DEVELOPMENT OF A REAL TIME IMMUNO-PCR ASSAY FOR THE DETECTION OF PEA ROOT ROT CAUSAL AGENT, *Aphanomyces euteiches*

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Bachelor of Science in Agriculture, **Tribhuvan University (Nepal), 2014**

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DEDICATION

This thesis is dedicated to my supervisor late Dr. Claudia Sheedy. Thank you for being an integral part of my life as a mentor, providing me the invaluable guidance, support and encouragement throughout my master's journey. I will terribly miss you Claudia, your contagious smile and your positive attitude.

ABSTRACT

Effective measures to detect *Aphanomyces euteiches*, one of the devastating pathogen of pea, are lacking. An indirect ELISA and a real time immuno-PCR (RT-iPCR) assay for the timely and sensitive detection of *A. euteiches* were developed using antiserum specific to oospores of *A. euteiches* isolates. RT-iPCR was 100 times more sensitive over the linear working range than indirect ELISA. To assess the performance of the RT-iPCR assay, non-infested soils representing a range of soil textures were collected from Alberta fields. An extraction protocol was developed by spiking the soils with *A. euteiches* oospores and the RT-iPCR assay was used to quantify oospores in the soil extracts. The method performed well at the concentration 100 oospores/g and above. A rolled towel bioassay and root PCR were performed on infested field samples which showed a good agreement with our RT-iPCR results. Thus, RT-iPCR may be an invaluable tool for field diagnostics.

ACKNOWLEDGEMENTS

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A. euteiches	*Aphanomyces euteiches*
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
Ae	*Aphanomyces euteiches*
ANOVA	Analysis of variance
BC	Background control
CA	California
CAD	Canadian dollar
Ct	Cycle threshold
CV	Coefficient of variability
DAOMC	Canadian collection of fungal cultures
DAS-ELISA	Double antibody sandwich ELISA
DNA	Deoxyribonucleic acid
DSI	Disease severity index
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FWD	Forward
g	Gram
GAM	Gallic acid medium
GAR	Goat anti-rabbit
HIV	Human immuno deficiency virus
HRPO	Horseradish peroxidase
HSGS	High specific gravity separation
IC_{50}	The half maximal inhibitory concentration
IP	Inoculum potential
iPCR	Immuno-PCR
LCB	Low cross buffer
LOD	Limit of detection
Log_{10}	Logarithmic base 10
MBV	Metalaxyl benomyl vancocin
Min	Minute
Ml	Milliliter
MPN	Most probable number
MPVM	Mircetich's pimaricin-vancomycin
NC	Negative control
ON	Ontario
OVA	Oval albumin
PAB	Polyromantic hydrocarbon

pAbs	Polyclonal antibody
PAHs	Polyaromatic hydrocarbons
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline + Tween 20
PCB	Polychlorinatedbiphenyl
PCNB	Pentachloronitrobenzene
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
Penn G	Penicillin G
qPCR	Quantitative PCR
REV	Reverse
RH	Relative humidity
RNA	Ribonucleic acid
RT	Rolled towel
RT-iPCR	Real time immuno-PCR
SAS	Statistical analysis
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SI	Soil indexing
T-L	Thunder-link
US	United States
x g	Relative centrifugal force (RCF)
µm	Micrometer

CHAPTER 1 : INTRODUCTION

1.1.   Oomycete plant pathogens

Oomycetes are eukaryotic microorganisms that exhibit morphological characteristics and lifecycles similar to those of fungi (Sapkota and Nicolaisen, 2015). Recent phylogenetic and molecular studies have unearthed many new oomycetes which are distinct from true fungi (McGowan and Fitzpatrick, 2017). Some of their distinctive features are the presence of cellulose and beta glucan instead of the chitin found in true fungi, non-septate hyphae and diploid life cycle (Raffaele and Kamoun, 2012). Among oomycetes, the genera *Pythium, Phytophthora* and *Aphanomyces* are known to threaten major agricultural crops like potato, tomato, alfalfa and pulses (Gaulin *et al.*, 2007), including *Phytopthora infestans*, the potato blight pathogen responsible for the Irish famine (Kamoun, 2003). Regardless of their unique phylogenetic affinities as eukaryotes and huge economic impacts, those pathogenic oomycetes were historically overlooked due to their filamentous growth habit and the lack of understanding with regards to their pathogenic interactions at the molecular level (Kamoun, 2003). More recently, significant research has been conducted with the genus *Aphanomyces*, which includes 35 to 40 described species responsible for causing important diseases in animals and plants including freshwater crayfish (Diéguez-Uribeondo *et al.*, 2009). More relevant to this thesis, the species *Aphanomyces euteiches* accounts for root rot in the Fabaceae (pea and bean) family (Diéguez-Uribeondo *et al.*, 2009).

1.2.   Pea root rot

Root rot in pea can be caused by multiple pathogens including *A. euteiches*, *Pythium* spp, *Fusarium* spp and *Rhizoctonia solani* (Gossen *et al.*, 2016). All of these

pathogens have been isolated from infected pea roots (Gossen *et al.*, 2016) and are known to cause damping off, seedling blight, as well as a reduction in a number of parameters: nitrogen fixation, root vigor, root establishment and germination (Gossen *et al.*, 2016). Root rot caused by *Pythium*, *Fusarium* spp. and *A. euteiches* has currently no effective control measures, thus leading to devastating losses in field pea (Chatterton *et al.*, 2019).

1.2.1. *Aphanomyces euteiches*

Aphanomyces euteiches is a strict soil-borne pathogen which has sexual and asexual stages in its life cycle. As a sexual part of life cycle, the pathogen can survive in soil for years by forming thick-walled oospores (15-25 µm in diameter). Upon favorable conditions: presence of host, soil saturation and temperature above 24°C (Papavizas and Ayers, 1974), dormant oospores form germ tubes to produce zoosporangia which produce zoospores. The zoospores are attracted to root exudates released from the host and swim short distance to find host roots (Judelson and Blanco, 2005). The motile zoospores encyst on the root forming an infection peg to penetrate the host. Inside the host tissue, mycelium is formed and grows throughout the cortex of entire root system At a later stage, when the nutrients are used up, mycelium differentiates to form oogonia and antheridia, the antheridia fertilize the oogonia to form oospores which in turn become dormant and remain in the soil (Gaulin *et al.*, 2007; Papavizas and Ayers, 1974).

Aphanomyces euteiches can infect pea plants at any growth stage but usually it infects the plant when they are around 6 weeks old favoured by soil moisture and temperature. Initial symptoms are characterized by softened, water-soaked and slightly discoloured lesions in the cortical region of the roots (Gaulin *et al.*, 2007). As the disease progresses, the tissue becomes soft, the epicotyl area darkens and finally collapses

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(Papavizas and Ayers, 1974). At a later stage, the pathogen is difficult to identify merely by looking at symptoms as root rot in pea can be caused by multiple pathogens including *Pythium*, *Fusarium* spp, *Rhizoctonia solani*, as well as *A. euteiches*. Besides pea, *A. euteiches* can damage several other legume crops such as alfalfa, faba bean, vetch, lentil, and red clover (Gaulin *et al.*, 2007).

1.3.   Origin, history and distribution

In 1924, Jones and Linford surveyed fields to determine the most important pea diseases of Wisconsin (United States) and found that pea root rot caused by *A. euteiches* was significant. In 1925, (Jones and Drechsler) described *A. euteiches* for the first time. They found that *A. euteiches'* thick-walled oospores could survive in soil for several years and hypothesized that *A. euteiches* was likely present in the United States (US) before they first reported its presence in 1925. Following its identification, the presence of *A. euteiches* was indeed discovered in several parts of the US, and more than 80% of yield losses in pea were attributed to root rot caused by *A. euteiches* (Papavizas and Ayers, 1974; Holub and Grau, 1990). Since that time, *A. euteiches* has been reported in Europe, Australia, Japan and Canada (Kraft and Pfleger, 2001), where it is known to cause heavy losses in pea fields (Gaulin *et al.*, 2007).

In Canada, *A. euteiches* was first reported in Ontario and Quebec (Basu *et al.*, 1973; Slusarenko, 2004) but was not considered as problematic in pea as other pathogens such as *Fusarium* spp, *Ascochyta pisi, Erysiphe pisi, Pernosporae viciae* and *Uromyces pisi*. In Alberta, *A. euteiches* was first reported in 2013 as part of routine field surveys (Chatterton *et al.*, 2015b). Pea root rot was prevalent in Alberta in 2014 due to prolonged cool and wet spring conditions conducive to the establishment of *A. euteiches,* and was

still observed throughout Alberta under drier conditions in 2015 (Chatterton *et al.*, 2019). Less than five years after being first reported in Alberta this pathogen was widespread across Western Canada provinces and complete crop losses were observed in some fields in Saskatchewan and Alberta (Chatterton *et al.*, 2019). Given the steady increase in pea production in western Canada and the quickly expanding range of *A. euteiches*, pea root rot is now considered a serious threat to pea production.

1.4.   Status and importance of field pea in Canada

Cultivation of field pea in Canada began in the 1920s (Wu *et al.*, 2018). Pea production has been growing steadily since 1990 due to pea nutritional benefits for both human and animal consumption, the crop's climatic versatility and its export potential to Asian markets (Slusarenko, 2004). In 2014, pea production in Canada peaked and in 2018, Canada became the world's second largest producer of pea (3.9 million metric tons representing 21% of the worldwide pea production (Agriculture and Agri-Food Canada, 2018). Canada is also the world's largest exporter of pea, with a total export value of $1.2 billion CAD in 2016 (Agriculture and Agri-Food Canada, 2018).

Being a pulse, field pea is an invaluable rotational crop that can help break disease and insect cycles in cereals, resulting in better yields and increased profitability (Miller *et al.*, 2002). Field pea can fix atmospheric nitrogen through a symbiotic association with bacteria (*Rhizobium* spp), thereby reducing fertilization costs for the crop itself, and subsequent crops because of residual soil nitrogen (Miller *et al.*, 2002; Gan *et al.*, 2015).

Based on current field production (4.2 million tonnes) and price (CAD $265/tonne), 10% yield loss in field pea can result in a total economic loss estimated at

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millions per annum (Agriculture and Agri-Food Canada, 2020). In addition, should

disease reduce pea acreage, farm expenditures for mineral nitrogen are predicted to

increase due to the decline in nitrogen fixation capacity and overall diversity and

sustainability, potentially leading to further degradation of the soil's physical, chemical

and biological properties (Gan *et al.*, 2015).

1.5.   Detection of pea root rot pathogens in soil

The accurate and sensitive detection of a pathogen is fundamental to understand

its epidemiology and to implement effective control measures (Wakeham and Pettitt,

2017). In the case of pea root rot caused by *A. euteiches*, where the primary inoculum

(oospores) of the pathogen survives in soil for years, timely measures to prevent

epidemics can only be effective if the number of oospores is accurately quantified. The

early, accurate and specific detection of the disease would help in understanding the

pathogen threshold to start early infection process, details in life cycle, stages, host

pathogen interaction can be known, distribution of the pathogen can be monitored before

planting season which would assist in establishing the strategies to when and where, to

escape, provide specific treatments and particular control measures (Wakeham and

Pettitt, 2017; Sanzani *et al.*, 2014). While scouting for disease symptoms is a routine

practice to assess the presence of plant pathogens in grower's fields, it is only qualitative

(presence/absence). Similarly, a number of methods including bioassays and a range of

selective media have been used qualitatively to assess the presence of pathogens in soils.

More recently, with the advent of molecular techniques, quantitative detection methods

such as quantitative PCR (qPCR) have been developed. Methods to specifically quantify

or determine the presence of *A. euteiches* in soil are discussed briefly below.

1.6. Qualitative methods

Qualitative methods are those methods that provide a non-quantitative assessment of the presence of *A. euteiches*. Usually simpler and less technically demanding than quantitative methods, they also tend to take more time and may not provide an accurate assessment of the presence of fungal propagules in a field. The qualitative methods reviewed here include greenhouse bioassays and selective media methods.

1.6.1. Wet sieving/baiting assay

Kraft (1990) developed a wet sieving and baiting assay to determine the inoculum level of *A. euteiches* in soils from Northern Idaho, US. This method involves the separation of soil's organic and mineral fractions by sieving. The organic fraction is then evaluated for the presence of disease by inoculating the pea seedlings root with organic debris followed by incubation at optimal growth conditions (21°Cwith 16-hour daylight) in a growth chamber. Wet sieving and baiting assay provides results similar to those obtained using other greenhouse methods (SI, MPN), but is simpler (Kraft, 1990).

1.6.2. Greenhouse bioassays

Greenhouse bioassays are one of the most commonly used methods to determine soil borne pathogens presence and disease severity (Malvick, 1994). Seeds of a susceptible cultivar are grown in soil to be tested to determine the fungal pathogen's inoculum presence and potential (Malvick, 1994; Moussart *et al.*, 2013). The Inoculum Potential (IP) is defined as the optimal pathogen density to initiate and promote root infection considering pathogen infectivity and soil factors such as soil texture, structure and soil saturation (Gossen *et al.*, 2016). Greenhouse bioassays include a range of similar

methods, several of which have been used to detect *A. euteiches* in soil including soil indexing, wet sieving/baiting, rolled towel bioassay and most probable number assay.

1.6.2.1.   Soil indexing method

Soil indexing (SI) was developed by Reiling *et al.* (1960) to evaluate the potential of naturally infested field where peas were known to be susceptible to root rot caused by *Aphanomyces euteiches* to induce root rot. SI is mostly used as a recommendation tool based on IP i.e. fields with elevated IP/soil index are not ideal to grow pea plants. To detect *A. euteiches*, soil from infested fields is collected and pea seeds susceptible to *A. euteiches* are sown in the greenhouse. Although this technically simple method has been utilized as part of grower advisory tools in the past, it requires a large volume of soil and green house space, it is not accurate or specific.

