Monitoring Important Soil-Borne Plant Pathogens in Swedish Crop Production Using Real-Time PCR

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Abstract

The global demand for food will increase considerably in the nearest future and among the major constraints to agricultural productivity are biotic stresses caused by microorganisms. In this thesis, the causal agents of four of the most important soil-borne diseases threatening the Swedish production of oilseed rape, sugar beets and red clover were selected as targets for developing diagnostic assays using real-time PCR.

The disease risk assessment of clubroot in oilseed rape was improved by developing a real-time PCR assay for quantification of the causal pathogen *Plasmodiophora brassicae* directly in soil samples. Broad disease risk categories including a threshold level for growing resistant cultivars were established. A real-time PCR assay for quantification of *Aphanomyces cochlioides*, the oomycete causing root rot in sugar beets, was also developed in the present study. The potential use of this assay as a tool in disease risk assessment was demonstrated for fields with high risk of infection.

Real-time PCR was used to monitor the plant- and airborne inoculum of *Sclerotinia sclerotiorum*, the causal fungus of Sclerotinia stem rot, in spring oilseed rape. We found that determining the presence of *S. sclerotiorum* on petals was not useful for stem rot risk assessment since (i) the inoculum incidence on petals varied during flowering, (ii) there was no clear relationship between petal infection and stem rot incidence and (iii) spore release and flowering were not synchronized at one of the field experimental sites. Real-time PCR detection of the incidence of *S. sclerotiorum* DNA on leaves revealing the field-borne inoculum and quantification of the airborne inoculum are likely more reliable tools for predicting the potential risk of disease.

The pathogen complex causing red clover root rot was monitored over three years in two field experiments. *Fusarium avenaceum*, *Phoma* spp. and *Cylindrocarpon destructans* were detected in red clover roots early in the seeding year using real-time PCR and the levels of pathogen DNA generally increased during the following years. Significant linear relationships were found between the amount of pathogen DNA and disease severity index.

Keywords: Plasmodiophora brassicae, *Aphanomyces cochlioides*, *Sclerotinia sclerotiorum*, *Fusarium avenaceum*, *Fusarium culmorum*, *Phoma* spp., *Cylindrocarpon destructans*, real-time PCR, diagnostics, disease risk assessment

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Sammanfattning

Världens livsmedelsproduktion behöver nästan fördubblas under de närmaste årtiondena i takt med att jordens befolkning ständigt ökar och våra matvanor successivt förändras till följd av en ökad urbanisering. Eftersom större delen av den landareal som lämpar sig för odling redan används inom jordbrukssektorn innebär det att produktiviteten måste öka. Historiskt har produktionsökningar inom jordbruket skett genom ökad mekanisering, växtförädling och ökad mängd insatsvaror, som växtskyddsmedel och växtnäring. Framtidens utmaning består nu i att öka produktiviteten utan att öka mängden insatsvaror som har en negativ inverkan på den omgivande miljön. Ett viktigt led i denna strävan är att kunna diagnosticera och följa växtsjukdomar som påverkar avkastningen. Vissa studier talar för att minst en tiondel av skördeförlusterna beror på växtsjukdomar orsakade av svampar och bakterier. Flera av sjukdomarna som drabbar våra vanligaste grödor orsakas av växtpatogener som tillbringar delar av sin livscykel i jorden och många av dem bildar olika typer av vilostrukturer som kan överleva i marken under lång tid vilket gör dem extra svåra att kontrollera. För att kunna fatta korrekta beslut om lämpliga odlingsåtgärder behöver lantbruket tillgång till snabba och säkra analysmetoder.

Syftet med den här avhandlingen är att förbättra riskbedömningen för några av de viktigaste jordburna växtsjukdomarna vi har i Sverige; klumprotsjuka i oljeväxter, rotbrand i sockerbetor, bomullsmögel i raps och rotröta i rödklöver. Arbetet har fokuserat på utvecklingen av analysmetoder för att detektera de sjukdomsalstrande mikroorganismerna (patogenerna) med realtids-PCR, en teknik som utnyttjar värmetåliga enzymer och fluorescerande prober för att selektivt mångfaldiga och detektera unika DNA-sekvenser. Metoden, som är mycket specifik och känslig, möjliggör en objektiv bestämning av nivån av den mikroorganism som orsakar en viss växtsjukdom.

Denna studie har gjort det möjligt för lantbrukare att bedöma risken för skördesänkningar i oljeväxter orsakade av klumprotsjuka genom utvecklingen av den analysservice som numera finns tillgänglig kommersiellt. Jordtestet ger svar på förekomsten av den mikroorganism som orsakar sjukdomen (*Plasmodiophora brassicae*) och kopplat till resultatet finns en riskbedömning och rådgivning kring val av rapssort; mottaglig eller resistent. Avhandlingen har också lagt grunden för ett liknande test för den patogen som orsakar rotbrand i sockerbetor (*Aphanomyces cochlioides*), men mer utvecklingsarbete krävs för att göra metoden tillräckligt känslig då den i dagsläget endast kan påvisa patogenen i jordar med hög risk för infektion.

Många jordburna sjukdomar har en mycket ojämn fördelning i jorden. Utbredningen av *P. brassicae* studerades i denna avhandling och bekräftade att det förekom mycket stora variationer i nivån både mellan närliggande fält och inom enskilda fält.

Man har länge ansett att bomullsmögel huvudsakligen orsakas av luftburna sporer från den sjukdomsalstrande svampen (*Sclerotinia sclerotiorum*) som landar på rapsblommans kronblad. När de smittade kronbladen faller av fastnar de på blad och i bladfästen där svampen sedan utnyttjar näringen i kronbladen för att kunna infektera plantan. Även om det troligtvis är så att kronbladen behövs som näringskälla för att infektionen ska äga rum, visar våra fältförsök att de inte alltid är bärare av sporerna. Med hjälp av PCR-analys har vi konstaterat att det troligtvis är bättre att analysera rapsblad jämfört med blommans kronblad eftersom förekomsten på kronbladen varierar under blomningen. Det fanns heller inte någon tydlig koppling mellan förekomsten på kronbladen och sjukdomsangreppen i fält. Dessutom har vi genom fältförsök kunnat visa att höga halter av svampens sporer i luften inte alltid sammanfaller med tiden för blomningen.

PCR har också visat sig vara ett mycket lämpligt verktyg för att analysera de svampar som orsakar rotröta i rödklöver. Svamparna *Fusarium avenaceum, Phoma* spp. och *Cylindrocarpon destructans* kunde påvisas i rötterna redan tidigt under insåningsåret och nivåerna ökade sedan i allmänhet under de kommande vallåren. *F. culmorum* är troligtvis en svag patogen på rödklöver då den endast påvisades i ett fåtal prover och inte bidrog till sjukdomsutvecklingen i våra fältförsök. Ingen av de utvärderade rödklöversorterna var resistent och det fanns heller inga stora skillnader i mottaglighet bland olika sorter vilket bekräftar tidigare hypoteser om små skillnader i mottaglighet.

Sammanfattningsvis kan man dra slutsatsen att realtids-PCR är ett användbart verktyg inom den moderna riskbedömningen av växtsjukdomar. Tekniken är också ett värdefullt komplement till okulära metoder för att bedöma sjukdomsnivåer i olika typer av fältförsök.

Dedication

To Christian, Märta and Åke

If the person you are talking to doesn't appear to be listening, be patient. It may simply be that he has a small piece of fluff in his ear.

Winnie the Pooh

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Wallenhammar, A-C., Almquist, C., Söderström, M. & Jonsson A. (2012). In-field distribution of *Plasmodiophora brassicae* measured using quantitative real-time PCR. *Plant Pathology* 61, 16-28.

II Almquist, C., Persson, L., Olsson, Å., Sundström, J. & Jonsson, A. (2016). Disease risk assessment of sugar beet root rot using quantitative real-time PCR analysis of *Aphanomyces cochlioides* in naturally infested soil samples. *European Journal of Plant Pathology,* doi 10.1007/s10658-016-0862-5.

III Almquist, C. & Wallenhammar, A-C. (2015). Monitoring of plant and airborne inoculum of *Sclerotinia sclerotiorum* in spring oilseed rape using real-time PCR. *Plant Pathology* 64, 109-118.

IV Almquist, C., Wallenhammar, A-C., Stoltz, E. & Sundström, J. (2016). Monitoring disease development caused by soil-borne plant pathogens associated with root rot in red clover (*Trifolium pratense*) crop stands using quantitative real-time PCR (manuscript).

The contribution of Charlotta Almquist to the papers included in this thesis was as follows:

I Was responsible for the development and validation of the real-time PCR assay as well as the DNA extraction and real-time PCR analysis of soil samples. Collected some of the soil samples. Performed the bioassays together with the co-authors and laboratory assistants. Analyzed most of the data and wrote the manuscript in cooperation with the co-authors.

II Was responsible for the scope, aim, and planning of the study together with the co-authors. Was responsible for the development and validation of the real-time PCR assay as well as the DNA extraction and real-time PCR analysis of soil samples. Analyzed the data. Wrote the manuscript assisted by the co-authors.

III Participated in the planning of the project. Was responsible for the development and validation of the real-time PCR assay as well as the DNA extraction and real-time PCR analysis of field samples. Analyzed the data in cooperation with the co-author. Wrote the manuscript assisted by the co-author.

IV Participated in the planning of the project. Was responsible for the development and validation of the real-time PCR assays as well as the DNA extraction and real-time PCR analysis of field samples. Assisted with the root sampling and the visual disease assessment of roots. Analyzed the data. Wrote the manuscript in cooperation with the co-authors.

Abbreviations

COX	Cytochrome oxidase
Ct	Cycle of threshold
DSI	Disease severity index
ELISA	Enzyme-linked immunosorbent assay
IGS	Intergenic spacer
ipt	Isopentyltransferase
ITS	Internal transcribed spacer
LOD	Limit of detection
LOQ	Limit of quantification
NGS	Next-generation sequencing
PCR	Polymerase chain reaction
qPCR	Quantitative real-time PCR
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal DNA
SCAR	Sequence characterized amplified region
T-RFLP	Terminal restriction fragment length polymorphism

1 Introduction

The world's population is expected to reach 9.7 billion people in 2050 (United Nations, 2015). The increase of the population along with increased wealth and dietary changes mean that the global demand for food will increase considerably in the nearest future. The Food and Agriculture Organization of the United Nations (FAO, www.fao.org) estimates that the world food production must increase by 70% in order to feed this larger, richer and more urban population. Since most of the land suitable for crop production is already being cultivated, it is likely that more food and feed will need to be produced from the same amount of land (Oerke & Dehne, 2004). In addition, increasing the land area used for agriculture would likely have negative environmental consequences, e.g. deforestation (Bruce, 2010). According to FAO, as much as 90% of the increased agricultural production need to come from intensifications such as greater yields and increased cropping intensity. We now face the challenge of increasing agricultural productivity in ways that are environmentally, socially and economically sustainable.

In terms of area, Sweden is one of the largest countries in Europe and a little over 6% of the land area is arable land (Swedish Board of Agriculture, 2014). The most important agricultural crops are cereals, ley and green fodder, oilseeds, sugar beets, peas and potatoes. The distribution of crops varies greatly between the north and south of Sweden due to the very different climatic conditions. Sugar beets are for example only grown in the southernmost parts, whereas the crop production in the northern regions mainly relies on forages and coarse grains. The major part of the arable land is located on the fertile plains of central and southern Sweden where e.g. bread grain and oilseeds are common. In 2014, the organic arable area was 16.6% of the total arable land which is an increase of approximately 10 percentage points since 2005 (Statistics Sweden, Agricultural Statistics 2015).

Biotic stresses, including insects and microorganisms, are major constraints to agricultural productivity. Plant pathogenic fungi and bacteria alone are likely to cause more than 10% yield loss (Oerke & Dehne, 2004, Oerke, 2006). However, the information available on crop losses is very scarce and relies on estimates. A vast majority of the plant diseases are caused by soil-borne pathogens and in the United States more than 50% of the crop loss is due to diseases caused by soil inhabiting microorganisms (Lewis & Papavizas, 1991). Soil-borne plant pathogens complete part of their life cycle in the soil and many of them form resting structures that can persist in the soil for long periods of time, which make them particularly difficult to control. Furthermore, a positive latitudinal shift has been observed for many plant pathogens over the past decades (Bebber *et al.*, 2013). The ongoing global warming implies extended growing seasons and longer time periods suitable for infection which will likely increase the severity of soil-borne diseases in Sweden (Roos *et al.*, 2011).

Disease diagnosis and plant pathogen detection are fundamental to be able to make informed decisions about e.g. choice of cultivar or application of fungicides and also to prevent spreading of diseases. In addition, pathogen detection can be used in disease resistance breeding to easily determine the degree of host susceptibility by large-scale screening (Vandemark & Barker, 2003; Yu *et al.*, 2015). The ability to correctly diagnose and monitor plant diseases is also a part of the concept of integrated pest management (IPM) which now has to be implemented in practice in the EU according to the EU directive 2009/128. The main purpose of this directive is to achieve a sustainable use of pesticides by actions including (i) preventive cultural practices, (ii) monitoring of harmful organisms, (iii) needs-oriented and site-specific pest control and (iv) evaluation of the success of the applied plant protections measures. Conclusively, disease monitoring is an important part of a sustainable plant production.

This thesis focuses on the development and validation of real-time PCR methods for detection and quantification of soil-borne plant pathogens of importance in Sweden. Their use as tools in improved disease risk assessment is also evaluated and discussed.

1.1 Plant pathogen diagnostics

The traditional way to diagnose plant diseases is to observe and interpret visual symptoms. The visual assessment is often followed by isolation of the pathogen and culturing on selective media, and identification using microscopy. Although traditional methods are still a cornerstone of plant

pathology and fungal diagnostics, they also have a number of drawbacks. The accuracy of such methods largely relies on experienced and skilled staff making the diagnosis. In addition, traditional methods are time consuming, often non-quantitative and sometimes rely on the ability of the causal organism to be cultured. Moreover, it may be difficult to distinguish between closely related species solely based on morphology.

