptibility is due to the fact that Real Time PCR can detect very low levels of nucleic acids and PCR amplification function can generate billions of copies of the DNA sequence that is being analyzed, and especially at higher cycle numbers (Witt *et al.*, 2009). There were two suspected sources of contamination in this study: Carryover DNA contamination and/or airborne spore inoculation. Previous studies showed that fungal airborne inoculation is more unlikely (3.3%) (Loeffer *et al.*, 1999), however both sources were considered and contamination problem was finally avoided by changing laboratory, pipettes and reagents, by separating pre- and post-amplification areas and equipment, and by performing qPCR non-DNA steps in a sterilized laminar airflow bench as suggested by Sundquist et al (2005) and Thermo Scientific (2015). After carefully evaluation, it was considered that the principal source of contamination were the pipettes, even though filter tips were being used. Pipettes were suspected of containing DNA aerosols from PCR amplicons and from DNA isolation reactions. Once the contamination was avoided, all the reagents were tested for contamination in order to be able to reuse any in further reactions. A new dilution series of the plasmid DNA samples, used for the quantification curves were made.

Primer/Probe specificity

Primer/Probe specificity assay (Table 1) showed amplification only for *Sclerotinia sclerotiorum* samples, so primer and probe are considered to be specific for *Sclerotinia sclerotiorum.*

Table 1: Primer/probe specificity assay. Different fungi species abundant in soil assessed for detection and amplification for the primer SSFWD and SSREV and TaqMan probe designed for *Sclerotinia sclerotiorum* 278 bp targeted sequence detection. Assay showed amplification only for *Sclerotinia sclerotiorum* samples with a mean Cq value of ~14.00.

Specie	Detection/Non-detection SSFWD/SSREV. *Sclerotinia. scl* probe	Cq value
Sclerotinia sclerotiorum	+	14.00
Phoma exigua var exigua	_	Undetermined
Fusarium avenaceum	_	Undetermined
Fusarium colmorum	_	Undetermined
Verticillum longisporum	_	Undetermined
Botrytis fabae	_	Undetermined
Fusarium solani	_	Undetermined
Rhizoctonia solani	_	Undetermined
Pythium ultimum	_	Undetermined

Previous studies, where 17 different fungal species were analyzed, showed the same results of specify for *Sclerotinia sclerotiorum* with the same primer/probe system and with the presence of an excess of a closely related fungi, *Botyris cinerea* (Freeman, 2002). However, is not strictly specific for S. sclerotiorum since *S. trifoliorum*, *S. minor* and *S. glacialis* are detected by the primer/probe system (Freeman, 2002; Almquist & Wallenhammar, 2014). Although these species can be detected by the same primer/probe system, this may not be a problem since the host infected is different from oilseed rape plants and/or the country where the specie inhabits is different from Sweden (Bom and Boland, 2000). Hosts of *S. trifoliorum* are restricted to forage legumes and *S. minor* and *S. glacialis* only inhabit Australia and Switzerland respectively (Freeman, 2002).

Results of detection and quantification from quantitative real-time PCR

Analysis of results from quantification curves

In the present study, quantification curves from 10-fold dilution series of plasmid DNA, containing the target sequence from *Sclerotinia sclerotiorum*, were generated in order to quantify the amount (copy number) of *S. sclerotiorum* DNA present in petal and leaf samples. Quantification curves contained at least five dilution points and the target DNA was detected at the highest template dilution, representing only three plasmid DNA copies and corresponding to 0.01 fg of plasmid DNA (Figure 5). Regression analysis, between threshold cycle (Cq value) and logarithm of total DNA copies, showed a linear function in all the occasions. Correlation coefficient (R^2) ranged from 0.90 to 0.99 and the assays had low to very high amplification efficiency (73% to 147%) in the four experiments, one per field.