1.6.2.2.   Most Probable Number (MPN) bioassay

The Most Probable Number (MPN) bioassay determines the inoculum density of soil-borne pathogens. MPN involves the serial dilution of a likely infested soil with non-infested soil in a cavity tray and bait plants to determine the presence of a pathogen (Adams and Welham, 1995). The proportion of bait plants infected at the various dilutions is used to estimate the concentration of infectious propagules in the soil. Many fungal as well as oomycete pathogens such as *Phytophthora* spp*, Fusarium* spp*,* and *A. euteiches* have been assessed using MPN (Adams and Welham, 1995; Pfender *et al.*, 1981). MPN bioassays provide an estimate of the infective inoculum density rather than total population density in soil (Williams-Woodward *et al.*, 1998; Pfender *et al.*, 1981; Chan and Close, 2012). Although MPN bioassays do not precisely quantify propagule density, lower requirements for greenhouse space and soil make them easier than the SI

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bioassays. However, MPN assays are technically demanding, laborious and provide only an approximation of inoculum density in soil (Malvick, 1994; Pfender *et al.*, 1981).

1.6.2.3.   Rolled towel bioassay

The rolled towel (RT) bioassay was developed by Malvick (1994), based on a protocol previously developed by Mitchell *et al.* (1969). Five-day old pea seedlings and infected soil are placed in a wet paper towel, rolled into a column and placed in a growth chamber (22°C) for 21 days. Following incubation, plants are examined for the presence of typical symptoms of *A. euteiches* infection, primarily the honey-coloured discoloration of the roots. The presence of *A. euteiches* is then confirmed by culturing symptomatic roots in a semi-selective medium containing the antibiotic rifampicin. Williams-Woodward *et al.* (1998) developed a modified RT bioassay based on those published by Kraft (1990) and Malvick (1994) to determine the IP of *A. euteiches* in soil. Modifications included use of soil artificially infested with known concentrations of oospores, placement of the soil along the seedling roots (above or below root tip), placement of seedlings inside the plastic bags to reduce cross contamination and watering at the beginning with a pentachloronitrobenzene solution to reduce seedling contamination. These modifications reduced contamination and revealed that the taproot region just below the seed was more susceptible to *A. euteiches* infection than the root tip. In addition, the finding that increased concentrations of oospores caused a lower onset of disease led in suggesting that oospore clustering might affect disease onset, but failed to clarity how.

To determine the most effective greenhouse bioassay for the detection of oomycetes, Malvick (1994) compared three different bioassays (SI, RT and MPN), by

determining the IP of *A. euteiches* in artificially infested soils. The study revealed a high correlation between the number of oospores added and the soil IP for both MPN (R^2 =0.85) and RT (R^2 =0.99). However, inconsistency was observed for inter as well as intra-assay replicates for the estimated IP due to the uneven dispersion of the oospores in the soil as oospores clustered where the plant debris were dense. Moreover, IP estimates derived from each method did not necessarily correspond. However, a direct comparison was difficult as each method used different approaches to estimate the soil IP. The authors concluded that more accurate and sensitive methods to determine the IP of *A. euteiches* in soils were needed (Malvick, 1994).

1.6.3. Media

Qualitative studies of soil-borne fungal pathogens have mainly relied on the traditional use of culture media into which soil suspensions are plated, allowing fungi to grow and sporulate (Wakeham and Pettitt, 2017). Selective media are designed to promote the pathogen of interest's growth while inhibiting that of unwanted microorganisms (Tsao, 1970). The cultured pathogen is identified based on its morphological features (Schroeder *et al.*, 2006). Using selective media to isolate and grow pathogens is labour intensive, time-consuming and the identification relies mostly on the appearance of specific morphological characteristics of the culture to a given pathogen (Wakeham and Pettitt, 2017). In addition, the isolation of fungal pathogens on selective media and their microscopic identification based on morphology and taxonomy require expert knowledge and growing specific fungal pathogens is limited to those pathogens which can be routinely cultured (Atkins and Clark, 2004). Nonetheless, these

methods can be used as a confirmatory test for the presence of pathogens (Wakeham and Pettitt, 2017).

In the case of *A. euteiches,* the inability of the pathogen to germinate consistently in culture media made it impossible to identify it by conventional media techniques (Williams-Woodward *et al.*, 1998). The fact that pea root rot may be caused by multiple pathogens (*Fusarium* spp, *Pythium* spp, and common soil saprophytes) that grow faster than *A. euteiches* has also made the isolation of *A. euteiches* difficult (Chatterton *et al.*, 2019; Sanzani *et al.*, 2014; O'Brien *et al.*, 2009; Martin *et al.*, 2012).

Various pathogens including oomycetes such as *Pythium* and *Phytophthora capsici* (Papavizas *et al.*, 1981) have been isolated from soil using selective media (Tsao, 1970) such as Mircetich's pimaricin-vancomycin (MPV) and gallic acid (GA) (Tsao, 1970). These media are specifically designed to isolate and grow only a few pathogens. For example, the selective media MBV specifically designed to isolate and grow *Aphanomyces*, includes metalaxyl to inhibit other oomycetes, benomyl to inhibit *Fusarium*, vancomycin to inhibit bacteria and at times amphotericin B to inhibit *Rhizopus* or *Alternaria* if present (Pfender *et al.*, 1984). The antimicrobials mentioned above inhibited the growth of *Fusarium*, *Rhizoctonia* and *Phytophthora,* while decreasing the growth rate of *Pythium* spp. to that below *Aphanomyces*'. MBV medium was effective for the recovery of *Aphanomyces* actively growing in plant (pea, bean, alfalfa) tissues however, a lot of expertise is needed to isolate and timing of plant collection is important. MBV medium was not effective for the isolation of *Aphanomyces* oospores from naturally or artificially infested soil (Malvick, 1994).

1.7. Quantitative methods

Challenges encountered with traditional (media) methods were in part eliminated once molecular methods based on the polymerase chain reaction (PCR) became available. PCR is fast, sensitive, specific and is able to detect pathogens that are impossible to grow via cultural methods (Mirmajlessi *et al.*, 2015). Molecular methods based on PCR are very sensitive and have become a routine laboratory technique for the detection of plant pathogens (Wakeham and Pettitt, 2017). PCR methods were further enhanced with real-time or quantitative PCR (qPCR) (Sanzani *et al.*, 2014). Two quantitative detection methods are reviewed below: quantitative PCR (qPCR) and immuno-PCR.

1.7.1. Quantitative PCR

Real-time PCR (also referred to as quantitative PCR, qPCR) is one of the most sensitive techniques for the accurate quantification of deoxyribonucleic acid (DNA) and the most widely used for the quantification of plant pathogen DNA (Mirmajlessi *et al.*, 2015). In qPCR, a fluorescent probe such as SYBR Green, or a sequence-specific fluorophore probe such as Taqman, is added to the amplification reaction mixture. These probes bind to a specific sequence and emit fluorescence upon the generation of PCR products (Gofflot *et al.*, 2005). The major advantage of PCR is that it can amplify DNA over a broad range of concentrations with high sensitivity, thereby eliminating several challenges previously encountered with traditional cultural methods (Mirmajlessi *et al.*, 2015). With the advent of qPCR, quantification happens in real time and therefore eliminates the need for electrophoresis gels and UV visualization following conventional PCR.

For fungi and other organisms present in soil, DNA extraction kits such as the Power Soil® DNA Isolation kit, the Soil Master™ DNA Extraction kit, and the Fast DNA® SPIN kit are commercially available. Following extraction and purification, DNA can be amplified by qPCR and quantified. Quantitative PCR has been used to detect oomycetes in soil including *Phytopthora* spp, *Pythium* spp (Cooke *et al.*, 2007; Wakeham and Pettitt, 2017; Sanzani *et al.*, 2014; Schroeder *et al.*, 2013) and *A. euteiches* (Gangneux *et al.*, 2014; Sauvage *et al.*, 2007).

The molecular analysis of environmental samples requires the extraction and purification of DNA prior to PCR. When working with environmental matrices such as soil, various inhibitors are naturally present and may be co-extracted along with nucleic acids. Co-extractants like humic and fulvic acids, pesticides and organics may interact with the template DNA resulting in improper gene amplification, lower accuracy, false-negative results and lower sensitivity (Schrader *et al.*, 2012; Opel *et al.*, 2010; Wakeham and Pettitt, 2017). PCR interference by inhibitors as well as nucleic acid shearing during extraction are ongoing issues that may affect PCR efficiency (Lebuhn *et al.*, 2004). PCR techniques are currently in practice to detect *A. euteiches* from root samples in the Canadian prairies (Chatterton *et al.*, 2019). Soil testing labs in Alberta, Saskatchewan and Manitoba utilize qPCR as a standard method to detect the pathogen's DNA from soil samples directly from fields (S. Chatterton, personal communication). However, both methods (PCR and qPCR) have some ongoing limitations. Detection based on PCR methods are rather qualitative than quantitative where samples are rated as positive or negative (Chatterton *et al.*, 2019). Inconsistency in amplification for some soil extracts, decreased sensitivity due to the

presence of humic and fulvic acids leading to decreased sensitivity in qPCR are some ongoing constraints (S. Chatterton, personal communication).

1.7.2. Antibody-based assays

Antibody-based assays rely on the use of antibodies specific for a given antigen to detect and quantify the molecule or entity (fungal spore) of interest. These assays are simple to perform, reliable and versatile. Immunoassays are also fairly inexpensive compared to other methods and have been used extensively to detect fungal pathogens (Martinelli *et al.*, 2015). Several of these immunoassays and their reagents are commercially available. While the standard immunoassay has existed since the 1960s, a novel type of immunoassay, referred to as the real-time immuno-PCR assay, has been developed more recently (Ryazantsev *et al.*, 2016). These assays are reviewed in the section below.

1.7.2.1. Enzyme Linked ImmunoSorbent Assay

The immunoassay concept was initially developed by Yalow and Berson for the detection of insulin in 1959. Since then, immunoassays have become a routine quantification tool (Lind and Kubista, 2005). The core principle in immunoassays is the specific antigen-antibody interaction. The specificity of antibodies towards particular antigens and their epitopes allows for the highly specific detection of a large range of organisms (Lind and Kubista, 2005). Various antibody formats (polyclonal, monoclonal and recombinant antibodies) have been developed and utilized (Alvarez, 2004; Martinelli *et al.*, 2015). While there are numerous immunoassay formats as well, they typically all include immobilization of the antigen or the antibody on a solid phase, and detection via colorimetric or fluorescence means.

The Enzyme Linked ImmunoSorbent Assay (ELISA) is one of the most widely used immunoassay formats for the detection of plant viruses, bacterial and fungal pathogens worldwide (Martinelli *et al.*, 2015). The ELISA was developed by Engvall and Perlmann in 1971, who included an enzyme to label antibodies (Engvall and Perlmann, 1971). The enzyme gives rise to a colorimetric reaction upon addition of its substrate, indicating the presence of the antigen (Voller *et al.*, 1978). One of the greatest advantages of ELISA is its quantitative nature, sensitivity and replicability (Dewey *et al.*, 1997). While several formats of ELISA exist, the indirect ELISA (Figure. 1.1) and the double-antibody sandwich (DAS) ELISA have been used the most for the detection of plant pathogens (Yuen *et al.*, 1998). In indirect ELISA, the antigen is coated in a plate followed by addition of antigen specific antibody whereas in (DAS) ELISA, the capture antibody which binds with the antigen is added first in a plate followed by the addition of antigen afterwards. For both assay formats, the signal is detected using a labelled enzyme.

ELISAs have been used extensively to quantify oomycetes pathogens. Among others, an ELISA was developed from the pathogen mycelia and used for the detection of root disease caused by *Aphanomyces cochlioides* in the infected sugar beet seedling in the greenhouse (Weiland and Shelver, 2004). *Phytophthora* has been detected using ELISA and the results were compatible with other PCR-based methods (Martin *et al.*, 2012). ELISAs have also been used for the detection of a number of plant pathogenic oomycetes in soils including *Phytophthora* spp where the pathogen was detected as *Phytophthora* antigen units (PAU). The (PAU) approximately equivalent to one oospore calculated by a standard curve developed from the dilutions of mycelial extracts (Miller *et al.*, 1997).