Today there are several alternative techniques for pathogen detection available, such as immunological methods (e.g. ELISA) and nuclei acid-based detection methods (e.g. PCR). These techniques have several advantages over traditional methods in that they are much faster, more reproducible and do not rely on the personal judgment of visual observations. Several review papers on the implementation of these techniques in plant pathology have been presented (e.g. McCartney *et al.*, 2003; Ward *et al.*, 2004). A common conclusion drawn in these reviews seems to be that for bacteria and fungi, nucleic acid-based techniques are to be preferred since it is both difficult and expensive to produce antibodies of sufficient specificity. Immunological methods are generally also less sensitive due to the fact that proteins are less stable than DNA and easily degraded (Ward *et al.*, 2004).

1.1.1 Plant pathogen diagnosis using real-time PCR

In the late 1990's, the first examples of using real-time PCR for detection of plant pathogens were published (reviewed by Mumford *et al.*, 2006). Since then, a large number of assays for detection of soil-borne fungi, oomycetes, bacteria and viruses have been developed (e.g. reviewed by Bilodeau, 2011). Real-time PCR is fast, sensitive, reproducible, and may be used quantitatively (qPCR), which makes it a very interesting and useful technique for commercial laboratories. One successful example of using qPCR in routine diagnostic testing of plant pathogens is the DNA-based soil testing service developed by SARDI (South Australian Research and Development Institute) in Australia (Ophel-Keller *et al.*, 2008). Included in this diagnostic test is a risk assessment where pathogen DNA-levels are linked to risk categories to indicate the likely risk of crop losses. One of the diseases included in this testing service is take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici* and *G. graminis* var. *avenae* (http://pir.sa.gov.au/research/services/).

Three initial steps are involved in the development of an in-house diagnostic real-time PCR assay, as summarized in Figure 1. The first step is to find a suitable nucleic acid target sequence. An ideal target sequence is sufficiently variable to be able to achieve the desired specificity of the primers and at the same time sufficiently conserved so the assay includes all strains of the pathogen (Schena *et al.*, 2013). In addition, the amplicon should ideally not

exceed 150 bp to achieve high amplification efficiency (Thornton & Basu, 2011). Among the most commonly used target sequences for fungal plant pathogens are the ITS regions (ITS1 and ITS2) of the ribosomal DNA (Bilodeau, 2011). The ITS regions generally contain sufficient diversity at the species level. Also, these sequences are present in multiple copies, which can be very useful in order to develop a sensitive assay (Bridge *et al*., 1998; Ward *et al*., 2004). ITS sequences from a very large number of fungi and oomycetes are available in databases that make them easily accessible and provide useful reference material for the development of specific primers and probes. Other commonly used multi-copy sequences are the IGS regions (intergenic spacer or non-transcribed spacer) located between adjacent rDNA units, the ribosomal DNA genes (18S, 5.8S and 28S) as well as mitochondrial DNA (Bilodeau, 2011). In addition, the β-tubulin gene is also a widely used target sequence (McCartney *et al*., 2003). Sometimes the available sequence data is inadequate. In those cases specific targets can be identified by amplifying random regions of the genome using PCR-based techniques such as RAPD (random amplified polymorphic DNA) (McCartney *et al*., 2003; Ward *et al*., 2004).

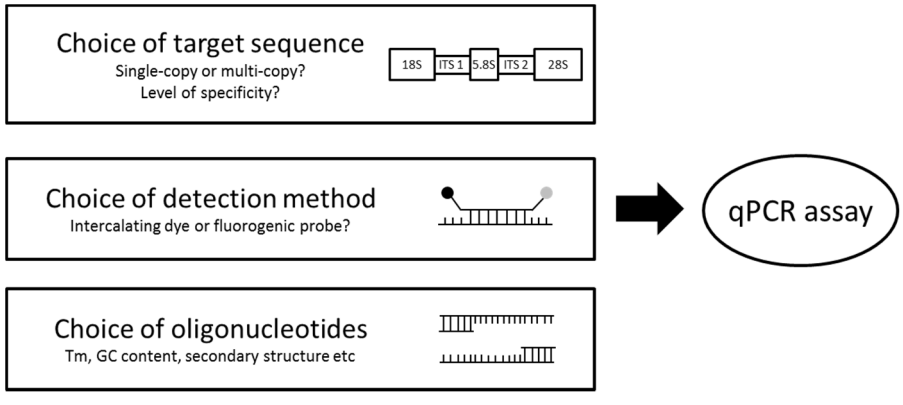

Figure 1. The three initial steps in the development of a diagnostic real-time PCR assay.

Next, the type of fluorescence chemistry format must be chosen. Common for all reporters (dyes and probes) is that they generate a fluorescent signal that corresponds to the amount of product formed. Since the fluorescence is measured in real-time, it is possible to correlate the fluorescent signal obtained during the exponential phase of the reaction to the initial amount of target. There are two categories of fluorescent reporters: sequence-specific and nonspecific. SYBR Green I, included in the nonspecific category, is the most frequently used intercalating dye (Wilhelm & Pingoud, 2003). This fluorescent dye binds sequence independently to double-stranded DNA. Unfortunately,

this also means that a fluorescent signal will be obtained in the presence of any double-stranded DNA including nonspecific products and primer dimers. In addition, fluorescent dyes cannot be used for multiplex assays since PCR products cannot be differentiated. The alternative to intercalating dyes is fluorogenic probes, which are oligonucleotides to which one or two fluorescent dyes are coupled. Such probes rely on hybridization to the correct target sequence, thus increasing the specificity of the assay (Bustin, 2000). The most frequently used fluorogenic probe for detection of soil-borne fungi is the TaqMan probe (Okubara *et al.*, 2005; Bilodeau, 2011) which utilizes the 5' exonuclease activity of the DNA polymerase to cleave the dual-labeled probe and thus releasing the fluorophore from the quencher to allow fluorescence (Wilhelm & Pingoud, 2003).

Finally, specific primers and probes need to be designed. The design can be made manually or by using appropriate software (e.g. Primer Express, Applied Biosystems) or web based tools (e.g. Primer3, Whitehead Institute) (Schena *et al.*, 2013). Factors such as secondary structure, annealing temperature and GC content should be considered in the design process (Edwards *et al.*, 2004). The specificity is of crucial importance when designing primers for routine diagnostic purposes. Generally, amplification is prevented when there is at least a two base mismatch at the 3' end of a primer (Ye *et al.*, 2012). A freely available tool for designing target-specific primers with specificity checking is now available online (Primer-BLAST, NCBI).

In addition to the routinely used PCR method, other nucleic acid-based techniques are available for pathogen diagnosis and some of them are suitable for detection of multiple targets simultaneously. One such method, used for multiplex detection of *Phytophthora* species, is target-specific primer extension combined with Luminex xTAG technology where labeled PCR-products are detected using color-coded beads (Kostov *et al*, 2015). Another example is high-throughput sequencing of environmental DNA using different next-generation sequencing (NGS) platforms (Shokralla *et al*., 2012; Lindahl *et al*., 2013). NGS was recently used to identify viruses causing maize lethal necrosis disease in Kenya and the results facilitated the development of specific real-time PCR assays (Adams *et al*., 2013).

The development of NGS techniques has also made sequencing and re-sequencing of whole genomes highly accessible and more affordable. A large number of plant pathogenic genomes are now available in databases and among the seven plant pathogens investigated in this thesis, entire genomes are available for four (Table 1).

Table 1. *Plant pathogens investigated in the present study and references on genome sequences when available.*

Pathogen	Genome sequence available (draft or complete)?	Estimated genome size (Mb)	Reference
Plasmodiophora brassicae	Yes	25.5	Schwelm *et al*., 2015
Aphanomyces cochlioides	No	-	-
Sclerotinia sclerotiorum	Yes	38.0	Amselem *et al*., 2011
Fusarium avenaceum	Yes[1]	41.6-43.1	Lysøe *et al*., 2014
Fusarium culmorum	No[1]	-	-
Phoma spp.	Yes[2]	45.1[2]	Rouxel *et al*., 2011
Cylindrocarpon destructans	No	-	-

1. Genome drafts or complete genomes of several other Fusarium species are available (Cuomo *et al*., 2007; Ma *et al*., 2010; Jeong *et al*., 2013; Moolhuijzen *et al*., 2013; Gardiner *et al*., 2014; Srivastava *et al*., 2014; King *et al*, 2015).

2. Genome sequence of *Leptosphaeria maculans*, the teleomorph (sexual reproductive stage) of *Phoma lingam*.

Digital PCR, a PCR method developed already during the 1990s (Sykes *et al.*, 1992; Vogelstein & Kinzler, 1999), is currently also on its way up into the area of plant pathogen diagnosis (Dreo *et al*., 2014). Even though the basic principle of this method is the PCR technique, digital PCR is quantitative without the need for standards. Instead, the sample is diluted and partitioned into a large number of chambers or droplets, and quantification is simply based on the number of positive and negative reactions using Poisson statistics. Digital PCR has great potential for plant pathogen diagnosis in environmental samples since it shows higher resilience to matrix inhibitors compared to real-time PCR (Rački *et al*., 2014).

1.1.2 DNA extraction from environmental samples

When analyzing plant pathogens in field samples such as plant tissue, soil or air samples, efficient DNA extraction and purification procedures are necessary to obtain high DNA yields and extracts free from inhibitors (Schena *et al.*, 2013). This step of the analysis is often the most challenging due to the complexity of environmental samples and the requirements to obtain DNA of good quality. In addition, the extraction procedure should be simple to use, cost-effective and reproducible.

There is a wide range of DNA extraction methods and commercial kits available that are suitable for DNA isolation from different types of plant tissue samples. Usually, plant samples are first homogenized through e.g. grinding in liquid nitrogen, using a knife mixer or by bead beating followed by chemical and enzymatic cell disruption (Schena *et al.*, 2013). Substances such as polysaccharides, polyphenols and proteins can be co-extracted with the DNA

and inhibit the qPCR assay (Ma & Michailides, 2007; Schena *et al.*, 2013). The effect of these inhibitors can often be minimized by diluting the extract. Even though dilution might reduce the assay sensitivity it is often necessary since the high concentration of plant DNA may inhibit the PCR (Edwards *et al.*, 2004).

Soil is one of the most difficult sample types to extract plant pathogen DNA from. First of all, soil is a very complex and divers matrix and properties such as organic matter content, texture and pH varies a lot between different soils and will affect the DNA extraction (Bürgman *et al.*, 2001; Frostegård *et al.*, 1999; Zhou *et al.*, 1996). Second, many soil-borne plant pathogens form survival structures with complex cell walls to be able to persist in the soil for long periods of time and these structures can be very difficult to lyse (Bilodeau, 2011). Third, all soils contain inhibitors, such as humic substances, that can inhibit the *Taq* DNA polymerase (Albers *et al.*, 2013) and bind to template DNA (Opel *et al.*, 2010). Recently, it was also shown that humic acid causes fluorescence inhibition in qPCR (Sidstedt *et al.*, 2015).

Direct methods for DNA extraction from soil samples, i.e. without the need for separation of the pathogen from soil particles, are the most commonly used protocols for DNA extraction from soil-borne fungi and oomycetes (Schena *et al.*, 2013). These methods provide higher yields and are often less laborious compared to indirect methods (Robe *et al.*, 2003). Several papers reporting evaluations of different DNA extraction protocols have been published and include both the comparison of in-house methods (e.g. Lakay *et al.*, 2007; Miller *et al.*, 1999) and commercial kits (Dineen *et al.*, 2010; Whitehouse & Hottel, 2007). A general conclusion from these studies is that mechanical cell lysis using bead beating results in the highest DNA yields. However, a drawback of this lysis method is that it also extracts inhibitory substances and might increase DNA shearing which may affect sensitivity especially in detection of long PCR products (Roose-Amsaleg *et al.*, 2001).

Among the published qPCR methods for detection of plant pathogenic fungi and oomycetes, only a minor share is developed for detection in air samples (Schena *et al.*, 2013). Air samples for PCR analysis are typically collected on adhesive tapes (e.g. Burkard 7-day volumetric spore sampler), in vials (e.g. Burkard multi-vial cyclone) or in liquid (e.g. multi-stage liquid impinge) and spore lysis is usually achieved by bead beating (Ma & Michailides, 2007; West *et al.*, 2008; Williams *et al.*, 2001). Both non-target DNA and pollen have been reported to inhibit the PCR reaction (Saunders & Parkes, 1999; Wilson, 1997).

1.1.3 Target quantification using real-time PCR

There are two basic principles for quantification of DNA using real-time PCR: relative quantification and absolute quantification. In absolute quantification, a

standard with known quantities is used to determine the amount of target in unknown samples (Bustin, 2000; Guiletti *et al.*, 2001). The standard curve is typically prepared using purified plasmid DNA or total genomic DNA of the pathogen of interest, but could also consist of artificially inoculated environmental samples (Schena *et al.*, 2013). The concentration of the standard is generally measured spectrophotometrically. By serial dilution of the standard the amount of target can be determined by interpolating Ct-values of unknown samples with the standard curve.

Standard curves can also be used in relative quantification, but in this case the quantity of the standard does not need to be known (Raymaekers *et al.*, 2009). Instead, a basis sample (calibrator) is used and all results are expressed relative to this sample as n-fold differences relative to the calibrator (Applied Biosystems). Often an endogenous control is used for normalization and therefore a separate standard is prepared for the control target as well.

In addition to the quantification techniques using standard curves, there are alternative comparative methods relying on arithmetic formulas. The comparative Ct method ($2^{-\Delta\Delta Ct}$ method) can be used when PCR efficiencies of the target and endogenous reference are equal (Livak & Schmittgen, 2001). A mathematical model to determine the relative amount of a target gene in comparison to a reference gene has also been presented by Pfaffl (2001) and this model takes differences in PCR efficiency into account.

In plant pathology, several examples using absolute quantification of soil-borne pathogens have been published and most of the assays use standards based on genomic DNA (e.g. Agustí-Brisach *et al.*, 2014; Lees *et al.*, 2002), purified PCR products (e.g. Scarlett *et al.*, 2013) or standards prepared from enumerated pathogen propagules (e.g. Hwang *et al.*, 2011; van de Graaf *et al.*, 2003; van Gent-Pelzer *et al.*, 2010). However, there are a few examples of methods using standards prepared from plasmids containing the target sequence (Gao *et al.*, 2015; Sauvage *et al.*, 2007; Ward *et al.*, 2005; Zhang *et al.*, 2013). The availability of commercially produced synthetic genes will probably make this option even more commonly used in the future.