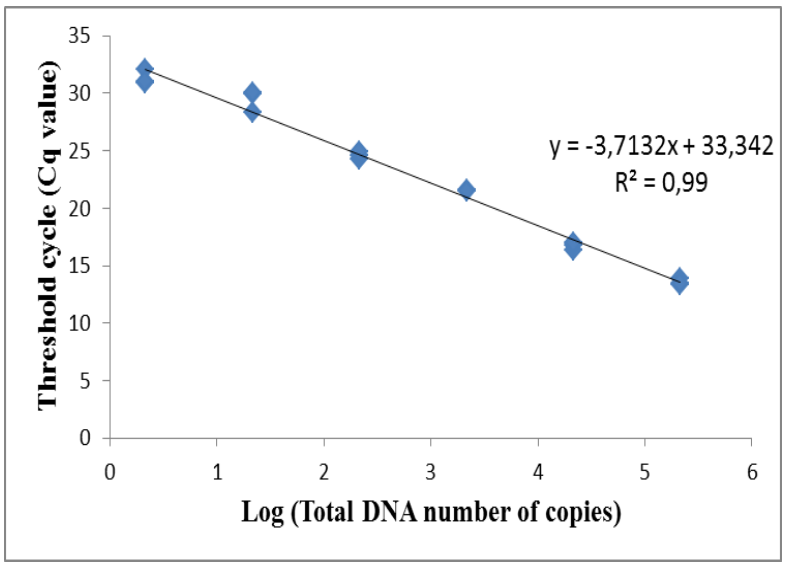

Fig.5: Quantification curves from 10-fold plasmid DNA serial dilutions, with *Sclerotinia sclerotiorum* target, generated for petal and leaf samples quantification from Tybble oilseed rape field. Linear regression was generated by plotting threshold cycle values (Cq), obtained in qPCR assay, and the logarithm of total DNA copies in each plasmid DNA dilution. Correlation value (R^2) was 0.99. One sample (0.33; 24.9) was regarded as an outlier and is not included in the regression analysis of the diagram. Results are shown in triplicates.

The hallmarks of an optimized qPCR assay are a linear quantification curve ($R^2 >$ 0.980), high amplification efficiency (90-105%) and consistency across replicates or precision (Bustin *et al.*, 2009; Bio-Rad, 2015).

Linearity close or higher than a correlation coefficient value (R^2) of 0.980 indicates how well the obtained experimentally Cq values correlate with the dilution series (Bio-Rad, 2015). In this study, linear quantification curves were obtained with a regression factor ranging from 0.90 to 0.99. From previous assays with *S. sclerotiorum* detection,

correlation coefficient was equal to or higher than 0.99 (Yin, 2009; Wallenhamar *et al.*, 2014). Results from this study, with R^2 values higher or equal value to 0.98, were considered more reliable. However, due to the lack of enough results, all the Cq values were analyzed and interpreted.

Amplification efficiency close to 100% (90-105%) is the best indicator of reproducibility of the assay. The values obtained in this study ranged from 73% to 147%. None of these values were within the range of 90% to 105% amplification efficiency. Several parameters can affect PCR efficiency, e.g. presence of PCR inhibitors, PCR primer and/or suboptimal probe design and improper analysis of standard curve (Life Technologies, 2015). Suboptimal design of PCR primer and/or probed was not probable because the primers were previously evaluated by bioinformatic and experimental methods by several authors (Freeman, 2012; Almquist & Wallenhammar, 2014). Presence of inhibitors was also unlikely since all the DNA samples, used as a template in the qPCR assay, showed A260/A280 ratios above 1.8 (See section Results & Discussion: DNA extraction). Finally, improper analysis of standard curve was unlikely since all qPCR assays were analyzed with auto Cq value and outliers were not included (Figure 4).

The most likely explanation of higher and lower amplification efficiency values may be pipetting error of the serial dilutions, template or master mix addition; and/or pipetting errors of standards. In serial dilutions, pipetting of excess diluent and/or pipetting of insufficient sample, will lead to a good R^2 value (≥ 0.99) but lower PCR efficiency; while pipetting of insufficient diluent and/or pipetting of excess standard sample, will lead to a good R^2 value but higher PCR efficiency. On the other side, pipetting errors of the standards lead to high Cq standard deviations and R^2 value < 0.99.

Consistency across replicates or precision is measured by standard deviation value (SD). Results of standard deviation (SD), between replicates, in quantitative curves ranged from 0.062 to 3, keeping most of the resulting values below 0.5 (Appendix D). Data sets, with a minimum number of three replicates, as the one in this study, forms an approximately normal distribution (Life Technologies, 2015). To be able to quantify two successive samples in a 95.4% of the cases, the standard deviation has to be $\leq$ 0.250; and in the 68.2% of the cases, ≤ 0.50 (Life Technologies, 2015). All the results were considered for evaluation and interpretation. The most plausible cause of big variation values was pipetting differences among replicates.