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Some other pathogens tested by ELISA includes *Septoria tritci* (Mittermeier *et al.*, 1990), *Plasmodiophora sojae* (Miller *et al.*, 1997) and *Pythium viole* (Wakeham and White, 1996). PCR-ELISA, where the labelled amplicons are captured into a microtitre plate and detected by immunoassay, and qPCR were developed for the quantification of *Fusarium* species causing dry rot (*F. avenaceum, F. coeruleum, F. culmorum* and *F. sulphureum).* Both assays (PCR-ELISA and qPCR) were similar in detecting the pathogens (Cullen *et al.*, 2005). Despite ELISA's relatively high sensitivity towards soil-borne pathogens (for example, *Plasmodiophora brassicae* could be detected at a concentration of 100 spores/g of soil (Wakeham and White, 1996) and *Phytopthora nicotinae* (1.7-2.3*10^2 zoospores/ml)) (Ali-Shtayeh *et al.*, 1991), some antigens that are present at very low concentrations can be missed (He *et al.*, 2011). ELISA's sensitivity can be further increased by combining its specificity with the amplification power of PCR, a method referred to as immuno-PCR.
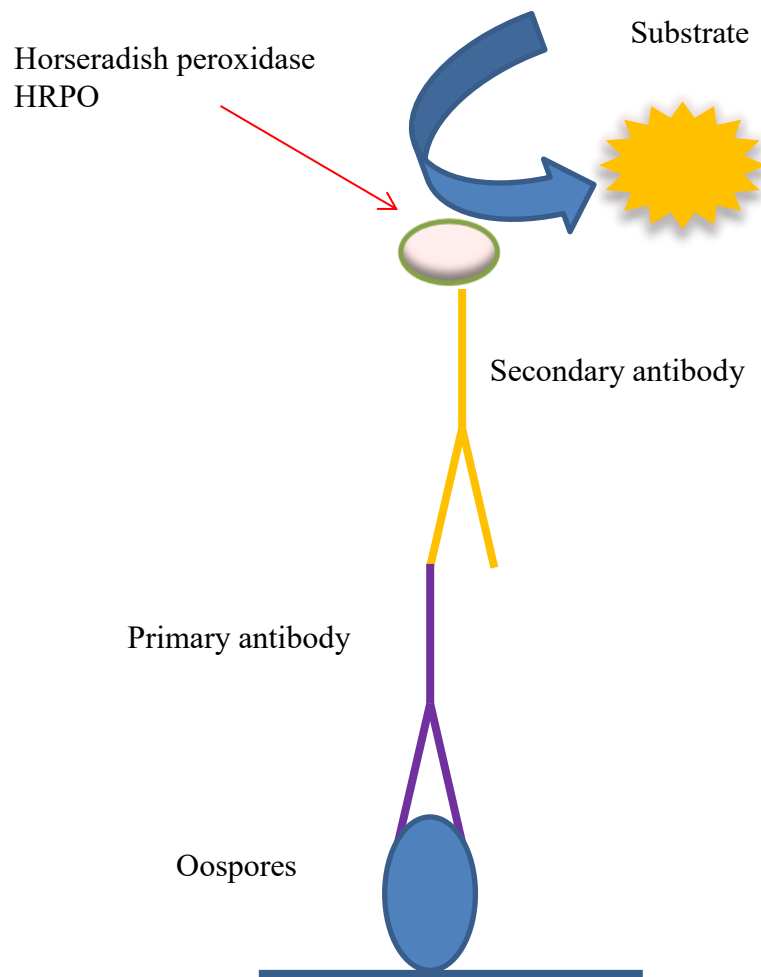
Figure 1. 1. Schematic representation of an indirect ELISA adapted from (Gaudet et al., 2015). Entity to be quantified (ex. *A. euteiches* oospores) is adsorbed onto the microtitre plate wells surface. The remaining active sites in the wells are blocked with a blocking agent such as milk to prevent non-specific binding. The primary antibody raised against the entity (ex. oospores) is added and binds to its antigenic epitope(s) followed by the addition of a secondary antibody conjugated to an enzyme (HRPO). Upon addition of the enzyme substrate, a colorimetric reaction arises and absorbance is measured by spectrophotometry.

1.7.2.2.   Immuno-PCR

Immuno-PCR (iPCR) was first developed by Sano, Smith and Cantor in 1992. In iPCR, the enzyme used in ELISA is replaced with DNA and the DNA is amplified by PCR following antibody binding. Because of its multistage signal amplification, iPCR can reach sensitivity up to 1000-10,000 times that of conventional ELISAs using the same antibodies (Niemeyer *et al.*, 2005).

1.7.2.3.   Real-Time Immuno-PCR

Real time-immuno-PCR (RT-iPCR) has been used to detect a wide range of antigens including viruses such as HIV (P24 antigen) with sensitivity up to 1000 times greater than that of ELISA and 25 times that of qPCR (Malou and Raoult, 2011). Similarly, a norovirus in food was detected with a sensitivity 1000 times greater than its corresponding ELISA and 10 times greater than qPCR (Malou and Raoult, 2011). Haptens such as estradiol in water (Gaudet *et al.*, 2015), shiga toxin (He *et al.*, 2011), polyaromatic hydrocarbons (PAHs) (Meng *et al.*, 2016) and 3,3′,4,4′-tetrachlorobiphenyl (PCB77) (Chen and Zhuang, 2011) were also quantified by RT-iPCR. These results demonstrate the high sensitivity and versatility of RT-iPCR assays for the detection of environmental contaminants.

DNA

PCR reaction

Secondary antibody
(GAR-TL-Conjugation)

Primary antibody/rabbit sera
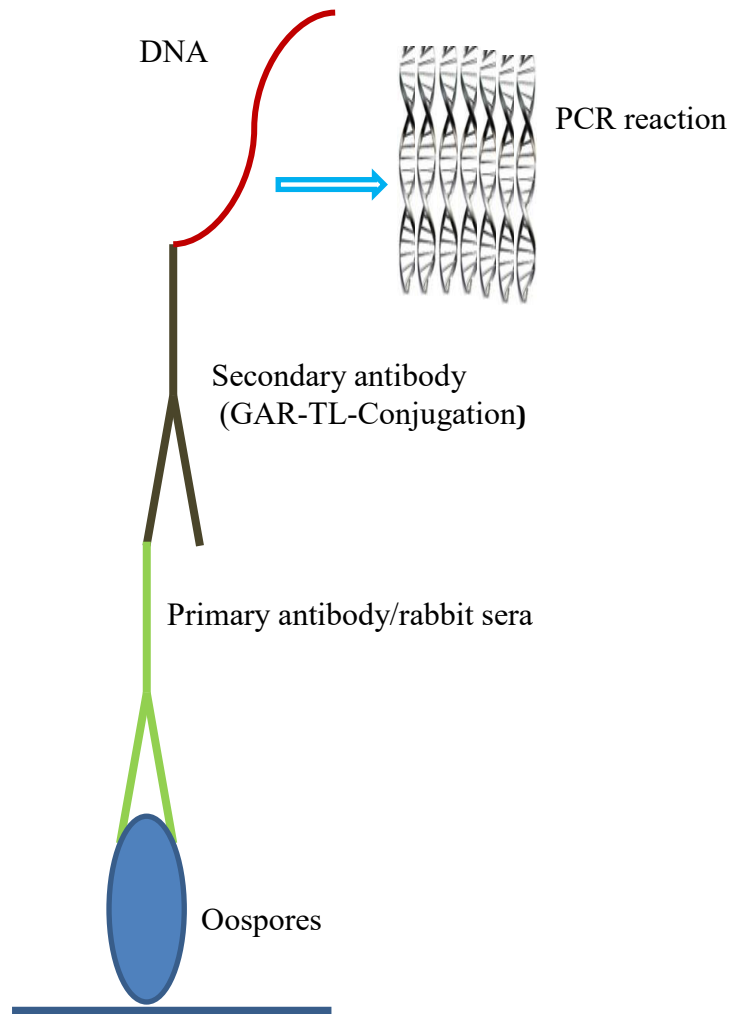
Oospores

Figure 1. 2. Schematic representation of RT-iPCR (adapted from Gaudet et al. (2015).
Aphanomyces euteiches oospores are added to PCR plate wells. The
remaining reactive sites in the wells are blocked using OVA to prevent any
non-specific binding. The antiserum (Primary antibody) is added, followed
by a secondary antibody (Goat anti-Rabbit) conjugated to template DNA by
Thunder Link conjugation. The template DNA is amplified by PCR.

While demonstrating extremely high sensitivity, the RT-iPCR also presents

several challenges including a complex multistep protocol, the need for customized

reagents, and the presence of high background noise due to matrix effects (interferences

arising from co-extractants) in some cases. Background noise can be minimized by

optimizing the RT-iPCR assay protocol and selecting reagents carefully. In addition, this

issue should be partly solved as commercial ready-to-use reagents and pre-conjugated antibody-DNA complexes are made available (Ryazantsev *et al.*, 2016). RT-iPCR can be a valuable option when one wants to detect an antigen (ex. oomycetes spore) or protein directly, rather than through its DNA (Bonot *et al.*, 2014) and is particularly useful when DNA extraction results in low yields. The numerous washing steps in the RT-iPCR also help remove inhibitors that may interfere via competition and mispriming with PCR quantification (Malou and Raoult, 2011; Niemeyer *et al.*, 2005).

The summary of qualitative and quantitative methods used to detect some soil borne pathogens is presented in Table 1.1.

Table 1. 1. Methods used for the detection of soil-borne pathogens in soil samples, their quantification limit, their advantages and limitations.

Methods	Quantitation Limit	Advantages	Limitations
Qualitative			
Greenhouse bioassays	Prediction based on correlation between disease development in greenhouse and field (Reiling *et al.*, 1960)		
Soil Indexing			Tedious
			Time consuming
Most Probable Number	Estimates the infective propagules (Pfender *et al.*, 1981)	Technically simple	Only provides measure of inoculum as severe, moderate or low.
Wet Sieving	Detection based on IP levels (Kraft, 1990)		
Selective Media	Not always effective		
Quantitative			
PCR/qPCR	For *Plamodiophora brassicae*, 500 resting spores/g of soil (Wallenhammar *et al.*, 2012)	Very sensitive	Matrix effects possible DNA extraction can be limiting
	For *A. euteiches,*10 oospores/g soil (Sauvage *et al.*, 2007; Gangneux *et al.*, 2014)		
ELISA	For *Plamodiophora brassicae,* 100 spores/g soil (Wakeham and White, 1996)	Well established, simple	Oospores/spores present in lower amount cannot be detected

1.8. Microorganism spore isolation from soil prior to RT-iPCR

The extraction of oomycete materials (spores, hyphae, etc) from soil prior to their quantification is a crucial step. The extraction removes debris, impurities and soil particles that may otherwise interfere with the quantification process (Silvestri *et al.*, 2014). Irrespective of the quantification method's sensitivity, its overall performance hinges on how clean (devoid of reactive co-extractants such as organic compounds such as humic, fulvic acids) the soil extract is.

Soil borne pathogens such as *A. euteiches, P. capsici* and *Fusarium* spp can adhere to soil aggregates for many years (Raaijmakers *et al.*, 2009; Moussart *et al.*, 2009), and their strong adherence makes dissociating their resting spores from soil particles challenging. This is why typically the extraction process consists of two steps: the spores must be dissociated from soil particles first, and then isolated from soil. Silvestri *et al.* (2014) reviewed various extraction processes to isolate *Bacillus anthracis* spores from soil including disrupting soil-spore aggregates chemically (with the addition of a buffer) and physically (sieving, manual shaking, vortexing, sonication, bead beating). Similar to *A. euteiches* oospores, the bacterial spores (*Bacillus anthracis)* are extremely resilient to environmental stresses and can survive in soil for several years.

1.8.1. Chemical separation

A variety of additives such as buffer (Phosphate Buffered Saline, PBS) (Fitzpatrick *et al.*, 2010), sterile water (Dabiré *et al.*, 2001) and surfactants such as TWEEN 20 (Marston *et al.*, 2008), sucrose (Pavón *et al.*, 2008), sodium dodecyl sulfate (SDS) and Triton™ X-100 (Lombard *et al.*, 2011) have been assessed as means to

dissociate spores from soil particles. However, information on use of surfactants in spore recovery efficiency is lacking (Silvestri *et al.*, 2014).

The combination of a buffer and surfactant (10XPBS + Tween 20: 10XPBST) added to soil to form a slurry which can be processed further using physical means has led to good recoveries for *B. anthracis* (Arduino *et al.*, 2011). The soil slurry can then undergo shaking, sonication or vortexing to separate and isolate the fungal spores from the soil (Silvestri *et al.*, 2014; Da Silva *et al.*, 2011). Overall, a combination of physical and chemical disruption resulted in higher recoveries of *Bacillus anthracis* spores than chemical disruption alone (Silvestri *et al.*, 2014).

1.8.2. Physical separation

Once a homogeneous soil slurry is obtained, it can be further processed to isolate the spores of interest. Centrifugation, high specific gravity separation (HSGS), affinity capture (immunomagnetic separation) or filtration have been used to isolate spores from soil slurries (Silvestri *et al.*, 2014). For *B. anthracis*, centrifugation at high speed (4000 × g) tended to perform better than at low speed in freeing spores from the soil slurry and aided in removing contaminants (Gulledge *et al.*, 2010). Filtration (following centrifugation) through various mesh sizes (depending on the average size of spores to be isolated) can also help isolate the spores from the soil slurry.

The principles involved in separating bacterial spores from soil have been utilized for the extraction of oomycetes spores as well. Van der Gaag and Frinking (1997) extracted *Peronosporae viciae* oospores from soil with a combination of sonication, a sucrose solution (70%), TWEEN-80 (1%), centrifugation (1000 × g for 3 min) and filtration (70 µm and 20 µm sizes) where they extracted 2-21 oospores per g of naturally

infested soil. The Van der Gaag and Frinking (1997) method was modified by Fernández-Pavía *et al.* (2004) for extraction of *Phytophthora infestans* oospores in naturally infested soil: soil was supplemented with water, the slurry mixed, filtered (100 µm sieve and 15 µm nylon mesh), supplemented with 70 % sucrose, centrifuged at 2100 × g for 5 min and filtered through a 15 µm nylon mesh. The centrifugation step was repeated with 15 ml of water. While the Fernández-Pavía study confirmed oospores as the principal source of inoculum in potato fields, recovery percentage was not calculated (Lees *et al.*, 2012). Pavón *et al.* (2008) used a sieving-centrifugation procedure and qPCR to quantify *Phytophthora capsici* oospores from artificially infested soil. Recovery rates varied from 21.4 to 79.8% in soil infested with 10 to 10^5 oospores per 10 g of soil (Pavón *et al.*, 2008). In naturally infested soils, *Phytophthora capsici* oospores could not be isolated from other propagules (hyphal fragments or sporangia) that were co-extracted when tested by microscopy (Lees *et al.*, 2012). As qPCR cannot differentiate the source of DNA, this can lead to overestimation of the pathogen actually present (Lievens *et al.*, 2006).

Detection of *A. euteiches* oospores is challenging. However it is important to accurately quantify oospores of this pathogen even before planting season to be able to quantify oospores and risk to pea production. This would allow producers to know beforehand whether to grow or avoid pea or other susceptible crops. If the quantity or level of the pathogen could be monitored before planting season this would also assist in establishing the strategies to provide specific treatments and effective control management options. Therefore this research project was initiated to detect and quantify the oospores of *A. euteiches* using an immunoassay based method called real time

immuno PCR (RT-iPCR). The outcome of the result will help in development of accurate and sensitive quantification methods for oospores.