Variation in DNA extraction yield and efficiency of amplification will affect the quantification and need to be corrected for in some way (Bustin *et al.*, 2009). There are a number of ways to normalize quantitative results but the most commonly used strategy is normalization to an internal reference gene (Bustin, 2000). Diagnostic assays for pathogen detection in the host plant usually utilize an endogenous plant gene for normalization (Schena *et al.*, 2013). However, this is not an option when analyzing air or soil samples. As an alternative, known quantities of exogenous DNA, such as DNA from another microorganism or plasmid DNA, can be added to the sample before or after

DNA extraction and purification (e.g. Dauch *et al.*, 2006; Park & Crowley, 2005; Li *et al.*, 2015; Schneider *et al.*, 2009). Another approach is to use a synthetic sequence that can be amplified using the same primers as the target sequence but has a different internal sequence between the primer sites and can thus be detected by a different probe (Bilodeau *et al.*, 2012; Deora *et al.*, 2015).

1.1.4 Validation of diagnostic real-time PCR methods

Analytical research PCR is mainly used during a limited time period for a specific project and often the main parameters related to assay validation that need to be addressed are the analytical sensitivity and specificity (Bustin *et al.*, 2009). In contrast, diagnostic PCR often requires high-throughput protocols and must perform reliably and consistently day after day. Therefore, a diagnostic PCR assay has to be thoroughly validated before being implemented in a routine analytical laboratory and this is of particular importance for in-house developed methods. There are a lot of standards, papers and guides regarding the validation of analytical methods available, both those that are more general (e.g. Thompson *et al.*, 2002; Tuomela *et al.*, 2005; Peters *et al.*, 2007; Magnusson & Örnemark, 2014) and those that specifically focus on real-time PCR methods (e.g. Mazzara *et al.*, 2008; Bustin *et al.*, 2009; Raymaekers *et al.*, 2009; Broeders *et al.*, 2014). The main performance characteristics to be evaluated during the validation of a qPCR method are the analytical sensitivity, specificity, amplification efficiency, linearity, accuracy, repeatability, reproducibility and robustness (Figure 2) (Tuomela *et al.*, 2005; Bustin *et al.*, 2009; Raymaekers *et al.*, 2009; Broeders *et al.*, 2014).

Selectivity or analytical specificity refers to the ability of the PCR assay to detect the appropriate target sequence rather than nonspecific targets or interfering substances present in the sample mixture (Mazzara *et al.*, 2008; Bustin *et al.*, 2009; Barwick & Pritchard, 2011). The specificity of a qPCR assay is mainly determined by the primers, but can also be enhanced by a fluorescent probe. Generally, the specificity is evaluated already during the development of an assay. First, this is done *in silico* using tools, such as BLAST, to search for sequence similarities against databases (Mazzara *et al.*, 2008; Raymaekers *et al.*, 2009). Second, the specificity of a diagnostic qPCR assay is evaluated experimentally by testing it against a set of non-target closely related species as well as other species likely to be found in the sample matrix (Broeders *et al.*, 2014). In addition, the inclusivity of a species-specific assay should also be evaluated by testing a set of targets of e.g. different origin, race or pathotype.

Sensitivity is the ability of the method to detect the target and often refers to the lowest concentration of target copies that can be detected by the method

(Bustin *et al.*, 2009). It is important to distinguish between the qPCR sensitivity, which is the minimum number of copies that can be detected per reaction, and the method sensitivity, which can for example be expressed as the number of copies or cells per g sample (Magnusson & Örnemark, 2014). The method sensitivity includes the whole protocol, i.e. sample weighing, DNA-extraction and purification and qPCR analysis. For qualitative real-time PCR methods, the sensitivity is generally expressed as the limit of detection (LOD), which can be defined as the lowest concentration at which 95% of the positive samples are detected (Tuomela *et al.*, 2005; Bustin *et al.*, 2009; Broeders *et al.*, 2014). For a pathogen diagnostic assay, this is determined experimentally by analyzing a series of samples containing different concentrations of the target. The limit of quantification (LOQ) is defined as the lowest concentration that can be reliably quantified with an acceptable level of precision and trueness (Mazzara *et al.*, 2008). In qPCR assays, this is easily determined by analysing multiple dilutions of the standard and determining when the assay stops being linear.

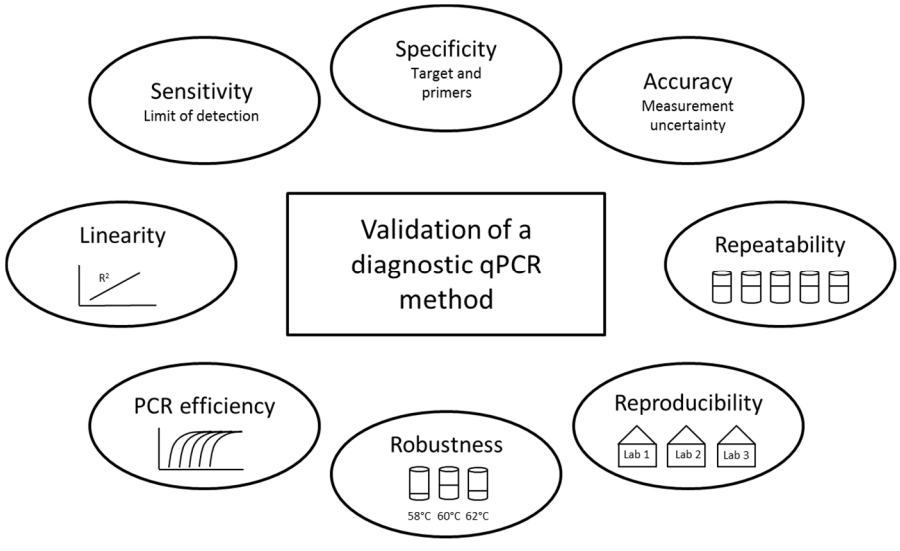

Figure 2. The main performance characteristics that should be evaluated during the validation of a diagnostic qPCR method.

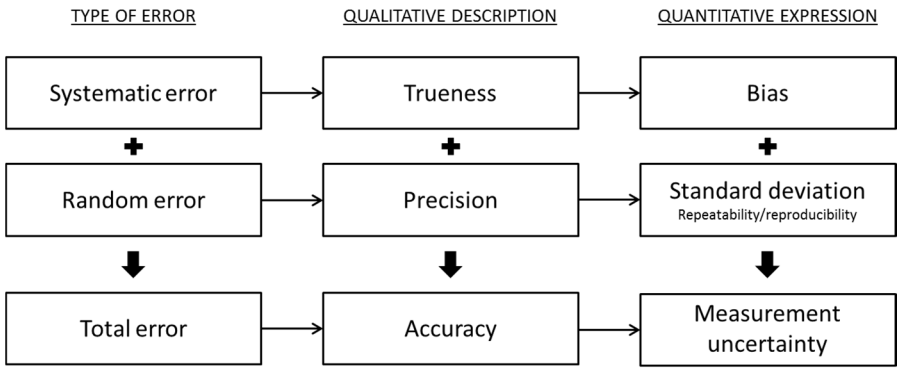

Figure 3. The three qualitative terms used to describe the quality of a measurement result (trueness, precision and accuracy), their quantitative expression and the type of error they are associated with (modified from Menditto *et al.*, 2007).

According to the MIQE Guidelines (Bustin *et al.*, 2009), the performance characteristics PCR efficiency and linear dynamic range of a qPCR method must be determined. The PCR amplification efficiency can easily be determined from the slope of the calibration curve (the Ct values plotted against the logarithm of the initial template concentration) using the equation: PCR efficiency=$10^{-1/\text{slope}}$-1. The dynamic range describes the linear range of a calibration curve and should ideally cover 5 to 6 orders of magnitude (Bustin *et al.*, 2009).

The general terminology used to describe the measurement quality still varies a lot between scientists and different sectors within science, which easily lead to a great deal of confusion. However, several guidelines are available to give a full understanding of the different concepts and the correct terminology to be used, e.g. VIM by the Joint Committee for Guides in Metrology (Joint committee for guides in metrology 200:2012, 2012) and the Eurachem Guide on terminology (Barwick & Pritchard, 2011). The quality of quantitative results obtained using an analytical assay can be described by three qualitative terms: trueness, precision and accuracy (Menditto *et al.*, 2007; Magnusson & Örnemark, 2014). Their quantitative expressions as well as the type of errors they are associated with are illustrated in Figure 3.

There are two types of errors related to a measurement: systematic error and random error (Joint committee for guides in metrology 200:2012, 2012). The systematic error tends to shift all measurements in a systematic way and can be corrected for if the true value is known. The bias is the quantitative measure of the total systematic error and the trueness is the qualitative description (Menditto *et al.*, 2007). Trueness (‘riktighet’ in Swedish) is defined as the closeness of agreement between the average value obtained from an infinite

number of replicate measured values and a reference value (Magnusson & Örnemark, 2014). This can of course not be measured, so the bias is more of a practical assessment of the trueness.

The random error varies in an unpredictable way and is not possible to correct for (Ellison & Williams, 2012), but effort should of course be put in to minimize it. Precision, which is the qualitative description of the random error, is defined as the closeness of agreement between independent test results obtained by performing the experimental procedure under specified conditions (Thompson *et al.*, 2002; Menditto *et al.*, 2007). It is usually expressed by statistical parameters, commonly as the standard deviation, and can be calculated by carrying out replicate measurements. The precision can be expressed as repeatability, intermediate precision and reproducibility (Menditto *et al.*, 2007; Magnusson & Örnemark, 2014). Repeatability measures the variation in results when the least changes are allowed, e.g. when the measurement is carried out by the same analyst using the same equipment over a short period of time. Reproducibility on the other hand, is expected to give a much larger variation and is generally a measure of the variation between laboratories. The intermediate precision, sometimes referred to as the within-laboratory reproducibility or the inter-assay variation, estimates the variation that is likely to occur in a single laboratory under routine conditions. This is usually achieved by performing the assay over a long period of time, by different analysts and using different batches of reagents. If more than one instrument is in use for the particular assay, also this factor should vary. The concept of precision and trueness is illustrated in Figure 4.

Accuracy (‘noggrannhet’ in Swedish) is defined as the closeness of agreement between the result of a single measurement and the true value or accepted reference value of the measurand (Joint committee for guides in metrology 200:2012, 2012). It should not be confused with the trueness which applies to the average values of an infinite number of measurements or with the precision which only relates to dispersion and not to deviation from a true value. Accuracy, which includes both the effect of precision (random error) and the effect of trueness (systematic error), cannot be given a numerical value (Magnusson & Örnemark, 2014). To be able to get an indication of the reliability of a measurement result, the measurement uncertainty needs to be estimated (Barwick & Pritchard, 2011).

The measurement uncertainty describes the dispersion of values that could reasonably be attributed to the measurand (Joint committee for guides in metrology 200:2012, 2012). There are two ways to estimate the uncertainty: using statistical analysis of measurement series and/or using other information, for instance information from past experience of the measurement, from

calculations, from parameters such as temperature or pressure that might have an effect on the result etc. By listing all possible sources of uncertainty and estimating the size of them (expressed as standard deviations), a combined uncertainty can be calculated as the positive square root of the total variance obtained by combining all the uncertainty components (Ellison & Williams, 2012). Usually, the measurement uncertainty is expressed as the expanded uncertainty, which corresponds to the product of the standard uncertainty and an appropriate coverage factor (Joint committee for guides in metrology 200:2012, 2012). This factor is often set to two to achieve an approximate level of confidence of 95%.

Finally, the robustness (or ruggedness) should be assessed. The robustness of an analytical method is the resistance to changes of the result when minor deviations are made from the standard experimental procedure (Magnusson & Örnemark, 2014). This is tested by examining the effects of deliberately introduced small changes to the procedure, e.g. minor changes of temperatures, volumes or concentrations. The robustness will provide an indication of the method's reliability during normal usage.

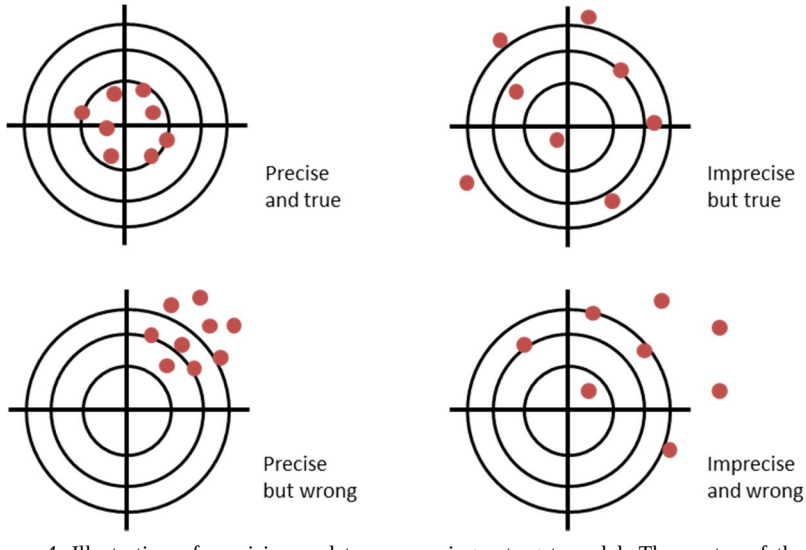

Figure 4. Illustration of precision and trueness using a target model. The center of the target symbolizes the true value.

1.2 Soil-borne pathogens threatening the Swedish agricultural productivity

As mentioned earlier in the introduction, the economically most important crops in Sweden are cereals, ley and green fodder, oilseed rape, sugar beets, peas and potatoes. Various pests pose a constant threat to the productivity of these crops and soil-borne diseases are often particularly difficult to control; many soil-borne pathogens form resting structures that survive in the soil for long periods of time. In this thesis, the causal agents of four of the most important soil-borne diseases threatening the Swedish production of oilseed rape, sugar beets and red clover have been selected as targets for developing routine diagnostic assays.