Another important variable is the analytical sensitivity of the qPCR assay which is measured by the limit of detection (LOD) (Burns & Valdivia, 2008). Recent studies with *S. sclerotiorum* quantification set the limit of detection as few as five copies of the target DNA (Almquist & Wallenhammar, 2014). In this study, the highest analytical sensitivity was obtained by the detection of three copies of target DNA. This value is supported by the fact that the lowest LOD theoretically possible is three copies per assay, considering a Poisson distribution, a 95% chance of including at least one copy in the PCR, and single-copy detection (Wittwer & Kusakawa, 2004; Bustin *et al.*, 2009).

Analysis of results from DNA samples from leaves and petals

Despite contamination problems, four oilseed rape fields were analyzed for detection and quantification of *Sclerotinia sclerotiorum* fungi. A total of 80 samples from the fields Tybble, Stora Valla, Frommesta and Joganbo, collected in the summer of 2014 in Örebro county, were analyzed. In each field ten DNA isolated samples from oilseed rape petals and ten DNA isolated samples from oilseed rape leaves were analyzed in triplicates, with the aim to establish differences in the fungi detection and quantification between leaves and petals. Real-time PCR results showed detection of the fungi in 69% of the samples, where the number of analyzed samples in total, taking into account triplicates, was 240. There were no big differences between petal samples and leaf samples detection, 75% of the leaf samples were positive while 64% of the petal samples were positive. In particular, Stora Valla and Joganbo showed higher percentage of fungi detection in leaves, while in the field of Frommesta was the opposite; and in Tybble field it was equal between leaves and petals (Figure 6).

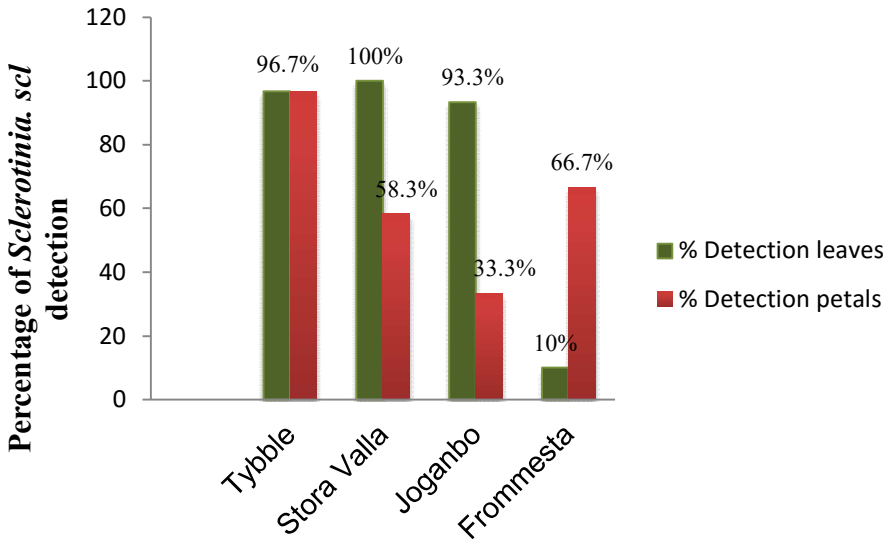

Fig.6: Results in percentage from qPCR *Sclerotinia sclerotiorum* detection in oilseed rape samples. Histogram shows the differences in percentage between detection of *Slerotinia sclerotiorum* in petals and in leaves samples from Tybble, Stora Valla, Joganbo and Frommesta oilseed rape fields. Percentage of detection was equal in Tybble field (96.7%), higher for leaves from Stora Valla and Joganbo (100% and 93.3% respectively) and higher for petals from Frommesta field (66.7%).

Almquist & Wallenhammar (2014) tried to search for advantages from leaf analysis compared with petal analysis, but no differences between detection in leaves and petals were found, only that senescent lower leaves showed higher detection of the fungi than higher leaves. Petals containing ascospores have been reported as the most effective source of leaf infection, but this does not mean differences between detection in leaves

and petals since the pathogen can be found in both structures at the same time (McCartney *et al.*, 2001).