The research described in this thesis includes the development of an extraction method modified from that of Silvestri *et al.* (2014) for the isolation of *A. euteiches* oospores from soil, and the quantification of oospores using an RT-iPCR assay. Hence, our research objectives were:

1. Development of an efficient method for extraction of *A. euteiches* oospores from soil;

2. Evaluation of the potential of RT-iPCR as a sensitive and reliable method for quantification of *A. euteiches* oospores.

The overall method (soil extraction and RT-iPCR quantification) was validated with soil samples collected from clean and infested fields of Alberta and Saskatchewan to determine its applicability and reproducibility. Finally, the RT-iPCR assay was compared to a standard PCR and a qualitative greenhouse bioassay.

CHAPTER 2: DEVELOPMENT OF A REAL TIME IMMUNO-PCR FOR THE DETECTION OF PEA ROOT ROT CAUSAL AGENT, *Aphanomyces euteiches*

2.1. Introduction

Root rot in pea can be caused by multiple pathogens including *Aphanomyces euteiches*, *Pythium* and *Fusarium* species, and *Rhizoctonia solani*. It is considered one of the most destructive diseases of pea worldwide (Gangneux *et al.*, 2014). Studies suggest that yield reductions of up to 100% can happen when fields are severely infested by *A. euteiches* (Kraft and Pfleger, 2001). Oospores of *A. euteiches* are strictly soil-borne and can withstand harsh weather conditions ranging from very dry to freezing cold; this allows disease inoculum to remain viable for up to 10 years (Gaulin *et al.*, 2007; Papavizas and Ayers, 1974). Pea root rot infestations may become severe when wet conditions prevail (Levenfors and Fatehi, 2004). Control of *A. euteiches* in the field is challenging as there are no effective pesticides for controlling *A. euteiches* in pulse crops. Furthermore, breeding efforts to obtain pulses resistant to this disease are only in their infancy and the genetic variability within *A. euteiches* populations has been slowing down progress in that regard (Pilet-Nayel *et al.*, 2002).

Canada is the world's largest producer and exporter of pea (Agriculture and Agri-Food Canada, 2020). Pea is therefore an economically important crop in Western Canada, occupying 1.7 million hectares of harvested land in 2019 (Agriculture and Agri-Food Canada, 2020). However, *A. euteiches* has emerged as a serious threat to pea production. It was first reported in Alberta in 2013 as part of routine field surveys (Chatterton *et al.*, 2015a; Armstrong-Cho *et al.*, 2014). The disease became more prevalent in 2013 and 2014 due to prolonged cool and wet spring conditions conducive to the establishment of this disease. Survey data from 2014-2017 however suggest that irrespective of weather

conditions (wet or dry), pea root rot was prevalent throughout Alberta (Chatterton *et al.*, 2015a; Armstrong-Cho *et al.*, 2014; Chatterton *et al.*, 2019). Based on current field production (3.65 million tonnes) and price ($265/tonne), it is estimated that 10% yield losses in pea production can result in economic losses of millions for Canadian pea producers (Agriculture and Agri-Food Canada, 2020). In addition to direct economic losses to the pea industry, limiting pulses in crop rotations is predicted to increase farm expenditures for mineral nitrogen and may negatively impact Alberta's cropping systems diversity and sustainability (Miller *et al.*, 2002). Given the lack of reliable control methods for *A. euteiches*, long rotations for minimum of six years with non host pulse crops such as chickpea, faba bean and soybean (Levenfors and Fatehi, 2004) can be used as practical options for growers to manage this disease where it is endemic. Sensitive and specific detection methods for *A. euteiches* are deemed necessary to determine infestation levels in a timely manner and to prevent or mitigate disease dissemination.

Detection of the presence of *A. euteiches* in soil is possible using quantitative PCR (qPCR) although accurate pathogen levels in soil cannot be determined in this manner (Gossen *et al.*, 2016). Moreover, extraction and purification of DNA prior to qPCR quantification remains challenging (Iker *et al.*, 2013) due to the complexity and heterogeneity of soil, which may reduce DNA recovery and lead to underestimated pathogen concentration (Iker *et al.*, 2013). Moreover, the presence of humic and fulvic acids in soil can lead to decreased sensitivity in qPCR or false-positive results (Watson and Blackwell, 2000).

The Enzyme Linked ImmunoSorbent Assay (ELISA) technique is antibody-based and uses the specificity of antigen-antibody interactions to detect targets of interest.

Despite ELISA's relatively high sensitivity, some antigens present in very low concentrations can be missed (Malou and Raoult, 2011). An ELISA's sensitivity can be further increased by combining its antibody specificity with the amplification power of PCR, a method referred to as immuno-PCR, which can detect extremely low levels of an antigen. Originally developed by Sano *et al.* (1992), further advancement of the PCR technology has led to real-time (RT) detection, which can be also combined with iPCR to result in very high sensitivity, specificity and extensive linear working ranges (Niemeyer *et al.*, 2005). Through coupling with PCR, a conventional ELISA's sensitivity can increase by up to 10,000 fold (Niemeyer *et al.*, 2005). Immuno-PCR has been successfully used for the quantification of proteins (Zhou *et al.*, 1993), viruses (Barletta, 2006; Mweene *et al.*, 1996) and bacteria (Kakizaki *et al.*, 1996), enabling their detection at levels as low as femtograms. As commercial reagents become available, the development of universal RT-iPCR (Zhou *et al.*, 1993; Malou and Raoult, 2011) will be useful to detect and quantify agriculturally important plant pathogens using this technology in the future.

In this chapter, we describe the development of a RT-iPCR assay for the detection of *A. euteiches* oospores. This RT-iPCR assay was adapted from an assay previously developed in our laboratory for the quantification of estrogens in water (Gaudet *et al.*, 2015). Direct conjugation of the antibody to template DNA was used in this novel assay instead of using a streptavidin/biotin conjugate as described and used previously (Gaudet *et al.*, 2015). The RT-iPCR assay performance was assessed (sensitivity, linear working range and reproducibility), and its cross-reactivity determined against related oomycete

pathogens commonly present along with *A. euteiches* including *A. cladogamus, Pythium ultimum, Pythium heterothallicum* and *Pythium irregulare.*

2.2.   Materials and methods

All chemicals including ovalbumin (OVA; M_r =45,000), Tween 20, primers and probe were obtained from Sigma-Aldrich (St. Louis, MO). Hot Start Fluorescent Quantitect probe PCR (Taqman) was from Qiagen (Mississauga, ON). Affinity pure goat anti-rabbit IgG (H+L) was purchased from Jackson Immuno Research Laboratory (West Grove, USA). Non-fat dry milk was from Bio-Rad (Hercules, CA). Low cross buffer (LCB) was purchased from Candor Bioscience GmbH, Wagen, Germany), Horseradish Peroxidase (HRPO)-conjugated goat anti-rabbit antibody was from Cedarlane Laboratories (Hornby, ON) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was from Kirkegaard and Perry Laboratories (Gaithersburg, MD). RNase-free water was from Sigma. Phosphate buffered saline (PBS) was prepared in Milli-Q water and contained 0.755 g L^{-1} of $K_2HPO_4 \cdot 3H_2O$, 0.25 g L^{-1} of KH_2PO_4 and 8.2 g L^{-1} of NaCl. *Aphanomyces euteiches* isolates were collected from different locations in western Canada: Ae 1 (Saskatchewan), Ae 206, Ae 1309, Ae 1352, Ae 315 (southern Alberta) and Morden (Manitoba). Other oomycete pathogens *A. cladogamus* (Br 693)*, Pythium ultimum* (DAOMC 144Br)*, Pythium heterothallicum* (DAOMC 720Br) and *Pythium irregulare* (DAOMC 870Br) were obtained from the Canadian collection of fungal cultures (DAOMC), Ottawa.

2.2.1. Oospore production

Isolates of *A. euteiches* Ae 1 (Saskatchewan), Ae 206, Ae 1309, Ae 1352, Ae 315 (southern Alberta) and Morden (Manitoba) were collected from *A. euteiches* infested

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fields from each province of western Canada (Chatterton *et al.*, 2015a). These isolates were grown for 3 days in media containing Potato Dextrose Agar (PDA) and antibiotics (streptomycin sulphate at 0.375g/ml and penicillin G procaine at 0.375g/ml), and colonies were transferred to oatmeal broth. The oatmeal broth was prepared by blending 5 g of oatmeal in 300 ml of water for 5 min, straining the oatmeal slurry through cheesecloth, followed by autoclaving. The cooled broth was inoculated with five mycelia plugs and incubated for 30 days at room temperature (21°C) in the dark (Windels, 2000). After incubation, the mycelial mat was mixed with sterile water in a Waring commercial blender (Waring Products Division, New Hartford, Connecticut, 0657, U.S.A) for 10 minutes and strained through four layers of cheesecloth. The suspension of oospores was made by adding 50 ml distilled water. Concentration of oospores was determined using a haemocytometer.

2.2.2. Animal immunizations for polyclonal antibody (pAbs) production

Polyclonal antibodies specific to *A. euteiches* were produced by immunizing New Zealand White rabbits (Charles River Montreal, Canada) with a mixture of *A. euteiches* isolates (Ae 206, Ae 315, Ae 1309, Ae 1352). For the primary injection, the *A. euteiches* oospore suspension was mixed with an equal volume of TiterMax Gold adjuvant to a final concentration of 1.0 mg/ml. For secondary injections or "boosts", the suspension was mixed with an equal volume of TiterMax Gold adjuvant to a final concentration of 0.5 mg/ml. Injections were subcutaneous and intermuscular with a single injection in each rear leg and two injections at the back of the neck (of each rabbit). Two weeks after each booster was administered, blood samples were collected from the marginal ear vein

into sterile 7 ml vacutainers (Becton Dickinson, Franklin Lakes, USA) and placed at 37°C for one hour prior to incubation at 4ºC overnight.

To determine the optimal concentration of polyclonal antibody for a particular oospore concentrations, immunoassays in checkerboard format, where the concentration of oospores (1 - 10,000) oospore/ml in a serial suspension was tested in pairwise combinations with different dilutions of polyclonal antibody (1 in 1000 to 1 in 5000). The checkerboard ELISA optimizes the concentration of polyclonal antibody (sera) for oospore detection. The polyclonal antibodies (sera) sensitivity was assessed by ELISA, and the assay was characterized [limit of detection (LOD) and linear working range]. The ELISA's LOD was calculated as the mean of 9 replicates of the zero oospore standard plus twice the standard deviation, as reported in Caron *et al.* (2010). Once a high titer was reached, terminal bleed and rabbit euthanization was performed by animal care staff.

2.2.3. Indirect ELISA

Oospore suspensions for all *A. euteiches* isolates (Ae 1, Ae 206, Ae 1309, Ae 1352, Morden) were stored at 4°C. Each isolate was diluted 10-fold in 1XPBS to reach concentrations ranging from 1 to 10,000 oospores/ml and detected by indirect ELISA. For each isolate, the indirect ELISA was performed three times with three replications each time, for a total of n=9 (replicates).

Microtiter plate (Nunc, Roskilde, Denmark) wells were coated with 100 µl/well of a serial dilution between 1 and 10,000 *A. euteiches* oospores in PBS for each of the isolates (Ae 1, Ae 206, Ae 1352, Ae 1309, Ae 315, Morden), and incubated for 16 hours at 37ºC. Wells were washed three times with PBS-Tween (0.05%), and blocked with 200 µl of 5% milk in PBS for 1 hour at 20ºC followed by three washes with PBS-Tween.

Next, 100 µl of primary antibody diluted in 1/2000 in LCB was then added to each well and incubated for 1 hour at 20ºC. Wells were washed three times with PBS-Tween. 100 µl/well of HRPO goat anti-rabbit antibody (diluted in 1/5000 in LCB) was added to each well and incubated at 20ºC for 1 hour. Wells were washed three times with PBS-Tween. 100 µl of ABTS solution was added to each well and incubated for 30 min at 20ºC prior to absorbance reading at 405 nm on a Spectramax 340PC plate reader. Assay completion time was 3.5 hours, in addition to the 16 hours incubation of the oospores, with a total time of 19.5 hours. The ELISA's LOD was calculated as the mean of 9 replicates of the zero oospore standard plus twice the standard deviation, as reported in Caron *et al.* (2010). The IC_{50} for each isolate was calculated: https://www.aatbio.com/tools/ic$_{50}$-calculator.

The precision and accuracy of the assay were determined by inter-assay and intra-assay coefficients of variability (CV), respectively. The CV was calculated as the ratio of the standard deviation (SD) to the mean. Intra-assay precision was estimated by performing three replicates (wells) per ELISA microtiter plate for the Ae 206 isolate. For inter-assay precision, data from three separate assays were used to calculate the CV. The outline of indirect ELISA is shown in Figure 2.1.

2.2.4. Antibody-DNA conjugate preparation

The secondary antibody, Affinity Pure Goat Anti-rabbit IgG (H+L), was purchased from Jackson Immuno Research Laboratory (West Grove, USA). Conjugation of secondary antibodies to synthetic oligonucleotides (Gaudet *et al.*, 2015) was performed using a commercial Thunder-Link PLUS Oligo Conjugation System (Innova

Bioscience) following manufacturer's instructions. Conjugation products were purified

using Microcon YM-100 centrifugal filters to remove unreacted oligonucleotides.