1.2.1 Clubroot

Clubroot is considered as one of the economically most important diseases of Brassica oilseed and vegetable crops world-wide (Dixon, 2009). The causal agent, the obligate biotroph protist *Plasmodiophora brassicae*, was historically classified as a primitive fungus but has in more recent time been included within the eukaryotic kingdom of Rhizaria (Adl *et al.*, 2012) and more specifically placed in the novel clade Phytorhiza (Sierra *et al.*, 2015). Just recently, the 25.5 Mb genome sequence of *P. brassicae* was presented (Schwelm *et al.*, 2015), which is the first pathogenic genome within this kingdom.

A proposed complete life cycle of *P. brassicae* was first described by Tommerup and Ingram (1971). Lately, Kageyama and Asano (2009) reviewed the current thinking regarding the different stages. The life cycle consists of two infection phases, where the primary phase occurs in the root hairs of the host plant and the secondary phase occurs in the cells of the root cortex. Primary zoospores are released from germinating resting spores, penetrate the root hairs and inject their cell contents into the host cell. After a series of endomitotic divisions, the pathogen develops into a multinucleate primary plasmodium, which in turn cleaves into zoosporangia. Haploid secondary zoospores are then released into the rhizosphere and infect root cortical cells. Secondary plasmodia are formed and spread inside the root which induces local hypertrophy and subsequently leads to gall formation (clubs, Figure 5b). These clubs affect the absorption and translocation of water and nutrients in the root which in turn leads to foliar wilting, stunted growth and premature ripening (Figure 5a). Early infection of seedlings may even lead to plant death. Finally, the secondary plasmodia develop into resting spores that are released in large numbers into the soil as the root decays. One gram of infected root tissue contains $1x10^{10}$ resting spores (Hwang *et al.*, 2013). The resting spores

are very persistent and can remain viable in the soil for more than 17 years (Wallenhammar, 1996), which makes this disease especially difficult to control.

Resting spores of *P. brassicae* are dispersed by transport of contaminated soil, mainly via farm equipment and machinery (Cao *et al.*, 2009), but also via livestock manure (Chai *et al.*, 2016) and water (Datnoff & Lacy, 1984; Faggian *et al.*, 1999). In addition, *P. brassicae* may also be transmitted by transplants from infested soils (Wallenhammar *et al.*, 2014). Seed-borne transmission has been suggested, but although resting spores can be detected on seeds the level is generally below that required to cause disease symptoms (Rennie *et al.*, 2011). Furthermore, an investigation on wind-mediated dispersal by Rennie *et al.* (2015) indicates that the pathogen can also be spread by windblown dust.

Clubroot is found in Brassica producing countries all over the world (reviewed by Dixon, 2009) and new outbreaks are constantly being reported. One of the outbreaks of most economic concern started in Alberta, Canada, in 2003 (Tewari *et al.*, 2005). *P. brassicae* had previously been observed on vegetable crops, but this was the first finding of clubroot in the multi-billion dollar crop canola. The pathogen is now well-established in this region and continues to expand dramatically (Rempel *et al.*, 2014; Strelkov and Hwang, 2014). The history of clubroot in Sweden as well as the current status of the disease was reviewed by Wallenhammar *et al.* (2014). In short, the disease was already common in vegetable Brassica crops in the 1920s and severe outbreaks of clubroot in oilseed rape occurred in the 1980s. Since then, several severe attacks have been reported in various parts of the country (Wallenhammar 1996; Wallenhammar, 2014; Wallenhammar *et al.*, 2014).

Figure 5. (A) Clubroot infection in a winter oilseed rape field in Halland, Sweden, in April 2006. (B) Typical gall formation on the tap root of a winter oilseed rape plant. (Photos: A-C. Wallenhammar).

Since there are few pesticides that have proved to be effective against *P. brassicae* and since fumigants pose problems both economically and environmentally, growers are mainly referred to an integrated control of clubroot (Donald & Porter, 2009). Among the available strategies are for example farm hygiene, crop rotation, control of volunteer oilseed rape, liming and application of calcium and boron. In recent years, partly resistant Brassica crops, including cultivars of winter oilseed rape and cabbage, have also become available.

Detection of Plasmodiophora brassicae

Detection of *P. brassicae* infection has traditionally relied on field observations and bioassays using bait plants. Several bioassays have been developed and many of them are still in use to e.g. assess the disease pressure in soils, validate newly developed detection methods or evaluate susceptibility of different cultivars. (eg. Wallenhammar, 1996; Friberg, 2005; Cao *et al.*, 2007; Sundelin *et al.*, 2010; Hwang *et al.*, 2012). The major drawbacks of this technique are that it is very coarse, time consuming and rely on personal judgment of disease symptoms. Therefore, improved diagnostic methods are needed for rapid and reliable prediction of *P. brassicae* in field soils. Several alternatives to the traditional bioassay, including fluorescence microscopy, serological methods and PCR-based methods, have been developed (reviewed by Faggian & Strelkov, 2009). PCR-based assays have several advantages over the other techniques. They are fast, have high specificity and reproducibility and are highly objective.

A number of different PCR-based diagnostic assays for detection of *P. brassicae* have been presented (Table 2). Ito *et al.* (1997) identified a single-copy DNA sequence unique to *P. brassicae* and developed species-specific primers targeting this sequence. Later, these primers were used to develop a single-tube nested PCR assay for detection of the pathogen in soil (Ito *et al.*, 1999).

A nested PCR targeting the multi-copy ribosomal DNA (rDNA) was designed by Faggian *et al.* (1999) and was evaluated in artificially inoculated as well as in naturally infested soil. A few years later, yet another nested PCR technique for detection of *P. brassicae* was presented by Wallenhammar and Arwidsson (2001), but this time the results were also correlated with disease severity. The assay was able to detect the *P. brassicae* target in soils with a corresponding disease severity index higher than 21. By using a commercial DNA extraction procedure, the protocol was further improved and positive test results were obtained in soils with a DSI (disease severity index) of 3.1 (Wallenhammar, 2010).

Table 2. *Summary of PCR-based methods for detection of* Plasmodiophora brassicae

Target sequence	PCR or real-time PCR	Validated in soil	Correlation to disease (soil samples)	Reference
ipt-like sequence	PCR	No	No	Ito *et al.*, 1997
ipt-like sequence	PCR (nested)	Yes	No	Ito *et al.*, 1999
rDNA (18S and ITS1)	PCR (nested)	Yes	No	Faggian *et al.*, 1999
ipt-like sequence	PCR (nested)	Yes	Yes	Wallenhammar & Arvidsson 2001
rDNA (18S and ITS1)	PCR	Yes	Yes	Cao *et al.*, 2007
rDNA (18S and ITS1)	Real-time PCR (SYBR Green)	No	No	Agrarwal, 2008
rDNA (18S and ITS1)	Real-time PCR (SYBR Green)	No	No	Sundelin *et al.*, 2010
rDNA (18S)	Real-time PCR (SYBR Green)	No	No	Rennie *et al.*, 2011
rDNA (18S)	Real-time PCR (MGB probe)	Yes	No	Hwang *et al.*, 2011
rDNA (18S)	Real-time PCR (TaqMan probe)	No	No	Cao *et al.*, 2014
rDNA (ITS1)	Real-time PCR (MGB probe)	Yes	No	Deora *et al.*, 2015

Cao *et al.* (2007) were the first to develop a one-step PCR assay. The detection limit of this assay was 1000 resting spores/g soil, which is also considered as the generally accepted threshold for symptom development (Faggian & Strelkov, 2009), and results were correlated to disease severity.

The PCR primers presented by Faggian *et al.* (1999) were later used for the development of a qPCR assay for monitoring disease progression in roots of *Arabidopsis thaliana* (Agrarwal, 2008). Sundelin *et al.* (2010) also reports of in planta quantification of *P. brassicae*, this time in Chinese cabbage. *P. brassicae* surface infestation of seeds and tubers was determined using a qPCR protocol developed by Rennie *et al.* (2011). More recently, Cao *et al.* (2014) validated a probe-based assay for quantification of *P. brassicae* in plant root tissue.

In the probe-based qPCR assay presented by Hwang *et al.* (2011), standard curves were generated using known quantities of resting spores and were used to determine the amount of *P. brassicae* in soil after the cropping of resistant and susceptible canola varieties. However, the qPCR assay was not correlated with disease severity. Just one month after this paper was first available online; Paper I of this thesis was published and results from a probe-based quantitative real-time PCR assay for detection of *P. brassicae* in soil were correlated with

disease severity. Since then, one more probe-based qPCR assay has been described (Deora *et al.*, 2015). This multiplex qPCR assay developed by Deora and coworkers is an improvement compared to previous assays since it has the advantage of using a competitive internal positive control to normalize for inhibition. On the other hand, the limit of detection is higher compared to many other assays (4000 resting spores/g soil) and the assay has not yet been correlated with disease severity.

1.2.2 Sugar beet root rot

Sugar beet root rot or Aphanomyces root rot is an economically important disease of sugar beets present world-wide in sugar beet growing districts in countries including USA, Germany, Poland, UK, Sweden and Spain (Rush 1988; Payne *et al.* 1994; Ramirez *et al.*, 1994; Olsson & Olsson 2004; Piszczek 2004; Amein 2006; Windels *et al.* 2007; Moliszewska & Piszczek 2008; Nechwatal *et al.* 2012). The disease is caused by the soil-borne oomycete *Aphanomyces cochlioides*. Oomycetes include organisms resembling fungi through morphological traits, but modern DNA based research places them far from true fungi. They are phylogenetically related to diatoms, chromophyte algae and other heterokont protists and are classified within the eukaryotic kingdom of Chromista. The genus Aphanomyces contains three independent lineages: (i) plant parasitic, (ii) animal parasitic, and (iii) saprotrophic or opportunistic parasitic species (Diéguez-Uribeondo *et al.*, 2009).

The life cycle of *A. cochlioides* includes two types of spores (Dyer & Windels, 2003; Papavizas & Ayers 1974). The asexually produced zoospores are short-lived and motile spores able to swim through soil water to infect sugar beet roots. Oospores, on the other hand, are long-lived sexually produced spores. Hyphae from germinating oospores can infect roots directly, but infection by zoospores is more frequently occurring. *A. cochlioides* causes post-emergence damping-off and root rot of seedlings (Windels, 2000). This acute phase of the disease first appears as brown and water soaked hypocotyls and roots, which later become black and thread-like (Papavizas & Ayers, 1974; Windels, 2000). This phase is sometimes referred to as black root (Papavizas & Ayers, 1974). If the seedlings survive, the adult roots eventually become malformed and scarred (Windels, 2000). Chronic symptoms including yellowed leaves, wilting, rotted roots, root lesions and reduced root size, may also develop from new infections of older plants (Harveson, 2007). Both quantity and quality of the crop are affected by this disease, and the sugar content is usually reduced leading to great economic losses (Papavizas & Ayers, 1974). The disease is favored by wet soils and high temperatures (20-28°C) (Papavizas & Ayers 1974; Windels 2000; Amein 2006). The infection is

also more widespread in soils with low pH and low calcium content (Payne *et al.* 1994; Olsson *et al.* 2011).

The disease history of sugar beet root rot was reviewed by Papavizas and Ayers (1974). "Wurzelbrand" of sugar beets was first reported in 1906 in Germany and one of the causal pathogens was identified as *A. laevis*. However, in 1928 Drechsler named and described *A. cochlioides* and later stated that this species was probably the same as the Aphanomyces causing "Wurzelbrand" of sugar beets in Europe (Drechsler, 1929). The disease is now well-established in sugar beet growing districts around the world and is considered as one of the most severe pathogens of sugar beet in Sweden where approximately 25% of the fields have a medium to high risk of infection (Olsson *et al.*, 2011). Also, yield losses of 27% have been reported in highly infested fields in Sweden (Olsson *et al.*, 2011).

Disease management mainly includes the use of resistant varieties, application of lime, early planting, seed treatment and proper soil drainage (Harveson, 2007, Persson & Olsson, 2010, Persson & Olsson, 2014; Olsson & Persson, 2014). However, in highly infested fields, severe infections can still occur despite these preventive actions (Olsson *et al.*, 2011).

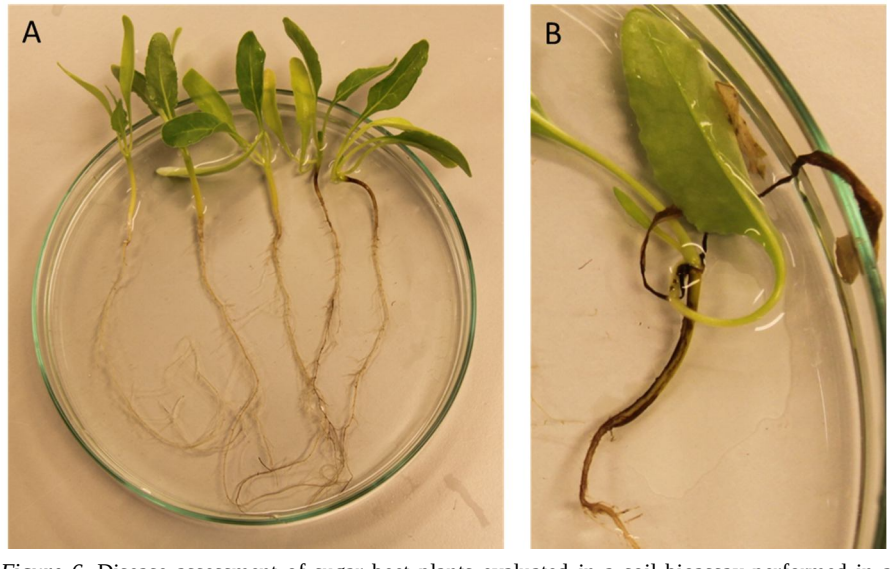

Figure 6. Disease assessment of sugar beet plants evaluated in a soil bioassay performed in a greenhouse. Classification of the plants according to Olsson *et al.* (2011). (A) Classes from the left: 0 (healthy plant), 10 (10% of the root system discolored), 25 (50% of the root system discolored), 50 (100% of the root system discolored) and 75 (100% of the root system and the hypocotyl discolored). (B) Class 75 (100% of the root system and the hypocotyl discolored).