In the case of the quantitative results (Appendix D) in the four fields analyzed: Tybble, Frommesta, Stora Valla and Joganbo; t-test showed no significant differences (p-value above 0.05) between number of copies detected from leaves and petals. Number of copies ranged from 0.012 to 59383 and the higher number of copies was observed more in the leaf samples than in petal samples (Appendix D). The highest number of copies was observed in a few number of samples, this could indicate that the plants corresponding to these samples would develop the disease. Furthermore, more than 80% of the samples showed a copy number below 215, corresponding with 1fg of fungi DNA, indicating that the fungi was present in very small amounts in the most of the plants analyzed, probably only fungi spores were present in the plants at the moment of recollecting of the samples. However, no previous studies have shown the approximate number of copies that will indicate the subsequent developing of the disease. Furthermore, quantitative results (Appendix D) do not show consistency among replicates. Consistency was measured by standard deviation value from the quantification value (Cq) and from the number of copies in 5µl for the three replicates. Standard deviation values were higher than expected and this could be explained by the existence of pipetting errors between replicates and/or quantification curve amplification efficiency distant from 100% (See Analysis of results from quantification curves). No previous studies has shown analysis and quantification outcomes for *Sclerotinia sclerotiorum* in leaf and petal isolated DNA samples from oilseed rape plants.

Previous studies (Almquist & Wallenhammar, 2014) affirm that the disease will be developed if *S. sclerotiorum* target sequence is detected in the sample, and if the conditions, such as weather, are optimal for the development of the disease. In this investigation 69% of the samples showed detection for *S. sclerotiorum* but the disease severity index (DSI) from oilseed rape plants, coming from the same fields, year and season of the samples collected for quantitative PCR; range from 0% to 1.25%. In particular, Stora Valla showed 0% of DSI, Frommesta and Joganbo 0.25% and Tybble 1.25%. A disease severity index of 0% indicates that most of the plants are healthy and a DSI of 1.25% indicates that very few plants have the rot enclosing <50% of the circumference of the stem and the rest of the plants is mostly completely healthy. These lower DSI values are due to the weather conditions during the period of the oilseed plants recollection, summer 2014. Spreading of the spores and stem rot disease development needs certain weather conditions, such as high moisture due to cumulative rainfall periods before flowering, and drying of the petals before falling (Almquist & Wallenhammar, 2014). Furthermore, increased mortality of ascospores has been correlated to number of hours above 21°C (Clarkson *et al.*, 2003). Local temperatures in

Örebro, during summer of 2014, were mostly above 22°C and precipitation was 0 mm most of the days and did not exceed 20 mm (AccuWeather, 2014). Due to the high temperatures and low moisture conditions the mortality of the ascospores was greater and thus the disease severity index was low even though *S. sclerotiorum* was detected in the plants of the field (Parker *et al*., 2014). In conclusion, it seems that the detection of the fungi, in the oilseed rape samples analyzed, is not strictly related with the development of the disease a posteriori. For the development of the disease there are many factors involved besides the presence of the fungus in the plant, such as the weather conditions and rainfall periods. Therefore detection of the fungi does not imply the development of the stem rot disease in the analyzed oilseed rape plant.

To summarize the results of the present study, the desired target sequence of a 278 bp long fragment of the ribosomal RNA gene from *S. sclerotiorum* fungi was successfully amplified, cloned and sequenced. During the set-up of the real-time PCR assay the initial problems of contamination, where the source was the target sequence of *S. sclerotiorum*, were later overcome. Plant material from four oilseed rape fields was analyzed for detection and quantification of *S. sclerotiorum* fungi. In addition, the specificity of the primers and probe used in the assay were verified. All quantification curves obtained were analyzed by assessing the hallmarks of an optimized qPCR assay. Furthermore, results of detection and quantification of the fungi between oilseed rape leaves and petals, were compared. These results showed that both sample material, petal or leaves, worked fine for the detection of the fungi since detection was equal between leaves and petals samples, depending on the field. Finally, quantitative results from qPCR assays were analyzed in four fields in terms of significant difference between petal and leaf material and consistency or precision, by the standard deviation, between replicates.

This is the second study showing quantitative results of *S. sclerotiorum* in oilseed rape plants and assessing differences in detection and quantification between petal and leaves. However, serious problems of contamination and time limitation made it impossible to perform additional qPCR assays and subsequently the obtaining and analysis of more data. Hereby, bearing in mind that real-time PCR has a strong sensibility to be affected by very low levels of contamination from its own product, it is important to avoid as soon as possible these problems by the change of all parts involved in the preparation and performance of the assay.

Moreover, there was no consistency in the results obtained due mostly to pipetting errors within replicates and the low reproducibility of the assay. Although it is easy to quantify the amount of a qPCR product, slight variations in the technique or in the amplification procedure can generate different amounts of DNA from the same starting material in the reaction. Hereby, it is important to properly establish an accurate pipetting method and attempt to repeat and optimize the assays considering the hallmarks of an optimized qPCR assay.