2.2.5. RT-iPCR

Quantification of *A. euteiches* oospores for isolates Ae 1, Ae 206, Ae 1309, Ae

1352, Ae 315 and Morden was carried out using RT-iPCR. Isolates were serially diluted

10-fold in PBS to reach 1-10,000 oospores/ml. A negative control (only PBS) was

included in each experiment. For each isolate, three independent assays were carried out

with three replications per assay (n=9). The RT-iPCR assay protocol was adapted and

modified from that of Gaudet *et al.* (2015). Thirty µl of each oospore dilution (1, 10, 100,

1000, 10,000 oospores) was added to each well of a 96 well Axygen PCR plate (Axygen,

Mississauga, Canada), covered with a PCR plate cover (Eppendorf, Mississauga, ON,

Canada), and incubated for 16 hours at 37°C. The plate was washed four times with 175

µl of washing buffer (20mM Tris, 150 mM NaCl, 5mM EDTA, 0.05% Tween, pH 7.3) in

a TECAN hydro speed plate washer with 10-second agitation. Plates were briefly

centrifuged for 10 seconds at 168 x g to remove remaining wash buffer. Wells were

blocked with 180 µl of 5% chicken ovalbumin (OVA) (Sigma-Aldrich, Oakville, Canada)

in PBS for 1 hour at 37°C. All subsequent washes were performed with 175 µl wash

buffer and 10 second agitation, except for the final wash (details below). *Aphanomyces*

euteiches – specific serum was diluted 1/2000 in LCB and 30 µl was added to each well

for 1-hour incubation at 20°C with gentle rocking. The wells were washed four times

with washing buffer, and 30 µl of secondary antibody diluted in 1/5000 in LCB was

added to each well and incubated for 1 hour at 20°C. For the final wash, wells were

washed 16 times in total: eight times with 175 µl of wash buffer with 60-second agitation

followed by rinse 8 times with 175 µl Milli-Q water with 10-second agitation, to remove

any unbound reagents. The PCR plate was briefly centrifuged to remove any residual

wash buffer. Thirty µl (Milli-Q water and PCR Master Mix) was added. RT-iPCR was

performed with a Quant Studio Applied Biosystem instrument. The PCR Master Mix

consisted of the following: 15 µl of 2x Quantitect Probe PCR Master Mix, 0.9 µM of

each primer (FWD 5' TCAGTCAGTCATGATATCGCAGT, REV 5'

GCTATATATCGGGCGTCTGC) as per Gaudet *et al.* (2015), 0.9 µM of Taqman probe

and RNase-free water. The PCR parameters were: 15 min at 95ºC followed by 35 cycles

of 18 seconds at 94°C and 60 seconds at 60°C. Assay completion time was 4.5 hours, in

addition to 16-hour prior incubation, for a total of 20.5 hours. The outline of the RT-iPCR

assay is shown is Figure 2.1.

2.2.6. RT-iPCR optimization and characterization

In order to minimize non-specific binding of the iPCR reagents (to the PCR plate

wells), different blocking agents (milk and OVA) were assessed at levels ranging from 3

to 10% w/v. To maximize PCR well coating with *A. euteiches* oospores, the assay's

performance was assessed with the oospores diluted in PBS or in LCB. A standard curve

was produced by plotting the Ct values (n=9) against known concentrations of *A.*

euteiches oospores. In addition to the standards, a negative control (NC) containing all

assay reagents except *A. euteiches* oospores was included, as well as a background

control (BC) containing RT-iPCR Master Mix reagents only, without the DNA template.

The Ct value was inversely proportional to the antigen concentration; samples containing

the highest oospore concentrations had the lowest Ct values. The LOD was defined as the

value of the NC minus three times the average standard deviation, as reported by

Niemeyer *et al.* (2005).

A)

Horseradish
peroxidase
HRPO

Substrate

Secondary antibody
conjugate

Primary antibody
(Rabbit sera)

Oospores

B)

PCR reaction

DNA

Secondary antibody (GAR-
TL-Conjugation)

Primary antibody
(Rabbit sera)

Oospores
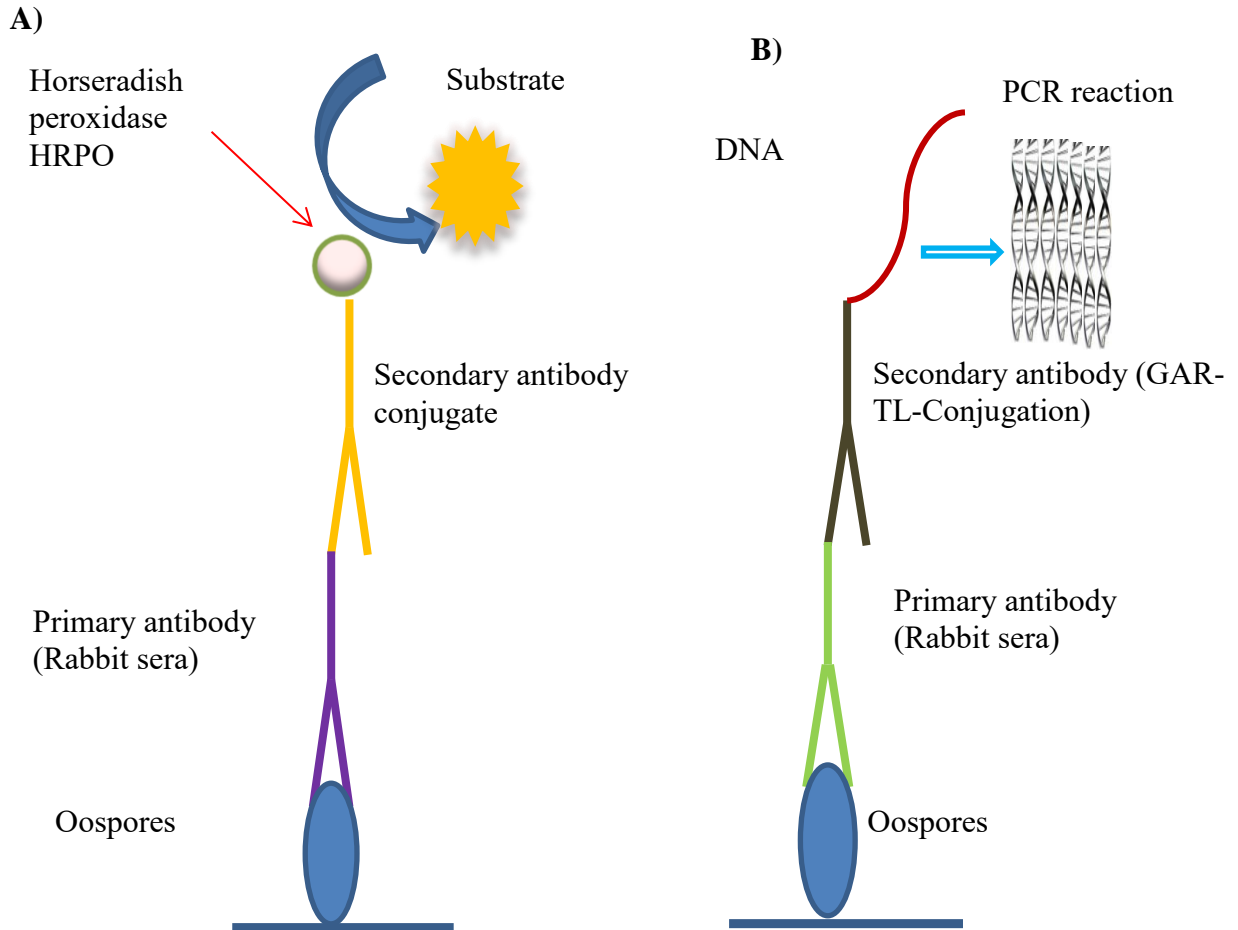
Figure 2. 1. Schematic representation of an indirect ELISA(A) and the corresponding RT-iPCR(B). The indirect ELISA uses a secondary antibody conjugated to an enzyme (for example HRPO) whereas in RT-iPCR, the same secondary antibody is conjugated to a template DNA which is amplified by PCR.

2.2.7. Cross-reactivity

To assess the specificity of the RT-iPCR assay, cross-reactivity experiments were performed against oomycetes species *A. cladogamus, P. ultimum, P. heterothallicum, P. irregulare* along with the *A. euteiches* 206 isolate (positive control). The same concentration of oospores (100 oospores/ml) for *Ae* 206 and all oomycete species was added to the PCR wells. A negative control (no oospore) was included in each experiment.

2.2.8. Statistical analysis

All data were subjected to normality and homogeneity testing via Shapiro-Wilk test and Levene's, respectively. RT-iPCR Ct values for cross-reactivity and ELISA absorbance values were non-normal and non-homogeneous. The RT-iPCR Ct values of the six *A. euteiches* isolates within concentration and across concentrations showed different results for normality and homogeneity of variance. A non-parametric Kruskal-Wallis test followed by pairwise Wilcoxon rank sum tests with the Benjamini-Hochberg corrections for multiple comparisons were used to compare the ELISA absorbance distribution and RT-iPCR Cts (for cross reactivity) using R studio version 3.4.3. A test with 10,000 permutations was used for the F statistic of two factors: isolate; concentration; and their interactions (R studio version 3.4.3, asbio package). A non-parametric Kruskal-Wallis test followed by pairwise Wilcoxon rank sum tests with the Benjamini-Hochberg corrections for multiple comparisons were used to compare RT-iPCR Cts within concentration for six isolates and across concentrations within an isolate. Linear regression analysis of RT-iPCR results for all six isolates and the added oospore concentration (log_{10}) was conducted using R studio version 3.4.3.

2.3. Results

2.3.1. Detection of *A. euteiches* by ELISA

A standard curve of the average absorbance values was plotted against the oospore concentration (log_{10}) per ml (Figure 2.2). The linear working range for isolates Ae 1, Ae 315, Ae 206 and Morden ranged from 100-10,000 oospores/ml while Ae 1309 and Ae 1352 ranged from 100-1000 oospores/ml (Figure 2.2).

A Kruskal-Wallis test comparing ELISA absorbance values of Ae 206 showed a significant difference for different oospore concentrations. A pairwise Wilcoxon rank sum test showed significance difference observed for 100, 1000 and 10,000 oospore/ml at $p<0.0001$ (Table 2.1).
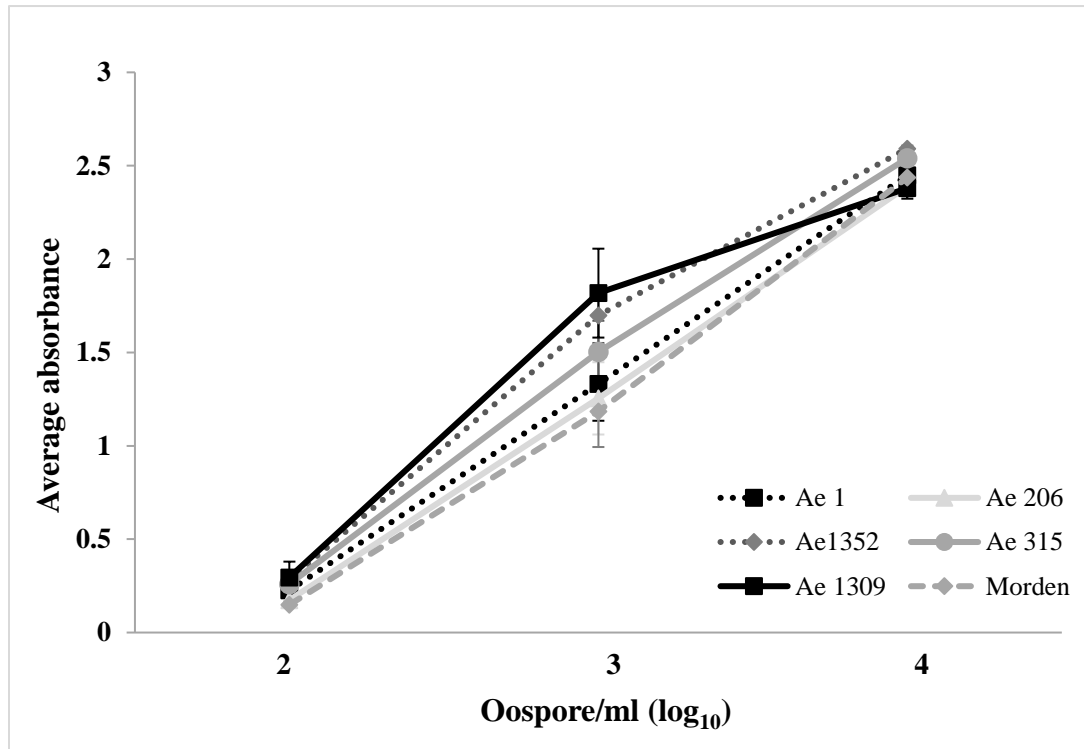
Figure 2. 2. Standard curves for the indirect ELISA with *A. euteiches* field isolates. Average absorbance values plotted against the oospore concentration ($\log_{10}$) per ml. Each data point represents the mean absorbance $\pm$ standard deviation (N=9).

2.3.2. Assay precision

The inter-assay and intra-assay coefficients of variability (CV) were determined for *A. euteiches* isolate 206 , the standard isolate for all experiments. The inter-assay CV ranged from 6.3 to 17.1% over the linear portion of the standard curve (Table 2.1). The intra-assay coefficient of variability ranged from 4.4 to 6.8% over the standard curve (Table 2.2).

The IC$_{50}$ (the number of oospores required to reduce the binding by 50%) and limit of detection (LOD) were calculated for each isolate and are presented in Table 2.3. IC$_{50}$ values for the different isolates tested ranged from 543 to 1689 oospores/ml. The LODs ranged from 4 to 12 oospores per ml (Table 2.3).

Table 2. 1. Inter-assay variability of the indirect ELISA.

Oospores /ml	Abs ± SD [*]	CV %
100	0.13^c ± 0.02	17.1
1000	1.32^b ± 0.08	6.3
10,000	2.38^a ± 0.16	6.6

[*]Mean of 3 independent replicates (N=9) a, b, c represent significance differences at $p<0.05$.

Table 2. 2. Intra-assay variability of the indirect ELISA.

Oospores /ml	Abs ± SD [p]	CV %
100	0.24^c ± 0.01	4.4
1000	1.50^b ± 0.07	4.8
10,000	2.21^a ± 0.15	6.8

[p]Mean of 9 separate wells. a, b, c represents significant difference ($p<0.05$).