Detection of Aphanomyces cochlioides

As with, *P. brassicae*, detection of *A. cochlioides* has conventionally relied on indirect estimations of inoculum densities by determining disease severity indices using root rot bioassays (e.g. Beale *et al.*, 2002; Harveson *et al.*, 2014; Olsson *et al.*, 2011) (Figure 6). Traditional PCR methods for detection of *A. cochlioides* have been developed. Weiland and Sundsbak (2000) presented a PCR assay targeting the actin coding sequence of *A. cochlioides*. However, these primers were not able to distinguish *A. cochlioides* from the closely related species *A. euteiches*. The two pathogens could only be differentiated by restriction enzyme digestion of the amplified products. Vandemark *et al.* (2000) developed a PCR assay using SCAR (sequence characterized amplified region) primers and were able to distinguish *A. cochlioides* from *A. euteiches*. Nevertheless, none of these methods were tested on soil samples and until now no quantitative PCR assays have been published.

1.2.3 Sclerotinia stem rot

Sclerotinia stem rot is caused by the plant pathogenic fungus *Sclerotinia sclerotiorum*. It has a broad host range and is capable of colonizing more than 400 different plant species (Boland & Hall, 1994) and is an important disease of spring oilseed rape in Sweden. Infected plants ripen prematurely and sclerotia (Figure 7a), which are hyphal aggregates formed inside infected tissue, can remain viable in the soil for up to 8 years (Adam & Ayers, 1979) and are able to germinate both carpogenically, producing apothecia (Figure 7b), and myceliogenically, producing mycelium (Bolton *et al.*, 2006). Sclerotinia stem rot of oilseed rape is initiated by airborne ascospores produced from apothecia and the timing of spore release is usually synchronized with flowering (West *et al.*, 2008). Ascospores cannot adhere to or germinate on the surface of healthy leaf tissue (Jamaux *et al.*, 1995); they need access to both a nutrient source and water to be able to infect the host plant. Typically, flower parts such as petals provide the primary source of nutrient and it is also believed that infected petals are the primary element of dissemination (Jamaux *et al.*, 1995).

Management of stem rot is challenging due to the wide host range of *S. sclerotiorum* and the longevity of sclerotia in soil. The control is mainly by foliar fungicide application, but systematic spraying is both cost-ineffective and has a negative impact on the environment (Koch *et al.*, 2007; Makowski *et al.*, 2005).

Figure 7. (A) Sclerotia of *S. sclerotiorum* formed inside the stem of an infected oilseed rape plant. (B) Apothecia of *S. sclerotiorum* germinating from sclerotia in the soil. (Photos: A-C. Wallenhammar).

Disease severity assessment and detection of Sclerotinia sclerotiorum

A large number of different stem rot forecasting schemes have been presented and most of them rely on risk-point tables based on field conditions and climate data, and sometimes also cropping history, to predict the risk of disease or onset of spore release (e.g. Makowski *et al.*, 2005; McLaren *et al.*, 2004; Twengström *et al.*, 1998). A German forecasting model based on climate, crop rotation and economy has also been presented to provide fungicide treatment recommendations (Koch *et al.*, 2007). However, even though the environmental conditions are favorable for disease development, *S. sclerotiorum* inoculum must be present for the disease to occur. Bom and Boland (2000) concluded that both the amount of inoculum present and environmental factors must be considered in an effective disease prediction model. In the UK, the AHDB (Agriculture and Horticulture Development Board) provides a risk forecast of Sclerotinia at monitoring sites using models based on petal tests, air sampling and weather data. Traditionally, agar tests have been used to determine the amount of *S. sclerotiorum* on petals (Turkington & Morall, 1993), but both serological (Wallenhammar *et al.*, 2007) and PCR-based methods (Table 3) have also been used to analyze field samples.

Table 3. *Summary of PCR-based methods for detection of* Sclerotinia sclerotiorum

Target sequence	PCR or real-time PCR	Sample matrix	Reference
rDNA (ITS region)	PCR	Petals and air samples	Freeman *et al.*, 2002
Mitochondrial small subunit rRNA	Real-time PCR (SYBR Green)	Air samples	Rogers *et al.*, 2009
Microsatellite	Real-time PCR (SYBR Green)	Petals	Yin *et al.*, 2009
rDNA (ITS region)	PCR	Petals	Qin *et al.*, 2011
Aspartyl protease	PCR (multiplex)	Tomato and peanut	Abd-Elmagid *et al.*, 2013
Mitochondrial small subunit rRNA	Real-time PCR (SYBR Green)	Air samples	Parker *et al.*, 2014
rDNA (IGS region)	PCR	Air samples	Jones *et al.*, 2015
SS1G_00263[1]	Real-time PCR (TaqMan MGB)	Petals	Ziesman *et al*., 2016

1. Single-copy gene encoding the hypothetical secreted protein ss263.

1.2.4 Root rot in red clover

Red clover (*Trifolium pratense*) is the most important forage legume in Sweden, and also used as a green manure crop in organic farming. In Sweden, forages are cultivated on approximately 45% of the arable land (www.sjv.se). Even though red clover is a perennial species, its yield generally declines over time and the plants rarely persist more than three years due to invasion of the roots by soil-borne pathogens (Rufelt, 1986; Wallenhammar *et al.*, 2008). Red clover root rot is caused by several fungal pathogens affecting the establishment, growth, persistence and overwintering capacity of clover plants. The pathogens causing root rot can also infect other legumes including white clover, lucerne and alsike clover, but the symptoms and disease progress is much more severe on red clover (Rufelt, 1986; Wallenhammar *et al*., 2008) and disease symptoms can be found already during the seeding year (Wallenhammar *et al.*, 2006).

The disease is sometimes referred to as *Fusarium* root rot, but numerous fungi have been reported as the cause of red clover root rot, e.g. species from the genera *Fusarium*, *Cylindrocarpon*, *Phoma*, *Rhizoctonia*, *Verticillium* and *Pythium* (Ylimäki, 1967; Rufelt, 1986; Skipp *et al.*, 1986; Lager & Gerhardsson, 2002; Wessén, 2006; Yli-Mattila *et al.*, 2010). However, the causal pathogens seem to vary geographically. Although several of these fungi

are considered as weak pathogens themselves, they can cause severe losses in stressed and damaged plants as a result from e.g. frequent cutting (Lager & Gerhardsson, 2002) or soil frost disruption (Ylimäki, 1967). Since stressed red clover plants are more susceptible to root rot, good management practices are required, and currently the only way, to control the disease (Rufelt, 1986).

Root rot is widely distributed on organic farms in south and central Sweden (Wallenhammar *et al.*, 2006) and some of the most commonly observed fungi causing the injuries are *Fusarium* spp., *Cylindrocarpon destructans* and *Phoma* spp. (Rufelt, 1986; Lager & Gerhardsson, 2002; Wessén, 2006). Rufelt (1986) conducted a large-scale survey of the root rot situation in Sweden in 1975-1977. *Fusarium* species were the most frequently isolated pathogens, but also *Phoma medicaginis* var. *pinodella* and *Cylindrocarpon destructans* were found. While evaluating the risk of legume-intensive crop rotations in organic farming, Lager and Gerhardsson (2002) isolated fungal pathogens from red and white clover roots. The isolated fungi were dominated by species of the genera *Fusarium*, *Phoma*, *Pythium* and *Sclerotinia*. Most of the *Pythium* strains were found to be mainly pathogenic to seedlings and *Sclerotinia trifoliorum* is the causal pathogen of clover rot or crown rot. The most pathogenic species associated with root rot in the different legumes were *F. culmorum*, *F. avenaceum*, *F. heterosporum*, *F. solani*, *C. destructans* and *P. medicaginis*. Isolation of fungi may give a skewed result since fast growing fungi may easily overgrow slow growing species. In a Swedish study performed by Wessén (2006), classical isolation was complemented by using T-RFLP (terminal restriction fragment length polymorphism) to potentially include more of the pathogens in the red clover root rot complex. The most frequently detected species in that study was *C. destructans*, *P. exigua*, *F. avenaceum* and *Ascomycota sp*.

Disease severity assessment of red clover root rot

A common way to assess red clover root rot disease severity is to examine the degree of vascular (internal) root symptoms (discoloration and root rot, Figures 8b and c), often followed by calculation of a disease severity index (Lager & Gerhardsson, 2002; Lager & Wallenhammar, 2003; Wallenhammar *et al.*, 2008; Stoltz & Wallenhammar, 2012). The external root symptoms (lesions and discoloration, Figure 8a) are also assessed (Wallenhammar *et al.*, 2006). This visual assessment and classification of root symptoms was first described by Rufelt (1986).

Although it is now known which species that cause red clover root rot under Swedish cultivation conditions, there are still a lot of unanswered questions regarding the disease and the causal pathogen complex. For example, there is a

lack of knowledge when it comes to which pathogens that infect already during the seeding year and which pathogens that are most prevalent in the following years. In addition, little is known about regional differences. Conclusively, there is a need for fast and specific quantitative diagnostic methods. A simple and reliable disease analysis could also be useful to evaluate the susceptibility among various cultivars of red clover.

Figure 8. Typical symptoms of red clover root rot. (A) Severe symptoms on the outside of the root. Almost the entire taproot is discolored with multiple damages on the upper parts of the root. (B) Severe inner root rot that appear to originate from damages on the outside of the root. (C) Roots with mild inner symptoms with discoloration mainly on the upper parts of the root (left) and more severe symptoms of root rot and discoloration along the central steele (right).

2 Aims of the study

The overall aim of this thesis was to develop real-time PCR assays with high specificity and repeatability for soil-borne plant pathogens of importance for Swedish farmers today, with the aim to improve disease risk assessment and management strategies. Specific objectives were to:

- Improve disease risk assessment of clubroot and sugar beet root rot by establishing DNA-based pathogen threshold levels for growing susceptible and resistant crop varieties.
- Study the in-field variation of *Plasmodiophora brassicae* using qPCR.
- Monitor naturally occurring inoculum of *Sclerotinia sclerotiorum* on plant material (oilseed rape petals and leaves) and in air samples using real-time PCR to increase knowledge about the infection process and to find potential tools for an improved disease risk assessment.
- Monitor the seasonal and year-to-year development of soil-borne red clover root rot pathogens using in planta qPCR.

3 Results and Discussion

This thesis covers monitoring of several different plant pathogens using real-time PCR. The aim is here to highlight the major findings including the performance of the assays, their reliability and the application and usefulness of the methods as tools in disease risk assessment and disease monitoring in field samples. Since method development and validation has been a major part of the work in this thesis, these results are also presented in this section rather than in a Materials and methods section.

3.1 Real-time PCR detection of selected plant pathogens

In this study, a total of eight different real-time PCR assays have been developed or adapted from previously published assays to be able to detect and quantify seven different plant pathogens: *P. brassicae* (Paper I), *A. cochlioides* (Paper II), *S. sclerotiorum* (Paper III) and *F. avenaceum*, *F. culmorum*, *C. destructans* and *Phoma* spp. (Paper IV). The target sequences and types of probes used in these assays are summarized in Table 4.

The specificity of the primers developed in this study was evaluated using BLAST and by analyzing closely related species as well as other common plant pathogens. For some assays, the experimental evaluation of the specificity had already been performed previously and in those cases the assays were mainly assessed on positive material. All pathogen assays, except the assays for detection of *S. sclerotiorum* and *Phoma* spp., were found to be species-specific. The *Phoma* spp. assay detects both *P. exigua* and *P. medicaginis*, as shown in the present study, and according to previous investigations the assay also amplifies DNA from *P. complanata*, *P. glomerata*, *P. herbarum*, *P. lingam* and *P. telepii* (Cullen *et al.*, 2007). This was not considered as a disadvantage, but rather as an advantage since several species within this genus have been shown to infect red clover (Rufelt, 1986;

Skipp *et al.*, 1986, Wessén, 2006). BLAST searches of the primers for detection of *S. sclerotiorum* showed that *S. trifoliorum* and *S. minor* are also likely to be detected in this assay. However, the primers were previously found not to detect the related airborne fungi *Botrytis cinerea* causing gray mold (Freeman *et al.*, 2002).

The amount of pathogen and reference DNA was quantified using plasmid standards. The reasons for choosing plasmid standards were multiple. First and foremost, a quantification standard suitable for routine use was required since some of the assays were intended to be applied as analysis services offered to farmers. Preparing standards based on enumerated spores, pure or added to soil, is tedious and the reproducibility of such standards is probably lower compared to DNA-based standards. In addition, using genomic DNA as standard for *P. brassicae* was not an option since this pathogen is an obligate biotroph and thus very problematic to obtain pure DNA from. Also, quality controlled plasmid standards can nowadays be ordered from life science companies at reasonable costs and can be stored in a standard freezer for several years without degradation.