Finally, results showed that not only the presence of the fungi in the plant will lead to the development of the stem rot disease caused by *S. sclerotiorum* since the samples, used for real-time PCR, showed a very low severity disease index. In conclusion, it would be important to properly establish a correlation between the incidence of the fungi in the plant and the quantification values, such as the number of copies; and the severity disease index. To achieve this correlation, further qPCR assays are required, where the quantitative values would be assigned to a specific severity disease index. Thus, after the recollection of all results, a correlation of all the possible quantitative values with its DSI will be generated as a reliable prediction model of the stem rot disease.

Impact of the research on the society

There are no ethical aspects to board on the present study. As a common pathogen of a wide range of plants, *Sclerotinia sclerotiorum*, should be managed following the general protocol of sterilization, storage and discard of biological samples. Furthermore, the creation of a prediction model, basing on recompilation of quantitative data in different conditions, would predict more efficiently the development of the disease and replace other alternative techniques currently performed, such as the creation of genetically modified rape oilseed plants to overcome stem rot disease development, or use of fungicides. However it is important to avoid possible modifications of the DNA of the pathogen that could occur during the process of cloning and transformation, to avoid contact of pathogen with natural material and also discarded it properly. As impact on the society this method of prediction by real-time PCR will avoid economic losses that currently affect many farmers greatly. The avoiding of these losses will allow a higher production of rape oil and subsequently a lowering of the prices of this oil since the farmers will not waste their fungicide applications meaningless. Moreover, if the fungi is not detected in the plant, there is no need to fungicide applications and this would contribute greatly to the maintenance of preservation of the environment. Finally, about the real-time PCR technique could not only help as an effective method for predicting plant diseases, such as stem rot disease, but could also serve as disease prediction model for human diseases or alterations, such as Parkinson disease.

Future perspectives

Foremost, it would be important to finalize the prediction model based on correlation of the quantitative results from real-time PCR with the specific severity disease index value. This prediction model would be a computer model where data, such as quantitative values and weather conditions, will be interpreted. This will allow the farmer knowing which is/will be the progression of the disease in the cultivar. Then, since the development of the disease depends on certain conditions, it will be important to further clarify how are related the quantitative values, given by qPCR assay; with these conditions. For this, an *in vitro* or *in situ* assay where the process of infection of the fungi is controlled in terms of moisture and temperature, could be conducted. This experiment would compare the quantitative values obtained from real-time PCR in

oilseed rape plants at different conditions: Uninfected plant with optimal temperature and moisture conditions for the growing of the fungi (control separated from the other plants), infected plants with the fungi and a grade of different temperature and moisture conditions, infected plants with optimal conditions and uninfected plants in contact with the infected plants. This experiment would find the quantitative values associated to optimal and different temperatures and how is the mechanism of naturally infection and the quantitative values associated to a recent infestation.

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Appendices

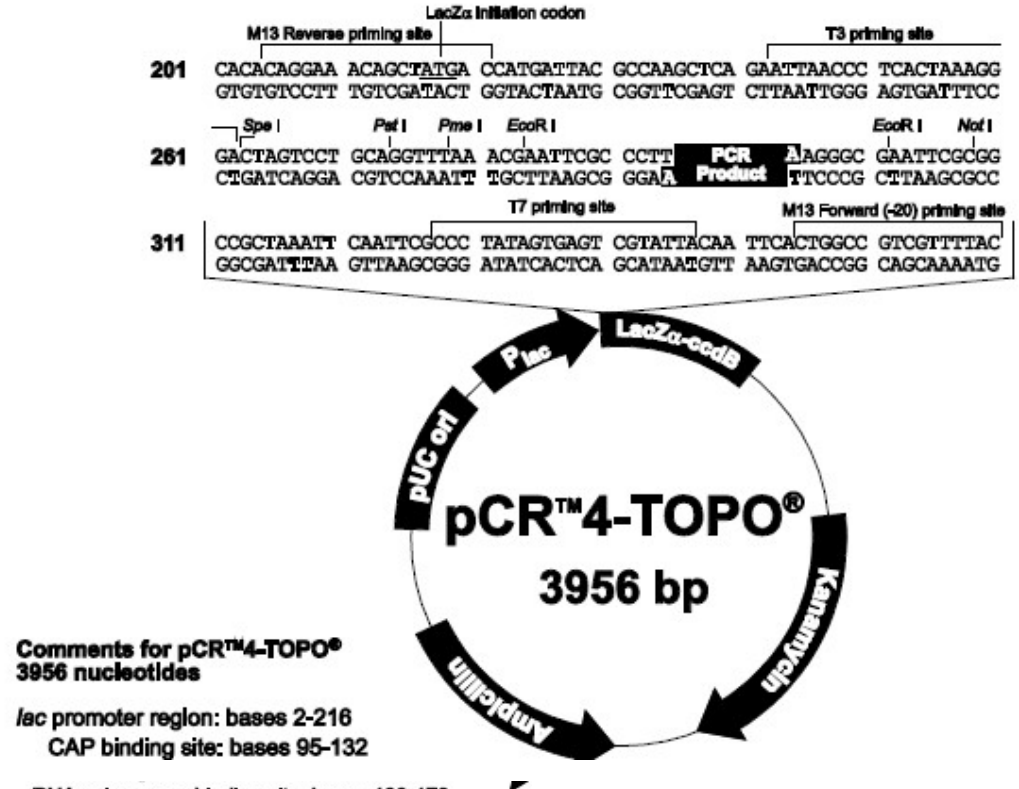
Appendix A: *Sclerotinia sclerotiorum* 5.8s DNA sequence from ribosomal RNA intervening transcriber spacer (481bp)