Table 2. 3. Inhibitory concentration (IC_{50}) and limit of detection (LOD) for field isolates of *A. euteiches,* presented in oospores/ml.

Isolate	IC_{50}	Limit of detection
Ae 1	1040	10
Ae 206	926	11
Ae 1309	543	4
Ae 1352	779	9
Ae 315	987	9
Morden	1689	12

2.3.3. Optimization and characterization of the RT-iPCR assay

One of the most crucial factors for RT-iPCR performance and sensitivity is the removal of matrix interferences (non-specific binding). Experiments were conducted to optimize assay conditions by improving assay resolution through interferences removal. Optimized conditions thereby established were used in all subsequent assays. Assay optimization resulted in selecting 5% OVA in PBS as blocking solution. The primary antibody (antiserum) concentration was optimal at a dilution of 1:2000 v/v (antiserum: buffer) based on the checkerboard titration with LCB. The secondary antibody binding was optimized at 1:5000 in LCB when incubated for 1 h at 21°C.

2.3.4. Detection of *A. euteiches* by real-time immuno-PCR

The results for RT-iPCR detection of *A. euteiches* oospores for different isolates are shown in Tables 2.4, 2.5 and Figures 2.3 and 2.4. The permutation test indicated a significant difference between *A. euteiches* isolates, between concentrations and a significant interaction between concentration and isolates ($p<0.001$) (Table 2.4). Table 2.5 shows how the isolates differed at each oospore concentration. For 10,000 oospores per ml, isolates Ae 206, Ae 1352, Ae 315 and Ae 1 showed similarity with each other while Ae 1309 and Morden varied from all others. For 1000 oospores per ml, only Ae

1309 varied significantly from all other isolates while all others were similar to each

other. For 100 oospores, Morden and Ae 1309 were significantly different with each

other as well as for the remaining four isolates. One thing was common for all oospore

concentrations: isolate Ae 206, Ae 315 and Ae 1352 were similar with each other, while

Ae 1309 and Morden varied mostly with the remaining isolates. Controls were similar for

all six isolates (Table 2.5).

Table 2. 4. Permutation test results for *A. euteiches* isolates for their $\log_{10}$ oospore concentration and interaction between them.

Source	Degree of freedom	F ratio	P value
Isolates	5	42.85	0.001
Concentration	5	2308.7	0.001
Isolates*Concentration	25	9.4	0.001
Residual	288	NA	NA

Table 2. 5. Comparison of six *A. euteiches* isolate Ct values obtained by RT-iPCR for each spiked oospore(s) concentration. Capital letters (A, B, C, D) denote significantly different results within concentration between isolates.

Isolates	Oospores/ml					
	10,000	1000	100	10	1	0
Morden	12.2^B	15.7^A	19.4^A	21.2^A	21.7^A	21.8^A
Ae 206	13.0^A	15.2^A	17.7^B	19.5^B	21.1^{BC}	21.7^A
Ae 315	12.7^A	15.0^A	17.2^B	19.4^{BC}	21.1^B	21.7^A
Ae 1352	12.9^A	15.2^A	17.4^B	19.4^{BC}	21.1^{BC}	22.1^A
Ae 1	13.5^A	15.3^A	17.3^B	18.2^D	20.5^{CD}	21.7^A
Ae 1309	11.9^B	14.0^B	16.4^C	18.8^{CD}	20.4^D	21.7^A

The pairwise Wilcoxon rank sum test showed that all isolates except Morden (no

significant difference for 0 and 1 oospore/ml) differed significantly ($p<0.001$) across all

oospore concentrations (Figure 2.3). A linear regression analysis was performed to determine correlation coefficient (R^2) values and verify the overall goodness of best fit. The analysis showed a significant linear relationship ($p<0.001$) between the $\log_{10}$ oospore concentration and RT-iPCR Ct values for each isolate (Figure 2.4). The R^2 values were: 0.99 except for Morden isolate (0.95). The linearity helped in determining the RT-iPCR assay's linear working ranges : For all isolates except Morden (10-10,000 oospores/ml), the linear working range was 1-10,000 oospores/ml.
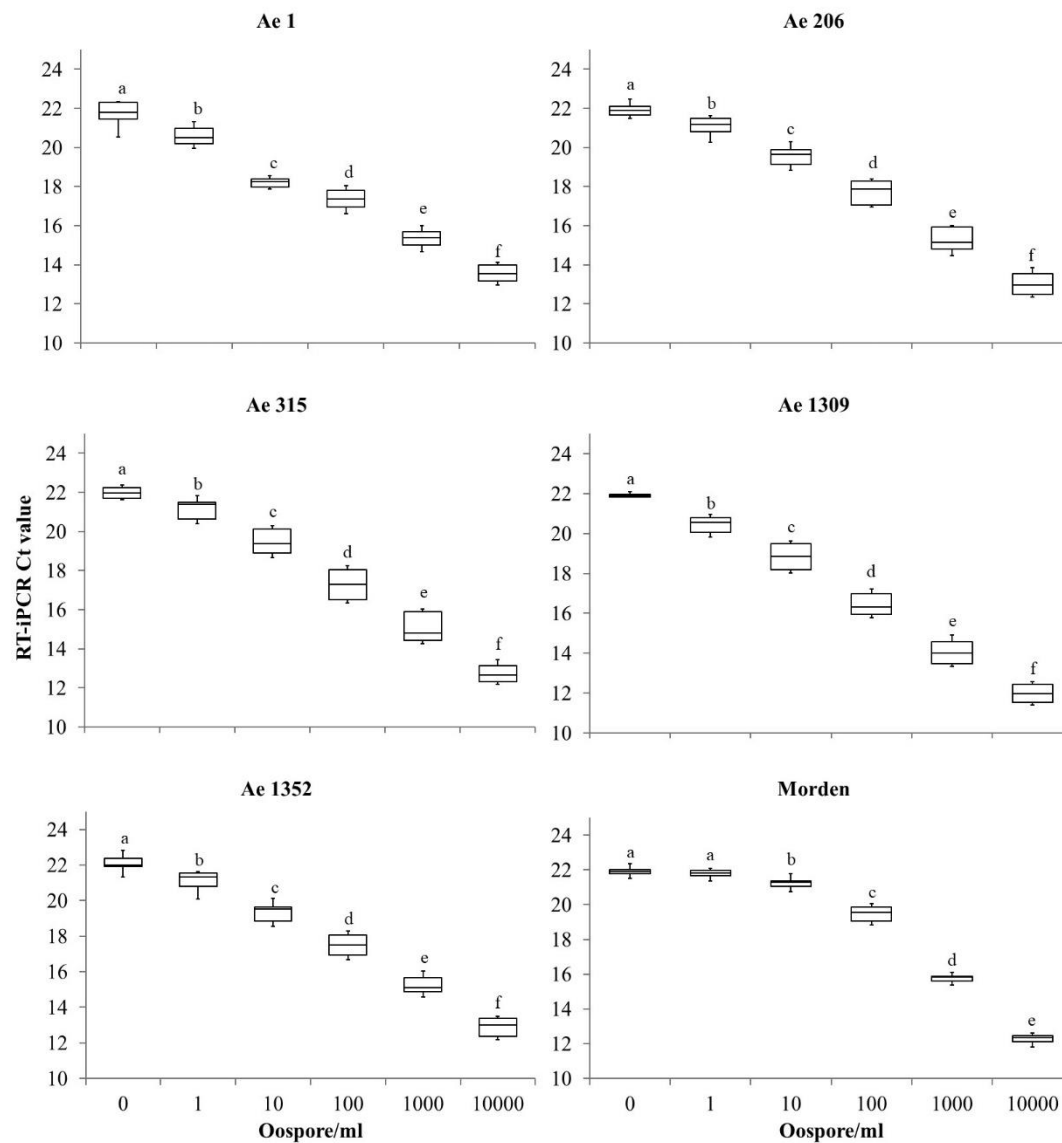
Figure 2. 3. Boxplots of the distribution of RT-iPCR Ct values across concentration for *A. euteiches* isolates. The shape, distribution and variability of RT-iPCR Cts across oospore concentration shown from five number summary (minimum and maximum denoted by the whiskers, first quartile, median and third quartile from the box). Letters a, b, c, d, e and f indicate a significance difference across oospore concentrations. Box plots with same letter are not significantly different.

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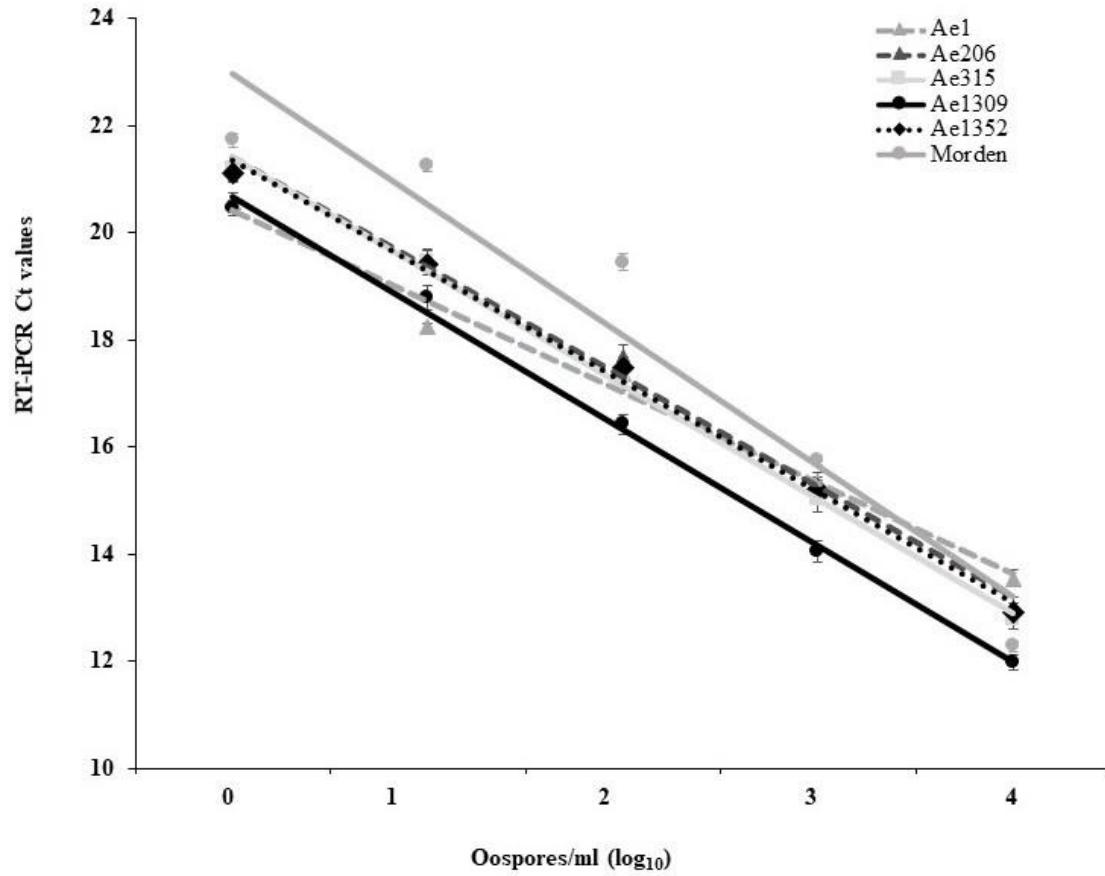
Figure 2. 4. Linear regression analysis of six *A. euteiches* isolates RT-iPCR Ct values. The relationship between oospore concentration ($\log_{10}$) and RT-iPCR Ct was linear and significant for all isolates ($p<0.001$). The correlation coefficient (R^2) for all isolates was 0.99 except for Morden (0.95). The linear working range was 1-10,000 oospore/ml for all isolates except for Morden.

Limits of detection for the isolates varied from 1 to 4 oospores (Table 2.6). The

negative control crossed the threshold cycle (Ct) at 31-33 Ct (the PCR reaction included a

total of 35 cycles) and the lowest positive control concentration crossed the threshold

cycle at 21.2 Ct. Nine Ct values separated the lowest positive control concentration (1

oospore) from the negative control, similar to results obtained by Barletta *et al.* (2004),

who observed a difference of 6 Ct cycles between both controls.