Table 4. *Target region, type of probe and reference for the real-time PCR assays used to detect and quantify seven different plant pathogens in environmental samples.*

Assay/species	Target region	Probe	Reference
P. brassicae	rDNA (18S)	TaqMan	Paper I
A. cochlioides	rDNA (ITS region)	TaqMan MGB	Paper II
S. sclerotiorum	rDNA (ITS region)	TaqMan MGB	Freeman *et al.* 2002 (primers) and Paper III (probe)
F. avenaceum	RAPD/SCAR	TaqMan MGB	Waalwijk *et al.* 2004
F. culmorum	RAPD/SCAR	TaqMan MGB	Waalwijk *et al.* 2004
C. destructans	β-tubulin gene	TaqMan	Tewoldemedhin *et al.* 2011 (forward primer), Dubrovsky & Fabritius 2007 (reverse primer) and Paper IV (probe)
Phoma spp.	rDNA (ITS region)	TaqMan	Cullen *et al.* 2007
Plant	COX gene	TaqMan	Weller *et al.* 2000 (forward primer) and Suarez *et al.* 2005 (revers primer and probe)

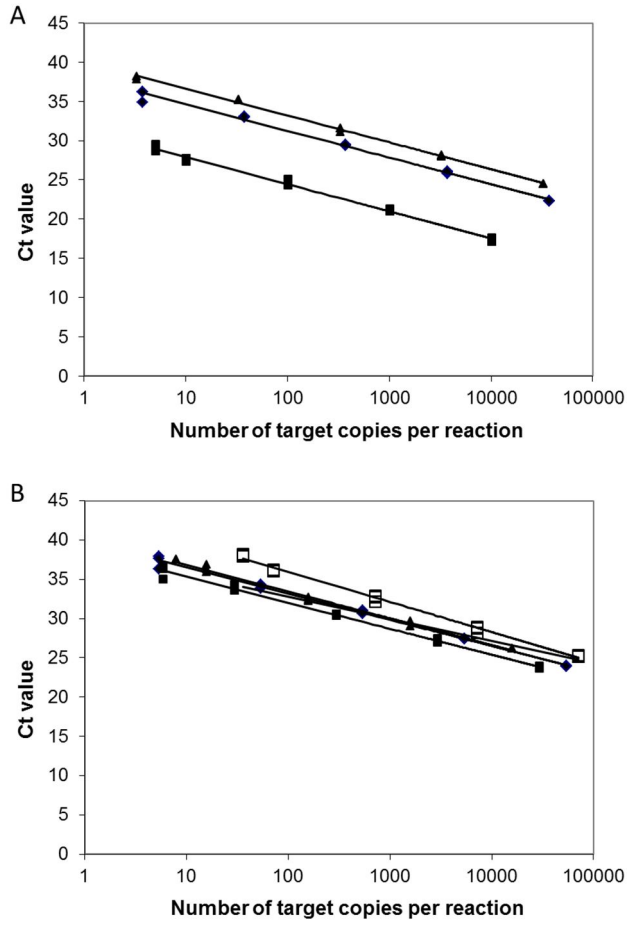

Figure 9. Examples of standard curves used in the qPCR assays for the seven plant pathogens included in this study. (A) Triangles: *A. cochlioides*, R^2=0.999, PCR-efficiency=95%, LOD=3.3 target copies. Diamonds: *P. brassicae*, R^2=0.994, PCR-efficiency=97%, LOD=3.8 target copies. Boxes: *S. sclerotiorum*, R^2=0.995, PCR-efficiency=95%, LOD=5.1 target copies. (B) Triangles: *F. avenaceum*, R^2=0.994, PCR-efficiency=96%, LOD=8.0 target copies. Diamonds: *Phoma* spp., R^2=0.995, PCR-efficiency=100%, LOD=5.4 target copies. Boxes: *F. culmorum*, R^2=0.993, PCR-efficiency=100%, LOD=5.9 target copies. Open boxes: *C. destructans*, R^2=0.994, PCR-efficiency=82%, LOD=35.7 target copies.

Standard curves were generated by analyzing reactions containing different amounts of plasmids carrying the target sequences. Examples of standard curves and corresponding amplification efficiencies as well as R^2 values of the assays are displayed in Figure 9. All assays were optimized to get high amplification efficiencies suitable for target quantification. It is commonly stated that the PCR efficiency should be >90% for a quantitative assay.

However, low efficiency assays can be used, but the major disadvantage of using an assay with poor reaction efficiency is that the dynamic range will most likely not include the lower copy numbers (Edwards *et al.*, 2004). Only the amplification efficiency of the *C. destructans* assay fell outside the optimum range, probably due to the rather long target sequence (233 bp). This assay also has the highest LOD of the qPCR of the assays investigated in this work; 36 target copies per reaction compared to 3-8 copies per reaction.

3.1.1 Quantification of *P. brassicae* and *A. cochlioides* in soil samples (Papers I and II)

To ensure high DNA yield from soils of various compositions, all soil samples were extracted using a commercial kit based on bead beating (Dineen *et al.*, 2010). The DNA extracts were, however, found to be very inhibitory to the qPCR and therefore further purification in two or three steps was necessary. The effect of inhibition was monitored by spiking an additional reaction with a small amount of the plasmid standard and proved to be a simple yet effective way to assess the inhibition in naturally infested soil samples. By comparing the amount of plasmid standard quantified in the presence of soil DNA to the amount quantified in a no template control, it was possible to determine whether the sample was highly affected by inhibition or not. This set up was only used as a rough estimate of the inhibition and not for normalization of the results. Another option is to add exogenous DNA or bacterial cells before the DNA extraction to be able to compensate for differences in extraction efficiency as well as inhibitory effects (Li *et al.*, 2015; Park & Crowley, 2005), but the accuracy and additional benefits of this technique is questionable since the DNA extraction procedure will likely function differently on purified DNA or bacterial cells compared to e.g. resting spores or oospores.

The repeatability of repeated extractions of a subsample can have a significant effect on the precision of the result, especially when it comes to complex procedures such as those involved in DNA extraction from soil. The repeatability of the assays for quantification of *P. brassicae* and *A. cochlioides* was almost the same, 22.5% and 25% respectively, and is similar to that of assays for other plant pathogens quantified in soil samples (Cullen *et al.* 2002; Atkins *et al.* 2003; Atkins *et al.* 2005; Zhang *et al.* 2006). To further evaluate the precision of these assays, measurement results of two *P. brassicae* positive control samples (field infested soils) were used to calculate the within-laboratory reproducibility. The first control sample was analyzed 21 times during 2010-2012 by different persons and with different batches of kits, reagents and standards. Results are visualized in a control chart (Figure 10a) and the relative standard deviation of these measurements was 43%. The

second control sample was analyzed 18 times during 2015 in a different laboratory by different persons and with different batches of kits and reagents. Results are visualized in Figure 10b and the relative standard deviation of these measurements was 42%. On the basis of these results alone, the expanded measurement uncertainty (k=2) of single sample measurements would be almost 90%. However, the actual measurement uncertainty is expected to be slightly lower since all analyses, both in this work and in the routine analysis service, are carried out as duplicate measurements (duplicate extractions). This might sound as an extremely high measurement uncertainty, and compared to most chemical methods this value is indeed very high. It is also high compared to the measurement uncertainty in quantitative analysis of genetically modified organisms (Žel *et al*., 2007). On the other hand, it is perhaps more correct to compare the results to microbiological methods since it is a microorganism that is being analyzed. Analytical results and measurement uncertainties of microbiological tests are usually expressed in logarithmic values, i.e. log cfu (colony forming units). Converting the results from the repeatability test in the present study to logarithmic values gives an expanded measurement uncertainty of approximately 0.4 log copies/g, which is in a similar range as many commercial microbiological tests (Eurofins Food & Feed Testing Sweden AB). Furthermore, the interpretation guidelines discussed in section 3.3.1 spans over a very wide range; from <1300 to >325 000 target copies. In this context, the measurement uncertainty lies on a reasonable level.

In addition, an interesting observation from these data is that long-term storage (≈2 years) of dried soil samples in room temperature does not seem to affect the level of detectable *P. brassicae* DNA. This was also demonstrated for two soil samples collected in 2006. The first analysis resulted in values of 25 000 and 590 000 copies/g soil. Results were similar when these two samples were re-analyzed after five years storage (dried samples, stored in -5-20 °C); 85 000 and 560 000 copies/g soil, respectively.

The LOD's of the diagnostic methods for both *P. brassicae* and *A. cochlioides* were determined by adding resting spores and oospores, respectively, to uninfested field soils. For *P. brassicae*, the LOD was approximately 500-1000 resting spores/g soil which is also considered as the generally accepted threshold for disease development (Faggian & Strelkov, 2009). It is also similar to LOD's reported in other studies (Faggian *et al.*, 1999; Cao *et al.*, 2007). For *A. cochlioides*, the LOD was evaluated in several different soil types and varied between 1 and 50 oospores/g soil. A low LOD was correlated to high clay content and low sand content and vice versa. DNA extraction from soils with high clay content is often especially difficult (Frostegård *et al.*, 1999) due to adsorption of nucleic acids to clay particles

(Cai *et al.*, 2006). Therefore, the results presented in Paper II were a bit unexpected. On the other hand, our results correspond to the outcomes from a study comparing several different commercial DNA extraction kits, including the kit used in this thesis that was found to yield similar amounts of DNA from both a clay soil and a sand soil (Dineen *et al.*, 2010).

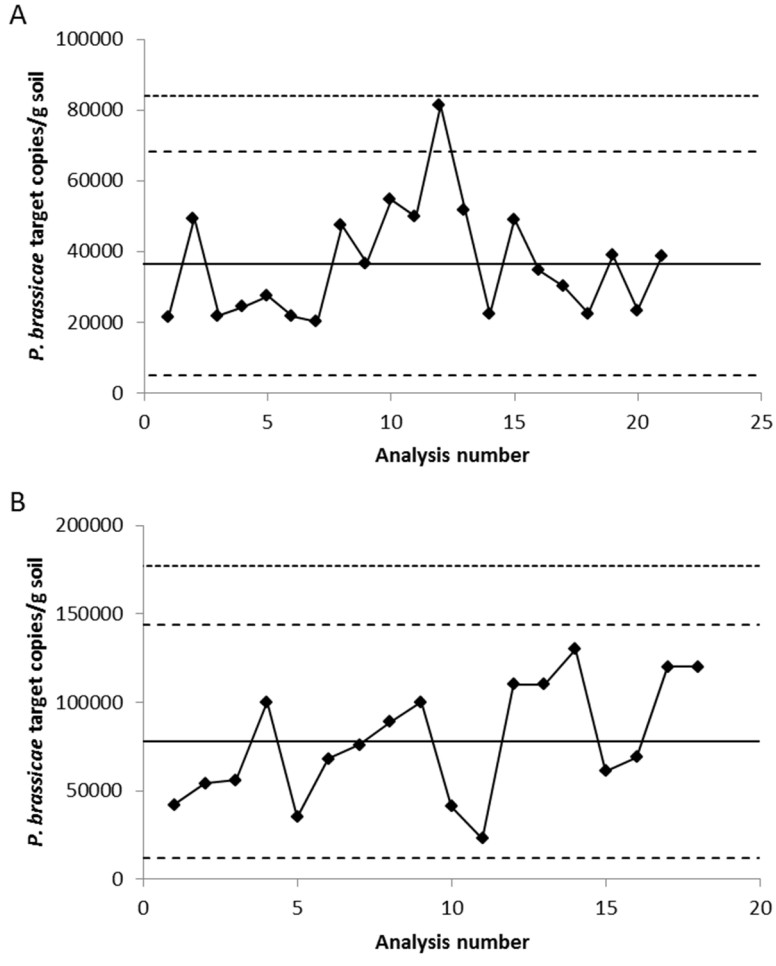

Figure 10. Reproducibility tests and construction of control charts. Two different control samples (naturally infested soils) were extracted and analyzed using the *P. brassicae* specific qPCR assay. (A) The first control sample was analyzed at different times during 2010-2012 (n=21). The mean value was 36 612 target copies/g soil with a relative standard deviation of 43%. (B) The second control sample was analyzed at different times during 2015 (n=18). The mean value was 78 000 target copies/g soil with a relative standard deviation of 42%. The means are displayed as solid lines. The dashed lines represent the mean ± 2 x the standard deviation (warning limit). The dotted lines represent the mean + 3 x the standard deviation (action limit).

Homogenization of the sample is an important part of the soil analysis procedure. In the initial phase of the clubroot project, we evaluated the possibility of using the soil mill currently used for grinding of soil samples for nutrient analysis at Eurofins. A simple contamination experiment was performed by first grinding an infested soil sample (180 000 copies/g soil) followed by grinding a soil samples free from *P. brassicae* DNA. This test showed that the risk of contamination was too high since the healthy soil contained approximately 1000 copies/g soil after grinding, although the mill was cleaned between the samples using a brush. Therefore, soil samples analyzed in this study were homogenized and pulverized either by hand using a mallet, in a ball mill or in single-use plastic jars containing stainless steel balls shaken in a standard paint shaker.

3.1.2 Detection of plant and airborne inoculum of *S. sclerotiorum* (Paper III)

In the present study we had the opportunity to work with S. *sclerotiorum* over several years and during this time we have successfully detected the pathogen in both plant and airborne inoculum using diagnostic real-time PCR. When we started working with *S. sclerotiorum* in 2006, the focus was entirely on detecting the pathogen on oilseed rape petals since the general opinion was that ascospores on petals constitute the primary source of inoculum (Jamaux *et al.*, 1995). First, we tried to correlate the amount of quantified *S. sclerotiorum* DNA in pooled petal samples to the number of infected petals determined by the agar test. However, no correlation was found, probably due to the fact that a large number of copies may be detected from just a few single petals that are well colonized by the fungus. On the other hand, a larger number of petals with low level of infection will likely cause a more widespread infection in the field and thus be more severe as only some of the petals are likely to stick to the lower leaves and stems to vector the infection. We decided to instead analyze individual petals and determine the incidence of *S. sclerotiorum* DNA. Extracting DNA from single petals required a protocol adapted to very small sample volumes. Therefore, a commercial lysis buffer suitable for this purpose was used and the extraction was carried out in 200 µl PCR-tubes (Figure 11a).

In the next part of this project (2008-2010), we started collecting and analyzing leaves in addition to the petals and to our knowledge this had not been attempted before. Oilseed rape leaves can be of varying size depending on age/leaf level (Figure 11b). A commercial plant DNA extraction kit with an up-scaled initial lysis step was used to be able to process leaves of different sizes. As for the petal analysis, we focused on determining the incidence rather than the quantitative amount of *S. sclerotiorum* DNA.

In the final year of the project (2010), we also collected air samples at one of the field trials to get a complete picture of the release of ascospores into the air. The air samples were collected on Melinex tape using a Burkard 7 day continuously recording spore trap (Figure 11c). To release the ascospores from the tapes and to efficiently lyse the cells we chose a similar DNA extraction kit as for soil samples using bead beating in a lysis buffer. However, further purification was necessary, probably due to inhibition by substances from the adhesive used on the tapes.

Figure 11. S.sclerotiorum DNA was monitored in three different sample types: (A) petals, (B) leaves from different leaf levels and (C) air samples collected using a Burkard 7 day recording volumetric spore trap. The photo of the spore trap is published with kind permission from Burkard Manufacturing Co. Ltd.