5′TCCGTAGGTGAACCTGCGGAAGGATCATTACAGAGTTCATGCCCGAAAGGGTAG
ACCTCCCACCCTTGTGTATTATTACTTTGTTGCTTTGGCGAGCTGCTCTTCGGGGCCT
TGTATGCTCGCCAGAGAATATCAAAACTCTTTTTATTAATGTCGTCTGAGTACTATA
TAATAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC
AGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTG
AACGCACATTGCGCCCCTTGGTATTCCGG GGGGCATGCCTGTTCGAGCGTCATTTC
AACCCTCAAGCTCAGCTTGGTATTGAGTCCATGTCAGTAATGGCAGGCTCTAAAAT
CAGTGGCGGCACCGCTGGGTCCTGAACGTAGTAATATCTCTCGTTACAGGTTCTCG
GTGTGCTTCTGCCAAAACCCAAATTTTCTATGGTTGACCTCGGATCAGGTAGGGATA
CCCGCTGAACTTAAGCATATCAATAAGCGGAGG3′

Sclerotinia sclerotiorum 5.8s DNA sequence of 481bp from ribosomal RNA intervening transcriber spacer (5'-3').

Target sequence in real-time PCR assay of 278bp amplified by SSFWD 5′-GCTGCTCTTCGGGGCCTTGTATGC-3′) and SSREV (5′ CAGCTTGGTATTGAGTCCATGTCA-3′) primers.
Part of the *Sclerotinia sclerotiorum* 5.8s DNA sequence sequence where SSFWD and SSREV bind and amplify the target sequence of 278bp used in this study.

Appendix B**:** Map of of pCRTM 4 - TOPO® vector (Life Technologies)



Comments for pCR™4-TOPO®
3956 nucleotides

lac promoter region: bases 2-216
CAP binding site: bases 95-132
RNA polymerase binding site: bases 133-178
Lac repressor binding site: bases 179-199
Start of transcription: base 179
M13 Reverse priming site: bases 205-221
LacZα-*ccdB* gene fusion: bases 217-810
LacZα portion of fusion: bases 217-497
*ccd*B portion of fusion: bases 508-810
T3 priming site: bases 243-262
TOPO® Cloning site: bases 294-295
T7 priming site: bases 328-347
M13 Forward (-20) priming site: bases 355-370
Kanamycin promoter: bases 1021-1070
Kanamycin resistance gene: bases 1159-1953
Ampicillin (*bla*) resistance gene: bases 2203-3063 (c)
Ampicillin (*bla*) promoter: bases 3064-3160 (c)
pUC origin: bases 3161-3834
(c) = complementary strand

Appendix C: Plasmid DNA sequence obtained from sequencing.