Table 2. 6. Sensitivity of assay towards *A. euteiches* isolates. Sensitivity was determined as the LOD in oospores/ml, and the ratio of the RT-iPCR LOD to that of the ELISA was used to determine the gain in sensitivity

Isolates	RT-iPCR (oospores/mL)	Gain in sensitivity
Ae 1	1	10
Ae 206	2	5.5
Ae 1309	1	4
Ae 1352	4	2.25
Ae 315	2	4.5
Morden	1	12

2.3.5. Cross-reactivity

The assay's specificity was assessed against other oomycete species including *A.*

cladogamus, P. ultimum, P. heterothallicum and *P. irregulare*. The oospore concentration

was 100 oospores/ml for all species tested including positive control (Ae 206). Cross

reactivity data were not normal ($p<0.01$). A Kruskal-Wallis statistical test indicated

significant differences between all species including positive as well as negative control

($p<0.001$). Post-hoc pairwise Wilcoxon rank sum tests showed significant differences

between the positive control (Ae 206) and the other oomycetes species tested for the

cross-reactivity study ($p<0.001$, Figure 2.5). *Pythium heterothallicum* did not vary

significantly from the negative control, while all other species did. No significant

difference was observed between *A. cladogamus,* and *P. heterothallicum* ($p>0.05$), while

all other species varied from each other (Figure 2.5).
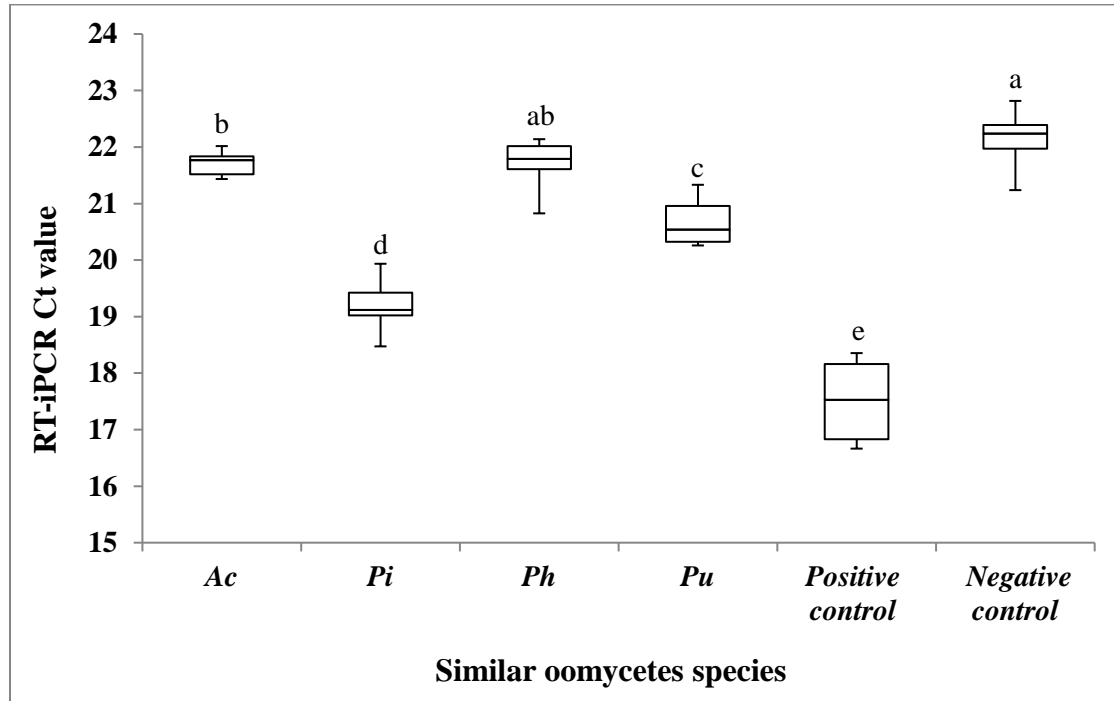
Figure 2. 5. RT-iPCR assay's cross-reactivity with oomycetes *A. cladogamus (Ac),*
Pythium irregulare (Pi), Pythium heterothallicum (Ph), and *Pythium ultimum*
(Pu) compared to *A. euteiches* (*Ae* 206*)* positive control. Significant
difference observed between positive control (Ae 206) and oomycetes
pathogen *p*<0.001. The shape, distribution and variability of RT-iPCR Cts for
oomycetes species shown from five number summary (minimum and
maximum denoted by the whiskers, first quartile, median and third quartile
from the box). Boxplot with same letter in common are not significantly
different.

2.4.   Discussion

A RT-iPCR assay was developed for the quantification of *A. euteiches* oospores. The relationship between the $\log_{10}$ oospore concentration and RT-iPCR Ct values were significant (p<0.001) at each oospore concentration for all isolates and linear. The linearity expressed in terms of coefficient of determination (R^2) of 0.99 for all isolates except for Morden (0.95) helped in determining the linear working range. For the field isolates including Ae 1, Ae 206, Ae 315, Ae 1352 and Ae 1309, the linear working range was from 1 oospore to 10,000 oospores/ml, whereas the linear working range for the Morden isolate was 10 to 10,000 oospores/ml. When the RT-iPCR results (Ct values) obtained for each isolate at each oospore concentration were analyzed, variability was observed between isolates within the same concentration. I observed that the oospores were clumped and aggregated in the suspension, which could prevent the exposure of the cells walls to the antibody, affecting the binding. The differences observed could be partly due to the inconsistent binding of polyclonal antibodies to the oospores.

Among the differences observed between isolates, isolates Ae 206, Ae 315, Ae 1352 were similar almost for all oospore concentrations, while isolates Ae 1309 and Morden behaved slightly differently. A polyclonal antibody was raised against oospores from a range of *A. euteiches* field isolates (Ae 206, Ae 315, Ae 1352, A e 1309) collected in southern Alberta. The antiserum could detect isolates collected from other provinces in Western Canada (Isolates Ae 1 from Saskatchewan and Morden from Manitoba). Although the antiserum was developed against the four isolates above, significant differences were observed among those isolates, especially Ae 1309. Genetic variation could, in part, explain some of the differences observed, however no genetic work was

included in the study. A study on the *A. euteiches* races on root rot of alfalfa in USA has revealed the prevalence of two races (race 1 and race 2) indifferent to the origin and geography (Malvick *et al.*, 2008). The same study disclosed the high genetic diversity among the isolates from different field and counties. Therefore, study on genetic diversity and races among the isolates of western Canada would definitely provide some knowledge to understand the differences observed.

Isolate Ae 1309, despite being significantly different almost from all other isolates, was detected with similar sensitivity of 1 oospore/well. In contrast, sensitivity of detection for the Morden isolate was 10 time less than other isolates i.e. 10 oospore/ml. There is lack of supporting evidence with regard to plant pathogens about their antigenic properties and variation which affects the antibody binding. However, in a study of Lipsitch and O'Hagan (2007) for the bacterial and viral antigens of human pathogen, hereditary or phenotypic changes or spatial variety in the pathogen's natural surrounding was identified as direct or indirect cause for antigenic diversity. Thus, geography and distance could be a function for the binding of the antibodies to the isolates. The polyclonal antibodies were raised against the isolates of Southern Alberta. Taking into consideration the geographical distance between the isolate to which the polyclonal antibodies were developed, Morden was geographically the most distant isolate therefore it could be sufficiently different to be poorly recognized by the antibodies.

When polyclonal antibodies are raised against an antigen or organism, cross-reactivity to similar antigens can arise (Lipman *et al.*, 2005). Cross-reactivity against relevant oomycete pathogens was considered negligible. The antiserum developed against *A. euteiches* discriminated ($p<0.001$) between the *A. euteiches* positive control (Ae 206)

and the other oomycetes. Thus, the antiserum is likely specific to the *A. euteiches*. Other polyclonal antibodies raised against *A. euteiches* (Petersen *et al.*, 1996) showed a similar lack of cross-reactivity towards *P. ultimum* and *P. irregulare*. However, some of these antibodies did bind to *A. cladogamus* (Kraft and Boge, 1994).

The RT-iPCR and the indirect ELISA were compared for their ability to detect and quantify *A. euteiches* oospores. The RT-iPCR was more sensitive than the indirect ELISA for all isolates (Table 2.6). The indirect ELISA had a linear working range of 100 to 10,000 oospores/ml, whereas the RT-iPCR exhibited a broader linear working range of 1 to 10,000 oospores/ml. The LODs of the RT-iPCR assay ranged from 1 to 4 oospores/ml whereas that of the indirect ELISA's LOD spanned 4 to 12 oospores/ml respectively (Table 2.6). The sensitivity of the RT-iPCR increased 4 to 12 fold compared to that of ELISA (Table 2.6). Similar sensitivity gain (i.e. 5 fold increase over ELISA) was observed in a study by Sims *et al.* (2000) for detecting vascular endothelial growth factor (VEGF) by RT-iPCR. Conjugation enabled by Thunder-Link PLUS Oligo Conjugation System (Innova Bioscience) and Imperacer® (Chimera Biotec) has been cited as increasing sensitivity up to 1000 times over the respective ELISAs (Spengler *et al.*, 2009), but such direct conjugation was not assessed as part of this thesis.

For pea root rot to develop in a field, the threshold level has been estimated as 100 oospores/g of soil by Gangneux *et al.* (2014). Since our RT-iPCR assay theoretically can detect as low as 1 oospore/ml, it can confirm the presence of *A. euteiches* oospores at levels well below 100 oospore/g of the soil, required for infection or at an early stage of infestation, allowing for timely treatment or management to minimize dissemination.

2.5.　Conclusions

An RT-iPCR assay to detect *A. euteiches* oospores was developed. The RT-iPCR can quantify oospores from all *A. euteiches* isolates tested, including those collected from different locations in Western Canada (Alberta, Saskatchewan and Manitoba).

While RT-iPCR assays have not been used to detect plant pathogens in soil, the increased sensitivity afforded by RT-iPCR i.e. 1 oospore/ml, and its direct quantification without requiring DNA extraction from soil represent some of its advantages that could help detect the threshold level of the pathogen presence i.e. 100 oospore/g of the soil and infestation levels under field conditions.

CHAPTER 3 : USING REAL TIME IMMUNO-PCR (RT-iPCR) FOR THE DETECTION OF *Aphanomyces euteiches* IN FIELD SAMPLES

3.1.   Introduction

Complete crop losses due to pea root rot have been reported worldwide in heavily infested fields (Gangneux *et al.*, 2014; Willsey *et al.*, 2018). Pea root rot in field pea can be caused by multiple pathogens including *Aphanomyces euteiches*, *Fusarium* spp, *Pythium* spp and *Rhizoctonia solani* (Gossen *et al.*, 2016). However, pea root rot caused by *A. euteiches* is of global significance (Gangneux *et al.*, 2014). *Aphanomyces euteiches* is a soil-borne oomycete responsible for pea root rot in pulses such as lentil, beans and alfalfa (Gangneux *et al.*, 2014). *Aphanomyces euteiches* was reported in Saskatchewan, Canada in 2012 and within five years it was found across Western Canada (Chatterton *et al.*, 2019). Complete crop losses were observed at some locations in Saskatchewan and Alberta (Chatterton *et al.*, 2019). Given the importance of pea production in Western Canada, *A. euteiches* is a serious threat, especially since there are no effective control measures to prevent this pathogen from infecting crops.

The accurate and sensitive detection of a pathogen is fundamental to implement effective control measures, and can also help understand its epidemiology (Wakeham and Pettitt, 2017). In the case of pea root rot caused by *A. euteiches*, the primary inoculum (oospores) may survive in soil for years (Gaulin *et al.*, 2007), therefore timely measures for disease management can only be effective if the number of oospores present is quantified at an early stage (Wakeham and Pettitt, 2017). Traditional detection methods, including selective media (Tsao, 1970), greenhouse assays such as Soil Indexing (SI) (Reiling *et al.*, 1960), Most Probable Number (MPN) (Chan and Close, 2012), wet sieving/baiting (Kraft, 1990), and Rolled Towel (RT) bioassays (Williams-Woodward *et*

al., 1998), have all been used to detect the presence of *A. euteiches* in soil, with mixed results. These methods are qualitative, labour-intensive, time-consuming and mostly rely on the appearance of symptoms specific to a given pathogen. These methods also require a certain expertise for the isolation of fungal and oomycete pathogens on selective media and their microscopic identification based on morphology and taxonomy (Sanzani *et al.*, 2014). Besides, growing specific oomycetes pathogens is limited to those pathogens that can be routinely cultured (Atkins and Clark, 2004), which is not the case for *A. euteiches (*Chatterton *et al.,* 2019*)*. While traditional methods can be used as a confirmatory test for the presence of pathogens (Wakeham and Pettitt, 2017), effective control measures require timely and sensitive pathogen detection.

PCR techniques are currently in practice to detect *A. euteiches* from root samples in the Canadian prairies (Chatterton *et al.*, 2019). Soil testing labs in Alberta, Saskatchewan and Manitoba utilizes the qPCR as a standard method to detect the pathogen DNA from the soil samples directly form the fields (S. Chatterton, personal communication). Both methods have some ongoing limitations, detection based on PCR methods are rather qualitative than quantitative where the samples are rated based on the PCR results as positive or negative especially to interpret the results for weaker bands as positive or confirmed negative (Chatterton *et al.*, 2019). Lack of amplification for some soil extracts, decreased sensitivity due to the presence of humic and fulvic acids leading to decreased sensitivity in qPCR are some ongoing constraints (S. Chatterton, personal communication).

The Enzyme-Linked Immunosorbent assay (ELISA) is one of the most widely used immunoassay formats for the detection of plant pathogens including viruses, bacteria and fungi (Martinelli *et al.*, 2015)**.** ELISAs have been developed for the

detection and quantification of a number of fungal plant pathogens such as *Phytophthora ramorum* (Kox *et al.*, 2007)*, Septoria tritici, Spetoria nodorum* (Mittermeier *et al.*, 1990)*, Pythium* spp, *Phytophthora* spp (Ali-Shtayeh *et al.*, 1991) and *Sclerotinia sclerotiorum* (Bom and Boland, 2000), and some of these assays are commercially available. Immunoassays have also been developed to detect fungal pathogens in soil including *Plasmodiophora brassicae* and *Pythium violae* (Wakeham and Pettitt, 2017), and *Phytophthora* spp (Miller *et al.*, 1997). Despite ELISA's relatively high sensitivity (ex. *Plasmodiophora brassicae* - 100 spores/g of soil (Wakeham and White, 1996)), it may not be sensitive enough when antigens are present at very low concentrations in environmental samples (He *et al.*, 2011). However, ELISA sensitivity can be increased further by combining antibody specificity with the amplification power of polymerase chain reaction (PCR); a method referred to as immuno-PCR (Niemeyer *et al.*, 2005). Immuno-PCR was first developed by Sano, Smith and Cantor in 1992. In immuno-PCR, the enzyme used in ELISA is replaced with DNA and the DNA is amplified by PCR. Because of its multistage signal amplification (ELISA, PCR), immuno-PCR can reach sensitivity up to 1000-10,000 times that of conventional ELISAs, with the same antibodies (Niemeyer *et al.*, 2005). Immuno-PCR's sensitivity can be further increased by real-time detection, a technique referred to as real time immuno-PCR (RT-iPCR) (Niemeyer *et al.*, 2005).