3.1.3 In planta quantification of red clover root rot pathogens (Paper IV)

Red clover roots sampled on several occasions at two different cultivar field trials were analyzed in this study. We decided to extract and analyze pooled samples, i.e. one pooled sample from each cultivar and block at each sampling occasion, to reduce the number of samples but still get representative results. The roots were mixed in a knife mixer and DNA was extracted from sub-samples of the homogenized pooled samples. As for both soil samples and air samples, we used a commercial kit based on bead beating to efficiently lyse the different types of pathogen cells present in the roots. Contrary to the analysis of soil and air inoculum, we now had the possibility to easily normalize the amount of pathogen target to a reference gene (relative quantification using standard curves). We chose an assay targeting the mitochondrial COX (cytochrome oxidase) gene (Weller *et al.*, 2000; Suarez *et al.*, 2005) for this purpose and expressed the number of pathogen target copies relative to the amount of this plant gene instead of relating it to e.g. sample weight.

3.2 Monitoring plant pathogens in field samples

The applications for real-time PCR detection of plant pathogens in field samples are numerous, e.g. disease diagnosis as a decision support to choose field, crop, cultivar or timing of fungicide application, early disease warning systems, disease resistance breeding and monitoring infection processes. The practical use, however, is still rather limited. In addition to the evaluation of qPCR as a tool in disease risk assessment in the present study (section 3.3), qPCR has also been used to study the spatial distribution of *P. brassicae* and to monitor seasonal variation of both *S. sclerotiorum* and the pathogen complex causing red clover root rot.

3.2.1 Spatial distribution of *P. brassicae* (Paper I)

Many soil-borne pathogens have a patchy distribution in the field (Gilligan *et al.*, 1996; Hornby, 1998), which was also demonstrated in the present study where the spatial distribution of *P. brassicae* was investigated on three Swedish farms. Soil samples were collected using the same strategy as applied for standard nutrient soil mapping, i.e. point sampling of one soil sample per hectare by pooling 10-30 randomly distributed subsamples (soil cores) taken from within a 3 m radius. In addition, an intensified randomized sampling consisting of 40 samples from an area of approximately 2.5 hectares was performed to further improve the description of in-field variation of the pathogen. These investigations clearly confirmed the in-field patchiness of *P. brassicae* (Figures 3 and 4, Paper I). Another illustrative way to visualize the in-field distribution is to create interpolated disease maps. An example is shown in Figure 12 using the data from Paper I. In the left field, the number of target copies/g soil varies between 1500 at one sampling point to >800 000 copies/g at the sampling point with the highest value. The distance between these two sampling point is less than 200 m.

Another example of the patchiness of *P. brassicae* is shown in Figure 13, where the highest amount of target copies/g was approximately 450 000 compared to a sampling point just 100 m away where the level of *P. brassicae* was undetectable using the qPCR assay. At the third location (Östergötland) where we studied the spatial distribution in 2007, there was less than 100 m between the highest level of >2 million target copies/g soil and one of the sampling points where no *P. brassicae* DNA was detected. This demonstrated patchiness of *P. brassicae* emphasizes one of the main challenges in biological soil mapping, i.e. the choice of soil sampling technique.

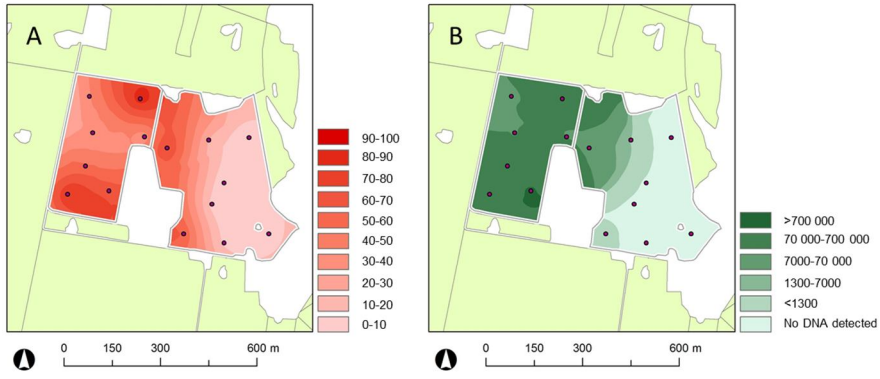

Figure 12. Interpolated disease maps showing the in-field distribution of *P. brassicae* at two fields in Halland, Sweden, sampled in 2006 (adopted from Paper I). (A) Disease severity index. (B) qPCR results (target copies/g soil).

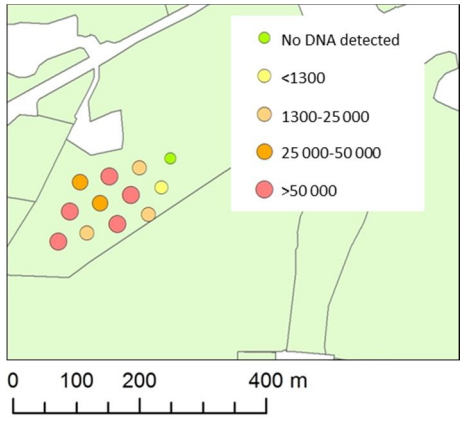

Figure 13. In-field distribution of *P. brassicae* DNA (target copies/g soil) at parts of a field in Västergötland, Sweden, sampled in 2006. (Construction of map: Mats Söderström)

3.2.2 Recommendations on soil sampling (Paper I)

The sampling strategy should always be carefully chosen and will depend on the purpose of the investigation. At present, due to the rather high cost of the qPCR analysis (approximately 200 EUR), farmers tend to prefer screening their fields by collecting and analyzing a composite sample representing the entire field. Some soil laboratories recommend collecting a composite sample along a diagonal line across the field (Figure 14b). However, this is not a recommended strategy when it comes to analyses of soil-borne plant pathogens. Due to their patchiness, areas with high inoculum density might not be sampled using the diagonal sampling. Instead, collecting sub-samples along

a ‘W’ transect is suggested to more likely include areas with high pathogen loads (Ophel-Keller *et al.*, 2008; Paper I) (Figure 14c).

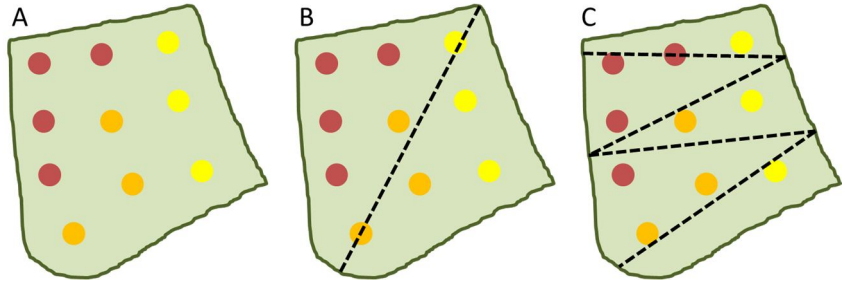

Figure 14. The soil sampling strategy will have a large impact on the result of the pathogen analysis. (A) Soil mapping of approximately one sample per hectare will show the variation in inoculum density across the field. (B) Sampling along a diagonal is often not suitable since areas with high inoculum density might not be included in the sampling. (C) For a more general survey, a composite sample of several cores should be collected along a ‘W’ transect. The different colors of the dots represent different disease risk categories (red=high, orange=medium and yellow=low).

3.2.3 Monitoring seasonal and year-to-year variation (Papers III and IV)

qPCR is an outstanding tool to monitor seasonal variation of plant pathogens and can be used for pathogen detection in a wide range of sample types. In the present study, the level of *S. sclerotiorum* was monitored in both plant material and air samples. Five field experiments were conducted from 2008 to 2010 and samples collected on different time points during the growing season were analyzed using real-time PCR to study the pathogen development. For both petals and leaves, real-time PCR proved to be a very useful tool to detect significant differences in incidence of *S. sclerotiorum* DNA between sampling occasions and the results gave us an indication of when the spore release took place. In addition, significant differences in disease incidence between leaf levels were found. Disease incidence was generally higher on basal leaves compared to leaves on apical levels (Tables 3 and 4, Paper III). The lower leaves are closer to the ascospore releasing apothecia, they are older compared to the more recently developed leaves higher up the plant and they also have a larger surface area. Conclusively, leaves on lower leaf levels are more likely to contain a higher number of ascospores, which was proven by real-time PCR analysis.

Using spore traps to monitor the changes of airborne ascospores have been used in e.g. the UK and results indicate that there is a correlation between the number of detected ascospores and disease incidence in the field (Rogers *et al.*, 2009). In the present work, we used a Burkard spore sampler to monitor the

airborne inoculum in one of the spring oilseed rape field trials in 2010. *S. sclerotiorum* DNA was first detected in the air samples collected on 27 May. In the beginning of June the amount started to increase and the spore release lasted for approximately three weeks (Figure 4, Paper III). Interestingly, the release of spores did not coincide with flowering, explaining why no DNA was detected on the petals collected in this field. Temperature as well as relative humidity in this region fulfilled the requirements for a regional risk of disease according to the SkleroPro model (Koch *et al.*, 2007) and the field assessment later showed that the level of Sclerotinia stem rot in this field was 7%. In contrast to the finding by Jamaux *et al.* (1995), our results suggest that the ascospores do not need to be present on the petals to cause an infection.

In the present study, pathogens causing red clover root rot were monitored in two field experiments to assess the potential seasonal and year-to-year variations. qPCR proved to be a very useful tool to identify the different pathogens in the red clover roots and also to monitor the changes in amount of pathogen DNA over time. Generally, the level of pathogen DNA increased over time and the highest levels were found on the last sampling occasions in the second harvest year (Figure 2 and 3, Paper IV). In one of the field trials, the level of both *F. avenaceum* and *Phoma* spp. DNA in the roots increased approximately 100-fold in the course of two years, whereas the level of *C. destructans* increased approximately 10-fold at both experimental sites. These results were also consistent with the disease severity indices determined using visual grading of internal and external symptoms of the roots.

The level of red clover root rot pathogens were significantly higher at one of the field experimental sites (Figure 4, Paper IV) and prevailing weather conditions is suggested as a possible source of the differences in disease development. By analyzing climate data from nearby weather stations, we found that the site with the highest levels of pathogen DNA received a greater amount of rainfall and had the coldest winters. Alternating freezing and thawing of the ground causes damages to the roots (Ylimäki, 1967) and it is thus likely that the red clover roots at this site suffered more severe and widespread winter injuries compared to the site with milder winter temperatures. Wounded roots have a higher incidence and severity of root rot (Stutz *et al.*, 1985) and therefore it is not so far-fetched to assume that the lower winter temperatures at one of the sites probably contributed to a greater disease progression.

3.2.4 Using real-time PCR in field trials (Papers IV)

Real-time PCR can be a time-saving method for pathogen diagnosis and quantification in field trials. qPCR was for example the method of choice in a

recent study evaluating Verticillium wilt susceptibility of different potato cultivars (Pasche *et al*., 2013) and has also been used in field trials to assess fungicide effects on wheat leaf blotch (Guo *et al*., 2007; Almquist *et al*., 2008). In the present study, the susceptibility of 18 different red clover cultivars to red clover root rot was evaluated in two field trials. The level of infection was assessed using qPCR and by grading internal and external symptoms. None of the evaluated cultivars were resistant to red clover root rot and only minor differences in disease susceptibility were found (Figure 1, Paper IV). Hence, the choice of red clover cultivar will probably have a negligible effect on the disease development.

In addition to the cultivar evaluation, red clover roots were collected from plots that were cut twice each year, representing two harvests, and from plots that were not cut at all to evaluate the possible effect of cutting on disease development. In November the first harvest year, the levels of *Phoma* spp. and *C. destructans* were significantly higher in the cut plots, whereas the level of *F. avenaceum* and the internal disease severity index were lower in the cut plots (Figure 5, Paper IV). However, no differences in external disease symptoms were found between cut and uncut plots. In root samples collected during the second harvest year, both the external and internal disease symptoms were more severe in the cut plots (Figure 6, Paper IV). Rufelt (1986) found that the disease severity was influenced both by the number of cuts and the time interval between successive cuts and suggests that only two harvests within a growing season might be possible under Swedish conditions based on greenhouse experiments. Results from field trials in the present study indicate that the severity of root rot damages may actually increase even at two cuts. However, the discrepancies between the visual grading and the qPCR assays demonstrate that cutting frequency in connection with red clover root rot needs to be further studied under field conditions.

3.3 Improved disease risk assessment

Validating diagnostic qPCR assays using traditional techniques, such as greenhouse bioassays (Papers I and II; Figure 15), agar tests (Paper III) or field assessments (Papers III and IV), is very important to assess the trueness of the analysis. It is also a crucial step to be able to interpret the meaning of the results or the impact a certain DNA-level will have on e.g. yield. However, the relationship between the number of pathogen target copies and the disease severity will be influenced by several other factors affecting disease development, e.g. soil type, environmental conditions and crop variety. Even though the number of factors affecting disease severity are reduced when

performing standardized greenhouse bioassays, the results will still be influenced by parameters that cannot be controlled, primarily soil type. This was clearly demonstrated for both *P. brassicae* and *A. cochlioides* as the relationships between qPCR results and DSI were weak (Figure 2, Paper I; Figure 3, Paper II). Therefore, care must be taken when interpreting the results of a DNA-test. The use of broad disease risk categories (e.g. low, medium or high) has proven to work well in a routine DNA-based testing service in Australia, but the final interpretations still have to be performed by specially trained advisors with the correct knowledge on which factors that affect the relationship between pathogen levels and disease expression (Ophel-Keller *et al.*, 2008). Another very important factor that will have a major effect on the outcome of both traditional techniques and qPCR tests of soil samples is the sampling strategy (see section 3.2.2).

Figure 15. Clubroot bioassay using Chinese cabbage as bait plant.

3.3.1 Pathogen threshold levels and interpretation guidelines for farmers (Papers I and II)

When we started working with a diagnostic assay for quantification of *P. brassicae* in 2006, there were no resistant oilseed rape varieties available for Swedish farmers. Therefore, the main aim was to determine threshold levels for growing susceptible varieties and when to completely avoid Brassica crops (Paper I). Since then, several clubroot resistant varieties of winter oilseed rape have been introduced to the Swedish market. It started with Mendel (NPZ-Lembke) and later came Mendelson (NPZ-Lembke), Mentor (NPZ-Lembke), SY Alister (Syngenta Seeds) and Andromeda (Limagrain). The resistance in Mendel is based on only one major gene (Diederichsen *et al.*, 2009) and since the other varieties are descendants from Mendel, it is likely that all these varieties use the same genetic source of resistance. In 2014, the Swedish seed and oilseed rape growers association evaluated the available partly resistant varieties in four field trials (www.svenskraps.se; www.sverigeforsoken.se).