5′CNNNTATANGGCGATTGATTTAGCGGCCGCGAATTCGCCCTTGCTGCTCTTCGGG
GCCTTGTATGCTCGCCAGAGAATATCAAAACTCTTTTTATTAATGTCGTCTGAGTAC
TATATAATAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGA
ACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATC
TTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCAT
TTCAACCCTCAAGCTCAGCTTGGTATTGAGTCCATGTCAAAGGGCGAATTCGTTTAA
ACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGTAATCATGGT
CATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCC 3′

Fragment of one of the sequenced plasmid DNA samples, by using the M13 forward primer [-20] (5′-GTAAAACGACGGCCAG-3′) and M13 reverse primer (5′-CAGGAAACAGCTATGAC-3′). The sequence is the forward sequence in forward sense (5′-3′) and M13 forward and M13reverse binding sites are not showed.

5′TNNNNATAACCTCACTAAAGGGACTAGTCCTGCAGGTTTAAACGAATTCGCCCTT
TGACATGGACTCAATACCAAGCTGAGCTTGAGGGTTGAAATGACGCTCGAACAGGC
ATGCCCCCCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACT
GAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGA
ACCAAGAGATCCGTTGTTGAAAGTTTTAACTATTATATAGTACTCAGACGACATTA
ATAAAAAGAGTTTTGATATTCTCTGGCGAGCATACAAGGCCCCGAAGAGCAGCAAG
GGCGAATTCGCGGCCGCTAAATTCAATTCGCCCTATAGTGAGTCGTATTACAATTCA
CTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTT 3′

Fragment of one of the sequenced plasmid DNA samples (5′-3′), by using the M13 forward primer [-20] (5′-GTAAAACGACGGCCAG-3′) and M13 reverse primer (5′-CAGGAAACAGCTATGAC-3′). The sequence is the reverse of the sequence above in forward sense (5′-3′) and M13 forward and M13reverse binding sites are not showed.

Fragment of pCRTM 4 - TOPO® vector amplified by the M13 forward primer [-20] (5′-GTAAAACGACGGCCAG-3′) and M13 reverse primer (5′-CAGGAAACAGCTATGAC-3′). Fragment shows vector cloning site (Appendix B), with the target gene inserted, and nearby regions.
Target sequence in real-time PCR assay of 278bp amplified by SSFWD 5′-GCTGCTCTTCGGGGCCTTGTATGC-3′) and SSREV (5′ CAGCTTGGTATTGAGTCCATGTCA-3′) primers.
Part of the *Sclerotinia sclerotiorum* 5.8s DNA sequence sequence where SSFWD and SSREV bind and amplify the target sequence of 278bp used in this study.

Appendix D: Results qPCR Assay.

Quantification curves

Field	Amount of DNA (fg)	Cq Value	Standard deviation of Cq	R^2	Field	Amount of DNA (fg)	Cq Value	Standard deviation of Cq	R^2
Tybble	1000	12.01	0.468	0.98	Tybble	1000	13.87	0.273	0.99
	1000	12.26	0.468			1000	13.34	0.273	
	1000	12.92	0.468			1000	13.48	0.273	
	100	17.19	0.785			100	16.36	0.335	
	100	15.67	0.785			100	16.78	0.335	
	100	16.07	0.785			100	17.03	0.335	
	10	20.24	0.497			10	21.64	0.137	
	10	21.11	0.497			10	21.44	0.137	
	10	20.25	0.497			1	24.3	0.288	
	1	23.34	0.641			1	24.57	0.288	
	1	23.61	0.641			1	24.88	0.288	
	1	24.56	0.641			0.1	29.91	0.948	
	0.1	29.44	0.937			0.1	28.32	0.948	
	0.1	27.74	0.937			0.1	30	0.948	
	0.1	29.28	0.937			0.01	32.03	0.641	
	0.01	36.02	3			0.01	30.88	0.641	
	0.01	31.24	3			0.01	30.98	0.641	
Joganbo	1000	14.91	0.210	0.91	Stora Valla	1000	14.16	0.524	0.90
	1000	14.72	0.210			1000	13.91	0.524	
	1000	14.49	0.210			1000	13.16	0.524	
	100	19.78	0.180			100	18.84	0.272	
	100	20.14	0.180			100	19.13	0.272	
	100	19.96	0.180			100	19.38	0.272	
	10	23.3	0.449			10	22.45	0.232	
	10	23.22	0.449			10	22.24	0.232	
	10	22.48	0.449			10	21.99	0.232	

	1	25.76	0.438			1	25.4	0.200	
	1	26.54	0.438			1	25.63	0.200	
	1	26.5	0.438			1	25.8	0.200	
	0.1	27.24	0.376			0.1	25.7	0.261	
	0.1	26.96	0.376			0.1	25.49	0.261	
	0.1	26.5	0.376			0.01	27.23	0.796	
	0.01	27.51	1.535			0.01	28.07	0.796	
	0.01	30.31	1.535						
	0.01	27.82	1.535						
Frommesta	100	16.75	0.062	0.97	**Frommesta**	1	24.41	1.433	0.97
	100	16.72	0.062			0.1	27.35	0.445	
	100	16.84	0.062			0.1	27.98	0.445	
	10	20.61	0.186			0.01	34.77	0.445	
	10	20.76	0.186			0.01	34.14	0.445	
	10	20.98	0.186						
	1	21.17	1.433						
	1	26.46	1.433						

Quantification results in leaf and petal samples

Field	Sample	Mean Cq values	Standard deviation	Mean nº of copies in 5µl	Standard deviation	pvalue (leaves vs petals)
	3	36.09	2.662	0.031	0.039	
	5	23.52	0.618	573.1	261.92	
	6	18.49	1.175	59383.87	63210.70	

Jogambo Leaves	7	30.21	0.686	1.84	1.00	**0.324195865**
	8	33.17	0.354	0.13	0.042	
	2	20.82	0.987	3565.54	1555.78	
	10	22.1	0.403	1408.55	301.39	
	13	36.3	3.82	1.58	2.00	
	15	27.62	1.031	80,53	44.07	
	17	21.22	0.346	2243.19	416.15	
Jogambo Petals	3 a	35.94		0.012		
	5 a	31.02		0.81		
	8 a	26.04		60.34		
	2	28.67	1.60	49.24	37.62	
	10 a	33.2		3.71		
	13	32.75	0.71	4.94	1.98	
	15 a	30.83		13.12		
	17	26.25	5.60	681.26	854.71	

Field	Sample	Mean Cq values	Standard deviation	Mean nº of copies in 5µl	Standard deviation	pvalue (leaves vs petals)
Frommesta Leaves	1	33.3	5.69	0.04	0.31	**0.398853**
	2	32.2	0.88	0.22	0.033	
	3	29.16	0.73	0.56	0.29	
	5	30.7	1.19	0.16	0.14	
	6	26.41	0.82	6.84	4.69	
	7	29.02	3.76	3.76	4.37	
	8	33.3	1.73	3.14	0.023	
	9	35.77	2.71	0.020	1.21	
	10	31.23	0.24	8.18	1.1	
Frommesta Petals	1 a	37.57		0.26		
	2 a	39.03		0.12		
	3 a	37.25		0.31		

Field	Sample	Mean Cq values	Standard deviation	Mean nº of copies in 5µl	Standard deviation	pvalue (leaves vs petals)
Tybble Petals	1	29.84	0.53	12.46	3.60	**0.488829483**
	2	28.75	1.69	28.42	20.76	
	6	29.5	0.93	15.77	6.55	
	13	25.9	1.141	120.50	59.34	
	12	28.23	0.59	30.74	10.54	
	7	28.04	1.003	30.44	18.64	
	14	29.68	0.90	10.61	4.97	
	17	27.49	1.43	52.04	33.67	
	19	28.88	1.02	17.91	9.88	
	20	25.4	0.31	139.43	25.61	
Tybble Leaves	1	29.47	1.286	17.18	9.52	
	2	25.88	1.035	117.31	63.49	
	6	24.89	0.18	188.22	18.63	
	12	27.67	1.57	52.76	49.20	
	13	29.37	1.081	17.48	8.43	
	7	29.38	0.51	12.04	3.72	
	14	29.04	0.69	15.37	7.09	
	17	27.01	0.39	51.69	13.36	

	19	29.75	0.30	10.64	1.99	
	20	30.30	0.29	6.65	1.17	

Field	Sample	Mean Cq values	Standard deviation	Mean nº of copies in 5µl	Standard deviation	pvalue (leaves vs petals)
Stora Valla Leaves	6	26.6	0.64	24.12	12.69	
	8	27.29	0.623	13.08	6.22	
	10	28.53	1.27	5.71	4.03	
	5	25.52	0.48	23.92	12.35	
Stora Valla Petals	6 a	34.41		0.03		
	8	29.53	1.08	2.36	1.92	
	10	25.72	1.39	61.21	58.88	**0.499832112**

Samples where the fungi was detected in 2 out 3 triplicates or 3 out 3 triplicates were considered as a positive detection.

a: Samples where detection in triplicates was 1 out 3. These samples were not considered as a positive detection of the fungi.