Three of the trials were set up at locations with very high *P. brassicae* infestation levels (determined using the qPCR assay) and the resistant varieties gave a much higher yield compared to the susceptible varieties. In addition, it was also shown that the resistant varieties only gave minor yield reductions in the field trial on healthy soil. These results led to an update of the cultivation recommendations based on the qPCR analysis of *P. brassicae* presented in Paper I. Using the previous findings by Wallenhammar *et al.* (2000) in combination with the more recent evaluations of the partly resistant varieties available today, improved interpretation guidelines were established within the BioSoM (Biological Soil Mapping) research project in 2015 (www.eurofins.se). This shows that the recommendations should never be considered as static, but instead be changing following the implementation of new cultivation practices, introduction of new varieties and report of new scientific findings. Even though oilseed rape growers now have the availability of partly resistant varieties, these varieties must be used only when necessary to avoid future breakdown of disease resistance. Therefore, the routine use of the diagnostic service is now even more important in order to determine the correct cultivation strategy.

It should also be noted that even though the trials in 2014 showed that the partly resistant varieties gave a high yield, approximately 20% of the plants were infected by clubroot (compared to almost 100% in the susceptible varieties). A similar result was obtained in a Canadian study where 14% of the resistant canola plants were infected compared to 100% of the susceptible plants (Hwang *et al*., 2013). Even though the gall mass produced in resistant varieties is significantly less compared to susceptible varieties (Hwang *et al*., 2013), these data still indicate that at very high infestation levels there will be an increase of the soil inoculum when growing resistant varieties and it is therefore not recommended to cultivate Brassica crops at these high levels. However, it is possible that this threshold will be reevaluated and set higher when we have more knowledge on the properties and effects of resistant varieties. Hwang *et al*. (2013) showed that there was no difference in the reduction of resting spore populations between repeated cropping of a resistant canola cultivar and continuous fallow. This type of field trial under Swedish conditions using cultivars available to Swedish farmers would increase our understanding of repeated cropping of resistant varieties and possibly yield data that could improve the interpretation guidelines.

A similar objective of creating threshold values was set for the diagnostic qPCR assay for quantification of *A. cochlioides*. In this case, disease risk categories were already determined previously based on the bioassay (Olsson *et al.*, 2011) and a few resistant varieties were also available on the market. In

contrast to the clubroot resistant varieties, the Aphanomyces root rot resistant varieties do not yield as much as the susceptible varieties. Therefore it is of great economic importance to know when to use a resistant variety or not to be able to avoid unnecessary yield losses. The bioassay for estimating potential disease losses has hitherto been used very sparsely. Therefore, the qPCR assay developed in the present study provides a valuable new tool to identify high-risk fields. Unfortunately, discrepancies between the bioassay and the qPCR assay were found as only one third of the field infested samples tested positive using the qPCR assay even though *A. cochlioides* was successfully isolated from approximately 80% of the soil samples. This inconsistency is not necessarily caused by low sensitivity of the qPCR method, but may be a result of other microorganisms causing similar symptoms on the root system. However, the consequence is that it is not yet possible to establish qPCR threshold values for the disease risk categories based on the present method and further efforts are now needed to develop an assay that is also useful to also identify medium-risk fields.

3.3.2 Real-time PCR as a potential tool in future disease forecasting of Sclerotinia stem rot (Paper III)

Today, the only Sclerotinia stem rot forecasting offered to Swedish farmers is a regional risk assessment based on field information and climate data (Twengström *et al*., 1998). In addition to environmental factors, information about the amount of inoculum present must be considered to improve the existing disease risk assessment (Bom & Boland, 2000). The most widely used way to estimate the inoculum has been to determine the pathogen incidence on petals (Turkington & Morall, 1993; Wallenhammar *et al.*, 2007; Table 3). However, even though the linear relationship between the PCR-method and the agar test was significant in the present study (Figure 2, Paper III), the relationship between infected petals and field incidence of Sclerotinia stem rot was not satisfactory (Figure 3, Paper III). Difficulties in finding a relationship between petal infection and stem rot disease incidence have also been reported in previous studies (Davies *et al.*, 1999; Young & Werner, 2012). Instead of petal analyses, determining the incidence of *S. sclerotiorum* ascospores on leaves or analyzing the amount of pathogen DNA in air samples seem like more reliable tools to be included in an improved disease forecasting in the future. Paper III is the first report of using real-time PCR to assess the incidence of *S. sclerotiorum* on field collected oilseed rape leaves and additional work is now needed to establish a link to field environmental conditions which lead to Sclerotinia stem rot.

One of the most important factors included in the currently used forecasting is the knowledge on disease incidence in the previous oilseed crop (Twengström *et al*., 1998). Today, many farm enterprises increase in size and as a result there is a risk that awareness of previous crop sequences is lost. Therefore, an additional improvement of the disease risk assessment might be to include a soil test to determine the soil inoculum potential. Initial tests in the present study revealed that it is possible to use real-time PCR to quantify the amount of *S. sclerotiorum* DNA directly in soil samples indicating the potential usefulness of this analysis in a future forecasting scheme.

3.3.3 Monitoring root rot pathogens in red clover – a first step towards an improved disease risk assessment (Paper IV)

Root rot in red clover is often referred to as Fusarium root rot, a name that does not quite give the full explanation of the causal agents of this disease. In the present study, *F. avenaceum* was frequently occurring, but our results indicate that *C. destructans* is the most serious pathogen since the qPCR results were significantly correlated to DSI at both investigated experimental sites (Figures 7 and 8, Paper IV). On the other hand, the relationship was much stronger at site 1, suggesting that other pathogens than the ones analyzed in the present study contribute to the disease symptoms at site 2. The pathogens chosen in the present study were selected based on previous studies where the causal pathogens of red clover root rot was determined mainly using classical isolation on agar (Rufelt, 1986; Lager & Gerhardsson, 2002; Öhberg, 2008), although one investigation also used T-RFLP analysis of roots (Wessén, 2006). Studies solely based on agar isolation techniques are often biased since fast growing fungi tend to overgrow slow growing species. Screening soil and root samples by high-throughput sequencing using different NGS platforms would give more information of other potential pathogens contributing to the disease development (Lindahl *et al*., 2013). When we have the complete picture of all pathogens involved in the disease, an accumulated value of individual qPCR results might be linked to disease severity and used in a disease risk assessment. An example of this is shown in Figure 16, where the sum of the n-fold increase of each investigated pathogen has been correlated to DSI at site 1 ($p<0.0001$). The lowest positive value of each pathogen was used as reference. At site 2, no significant relationship was found, indicating the presence of other soil-borne pathogens. Improved relationships would probably be achieved by including all causal pathogens and to use weighted qPCR data to increase the effect of fungi considered as particularly strong pathogens and decrease the effect of weak pathogens.

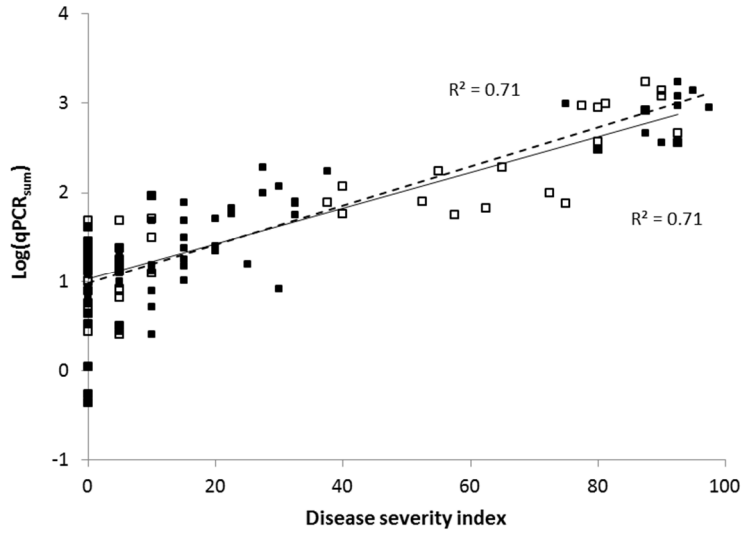

Figure 16. Relationship between qPCR results (n=69) and disease severity indices according to the visual grading of external (□, solid line) and internal (■, dashed line) root rot symptoms at field experimental site 1 (Götene). The PCR results are expressed as the logarithm of the sum of the n-fold increase compared to the lowest observed value (>0) for each pathogen (target copies of each pathogen/target copies of plant reference x 10^6). Regression analysis demonstrated significant linear relationships of y=1.028+0.020x (R^2=0.71, p<0.0001) and y=0.985+0.022x (R^2=0.71, p<0.0001) for external and internal index, respectively.

Reliable qPCR analyses of red clover root rot pathogens could be valuable instruments to assess host susceptibility in future resistance breeding. When less susceptible varieties of red clover are available, an improved disease risk assessment could aid farmers in their choice of cultivar either by qPCR analysis of roots to assess the risk in the next crop rotation or by further developing the assays for soil analysis to be able to evaluate the potential risk of disease prior to sowing. qPCR analysis might also serve as a decision support for harvest intensity.

4 Conclusions

- Specific and sensitive real-time PCR assays for quantification of pathogen DNA directly in soil samples were successfully developed for *Plasmodiophora brassicae* and *Aphanomyces cochlioides*.
- Cultivation guidelines, based on broad disease risk categories, can be established using a diagnostic qPCR assay combined with results from greenhouse bioassays and field trials. For clubroot, it was possible to identify soil samples with *P. brassicae* resting spore levels below the generally accepted threshold for symptom development (1000 resting spores/g soil). For sugar beet root rot, the newly developed qPCR assay can be used to identify high-risk fields.
- *P. brassicae* was shown to have a patchy distribution in the field and soil sampling can therefore have a major effect on the result of a field screening. The strategy of soil sampling should be chosen carefully depending on the purpose of the investigation. For a general screening of a larger area, collecting several sub-samples along a 'W' transect is suggested.
- Clay and sand content influence the LOD of the qPCR assay for *A. cochlioides*, probably by affecting the efficiency of the DNA extraction. High clay content is correlated with low LOD and vice versa.
- Measurement uncertainties of diagnostic qPCR methods for detection of plant pathogens in soil samples are high due to the complexity of soil samples and multi-step DNA extraction and purification procedures needed to efficiently lyse cells and obtain DNA of sufficient purity.
- Real-time PCR was successfully used to determine the disease incidence of *Sclerotinia sclerotiorum* in petal and leaf samples and to quantify the level of fungal DNA in air samples.
- Determining the presence of *S. sclerotiorum* on spring oilseed rape petals is not useful as a tool in stem rot risk assessment since (i) the inoculum incidence on petals varies during flowering, (ii) there is no clear

relationship between petal infection and stem rot incidence and (iii) spore release and flowering are not always synchronized. Real-time PCR detection of the incidence of *S. sclerotiorum* DNA on leaves revealing the field-borne inoculum and qPCR quantification of the airborne inoculum are more reliable tools for predicting the potential risk of disease.

- Sclerotinia stem rot can be a serious problem even though ascospore release and flowering do not coincide.
- *Fusarium avenaceum*, *Phoma* spp. and *Cylindrocarpon destructans* can be detected in red clover roots early in the seeding year using qPCR and the levels of pathogen DNA generally increase during the following years.
- *Fusarium culmorum* is likely a weak pathogen of red clover and does not seem to contribute to disease symptoms under the conditions in the field trials of the present study.
- There are only minor differences in disease susceptibility between red clover cultivars commonly used in Sweden and none of the cultivars are resistant to red clover root rot.
- Results from two field experiments indicate that the disease development of red clover root rot and the increase in pathogen DNA are related to weather conditions. It is likely that cold winters without snow cover cause more damage to the roots and consequently more severe disease progression.

5 Future perspectives

Future research includes the following ideas, possibilities and goals:

- Improve the clubroot diagnostic service by using the existing qPCR assay as a first screening followed by pathotype/race testing. The recently sequenced *P. brassicae* genome (Schwelm *et al.*, 2015) will facilitate the development of pathotype/race specific primers.

- Further investigations on why the qPCR assay developed in the present study cannot detect *A. cochlioides* in some naturally infested soils are needed. This could be addressed by (i) resampling the false negative soil samples, (ii) repeating the bioassay and saving roots for confirmation analysis using classical isolation and (iii) performing qPCR analysis on roots to reveal if *A. cochlioides* is the causal pathogen of the visible symptoms. In addition, screening the infected roots for other microorganisms, using e.g. NGS techniques, can reveal additional plant pathogens causing similar symptoms or secondary infections.

- Improve the limit of detection of the *A. cochlioides* qPCR assay, possibly by including a pre-enrichment step before the DNA-extraction.

- Reduce the cost of the DNA-based soil tests to make the analysis service more affordable for farmers. This could be achieved by offering a complete testing service including several important soil-borne plant pathogens. The cost per pathogen would decrease significantly if one single DNA extraction per soil sample was performed followed by several qPCR detections. Other interesting candidates to be included in this service are e.g. the soil-borne pathogens causing Verticillium wilt, pea root rot, take-all in wheat, soil-borne diseases in potatoes and damping off in various crops. Since the soil test rely on expensive commercial kits, the cost could also be

reduced by developing an in-house DNA extraction method or an automated DNA extraction procedure.

- Validate a disease forecasting model for Sclerotinia stem rot based on leaf and/or air analysis by creating a link between real-time PCR results and climatic conditions favoring the disease development.

- Screen red clover root samples and/or soil samples to find other potential pathogens contributing to the red clover root rot disease symptoms. NGS techniques have made such screening much more accessible and cost-effective.

- Identify which of the red clover root rot pathogens that are the most severe and determine relative levels of each pathogen contributing to the disease in order to develop threshold values. Since some of the pathogens found in infected red clover roots are considered as weak pathogens, it is likely that they only cause secondary infections.

- Today, Swedish breeders use different Fusarium species to breed for red clover root rot resistance. These breeding programs would probably benefit from including other fungal red clover pathogens, e.g. *C. destructans* and *Phoma* spp.

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