Irrespective of how sensitive a diagnostic assay is, the accurate quantification of an antigen (ex. fungal spores) requires its extraction from environmental samples. With soil, interferences can arise from organic matter, including humic and fulvic acids (Bürgmann *et al.*, 2001; Hargreaves *et al.*, 2013) which can be co-extracted along with

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the antigen of interest (spores). Due to the adherence of *A. euteiches* oospores to soil particles, their dissociation from soil is challenging. As part of this project, two means of dissociation were used: 1. Physical and chemical processes, and 2. Isolation of freed (dissociated) spores from the soil. Following their dissociation and isolation from soil, oospores can be quantified by various methods. In this chapter, our main objective was to determine the relationship between number of oospores added to soil and the corresponding RT-iPCR Ct values and to test the developed assay to detect and quantify oospores of *A. euteiches* from naturally infested field samples. RT-iPCR results were compared to those of a traditional rolled towel bioassay (soil) and PCR (root) for validation.

3.2.   Materials and methods

All chemicals including ovalbumin (OVA; M_r =45,000), Tween 20, primers and probe were obtained from Sigma-Aldrich (St. Louis, MO). Hot Start Fluorescent Quantitect probe PCR (Taqman) was from Qiagen (Mississauga, ON). The secondary antibody, Affinity Pure Goat Anti-rabbit IgG (H+L), was purchased from Jackson Immuno Research Laboratory (West Grove, USA). Thunder-Link PLUS Oligo Conjugation System was from Expedeon (Cambridge, U.K) . Phosphate buffered saline (PBS) was prepared in Milli-Q water and contained 0.755 g L^{-1} of $K_2HPO_4 \cdot 3H_2O$, 0.25 g L^{-1} of KH_2PO_4 and 8.2 g L^{-1} of NaCl. 10 litres of 10XPBST was prepared (800g NaCl, 20g KCl, 144g $Na_2HPO_4.2H_2O$, 24g KH_2PO_4, 8L of distilled water).

3.2.1. Production of oospores

Aphanomyces euteiches isolate Ae 206 was collected from a field in southern Alberta (Chatterton *et al.*, 2015a). The isolate was grown for 3 days in a medium containing Potato Dextrose Agar (PDA) and antibiotics (streptomycin sulphate and

penicillin G procaine at 0.375g/ml each), and colonies were transferred to oatmeal broth. The oatmeal broth was prepared by blending 5 g of oatmeal in 300 ml of water for 5 min, straining the oatmeal slurry through cheesecloth, followed by autoclaving (121°C for 50 min). The cooled broth was inoculated with 5 mycelia plugs and incubated for 30 days at room temperature (21°C) in the dark (Windels, 2000). After incubation, the mycelial mat was blended with sterile water in a Waring Commercial blender (Waring Products Division, Torrington, CT, U.S.A) for 10 minutes and strained through four layers of cheesecloth. Fifty ml of distilled water was added to the mycelial mat to form an oospore suspension. The concentration of oospores in the suspension was determined by using a haemocytometer, where the mean was calculated from five separate cell counts. The mean number of oospore suspension was found to be $445,000 \pm 5000$ oospores/ml.

3.2.2. Preparation and extraction of artificially infested soils

Soils with no history of pea production or *A. euteiches* presence were collected from fields located in Alberta (Rolling Hills and Cranford) and sent to a commercial laboratory (Down To Earth Labs Inc., Lethbridge, AB) for texture and composition analysis (Table 3.1). Soils were sieved (2 mm pore diameter), dried at 90°C for 24 hours, autoclaved (121°C) for 90 min and cooled down for 15-16 hours at 21°C. These soils are referred to as "reference" soils for the remainder of this chapter, and are assumed to be free of *A. euteiches* oospores. An oospore suspension was prepared with a serial dilution of *A. euteiches* isolate 206 (1, 10, 1000, 10,000 oospores/ml). One ml of the oospore suspension at each oospore concentration was added to each soil (1g). The oospore suspension was vortexed before adding it to the soil. For each soil, a control with no added oospore (MilliQ water only) was included.

Table 3. 1. Reference soils texture and composition.

Location	Texture	Composition		
		Sand %	Silt %	Clay %
Rolling Hills	Clay loam	44	28	28
Cranford	Sandy loam	66	22	12

Reference soils with added spores were incubated for 10 min at 21°C and vortexed for 1 min to incorporate the oospores into the soil prior to adding 3 ml of 10X PBST (10XPBS -0.3% Tween 20). The slurry was mixed in a rotatory shaker (Nutator, Innovative Medical Systems Ivyland, PA 16974, USA) for 80 min to homogenize it before centrifugation at $2683 \times g$ for 20 min (Eppendorf Centrifuge 5810 R). Supernatants were sieved with diminishing mesh sizes (PluriSelect, Germany) ranging from 100 to 15 µm. The final filtrates (2.5ml) were stored at 4°C until analyzed by RT-iPCR.

3.2.3. RT-iPCR

Three separate extractions were performed with each soil, and each extract was analyzed in three replicates. A negative control was included in each experiment, where 1 ml of sterile water and 3 ml of 10XPBST were added (to the soil) and the resulting slurry extracted using the protocol described above. Thirty µl of filtrate (soil extract) was mixed by vortexing before adding to Axygen PCR plate wells, the latter covered and incubated (Innova 42 Incubator Shaker Series) for 16 hours at 37°C. The wells were washed four times with 175 µl of washing buffer (20 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.05% Tween, pH 7.3) using a TECAN hydrospeed plate washer with 10-seconds agitation prior

to each washing cycle. Plates were centrifuged for 10 seconds at 168 x g to remove any

wash buffer remaining at the bottom of each well. Wells were blocked with 180 µl of 5%

OVA solution in PBS for 1 h at 37°C. All subsequent washes were performed with 175 µl

wash buffer and 20-seconds agitation, except the final wash. *A. euteiches*–specific

polyclonal rabbit antibodies serially diluted to 1/2000 in LCB and 30 µl was added to

each well and incubated for 1 hour at 20°C with gentle rocking. The wells were washed

four times with washing buffer. Thirty µl of secondary antibody (Goat Anti Rabbit

Thunderlink conjugation) diluted 1/5000 in LCB was added to each well and incubated

for 1 hour at 20°C. Finally, wells were washed 8 times with 175 µl wash buffer with 60

seconds agitation and rinsed 8 times with 175 µl of Milli-Q water with 10 seconds

agitation, in order to remove any unbound reagents. The PCR plate was then briefly

centrifuged to remove any residual wash buffer, prior to adding Milli-Q water and PCR

master mix (30 µl). RT- iPCR was performed using a Quant Studio Applied Biosystem

PCR instrument. The PCR master mix consisted of the following: 15 µl of 2x Quantitect

Probe PCR Master Mix (Quantitect Probe PCR kit, Qiagen, Mississauga, ON), 0.9 µl of

each primer (FWD 5' TCAGTCAGTCATGATATCGCAGT, REV 5'

GCTATATATCGGGCGTCTGC) (Gaudet *et al.*, 2015), 0.9 µl of Taqman probe and

12.3µl of RNase-free water). The PCR parameters were as follows: 15 min at 95°C

followed by 35 cycles at 94°C for 18 seconds and at 60°C for 60 seconds. Overall, the

assay was completed in 20.5 hours including 16 hour incubation of the oospores and 4.5

hours for running assay.

A negative control (NC) containing the soil extract and all assay reagents except

A. euteiches oospores was included, as well as a background control (BC) containing RT-

iPCR Master Mix only, without the DNA template. If the RT-iPCR Ct value of the sample was below the threshold (RT-iPCR Ct value for the negative control minus 3 standard deviation), the sample was considered *Aphanomyces* positive; otherwise, the sample was considered *Aphanomyces* negative as per He *et al.* (2011).

3.2.4. Quantification of oospores from field soils

Field soil samples (15) were collected to validate the extraction method and RT-iPCR assay. Five soils were collected as part of pea root rot surveys from southern Alberta and Saskatchewan (Chatterton *et al.*, 2019). Surveyed fields from which soil samples were collected in 2018 were designated as 8610, 8609, 8614, 8115 and 8122 (Figure 3.1). Sites 8610, 8609 and 8614 were from Saskatchewan and 8115 and 8122 were from Southern Alberta (Figure 3.1). RT-iPCR analysis was performed on these soil extracts and the results for root PCR were provided by Chatterton *et al.* (2019).The other five field soil samples were collected in 2018 from Taber, Alberta by Willsey *et al.* (2018) from a field infested by *A. euteiches* (pea plants were severely stunted, displayed yellow shoots and most pea plants were dead by mid-July) (Figure 3.1). These fields were coded as 415, 203, 803, 809 and 1011 as shown in Figure 3.1. RT-iPCR analysis was performed on these soil sample extracts, while root PCR results for those same soils were provided by Willsey *et al.* (2018). Other soil sampling locations consisted of sites where *A. euteiches* management options were being investigated, and soil samples were collected in 2019 (Chatterton's lab, 2019). Those sites were named according to their geographical location: Lomond, Lacombe, Taber, Drumheller and Saskatchewan (Figure 3.1). Root PCR results were provided by Chatterton's lab (2019).

For each site, a soil core from the top 20 cm was collected from 10 locations within the field following a U-shape pattern, each location being approximately 15 m apart. Soil cores were pooled, mixed to homogenize the sample, wet sieved (2 mm) and stored at 4°C until being processed.
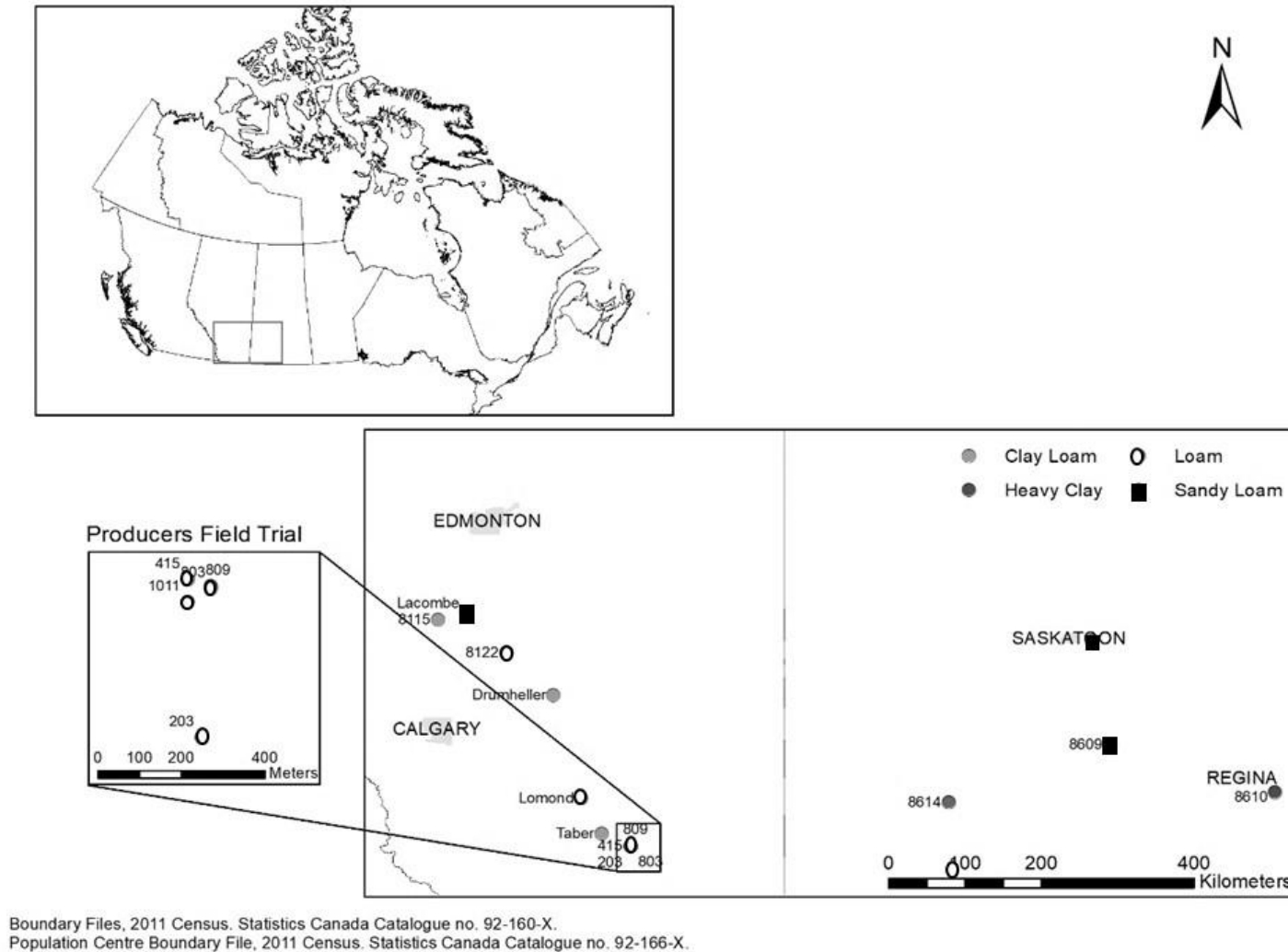
Figure 3. 1. Locations in Canada where soil samples were collected for this study: soils 8610, 8609, 8614, 8115 and 8122 were surveyed soils, soils 415, 203, 803, 809, and 1011 were field trial soils whereas those collected in 2019 were named according to their respective location (Lomond, Lacombe, Saskatoon, Drumheller and Taber).

3.2.5. Rolled towel bioassay

The rolled towel bioassay was modified from that of Williams-Woodward *et al.* (1998) and used to confirm RT-iPCR results. The rolled towel bioassay was selected (among other bioassays) because of its fewer logistical requirements (i.e. less greenhouse space, less soil). The rolled-towel bioassay was only performed on a subset of ten soils collected in 2018.

Pea seeds (CDC Meadow) were treated with Apronmax (1.1% metalaxyl and 1.1% fludioxonil) at the concentration of 325 ml/kg of seed before growing the seedlings in vermiculite. Five seeds were sown per pot and pots were kept in the greenhouse with day/night temperatures of 23°/18°C, 16 hours of daylight and relative humidity (RH) of 53.3%. After 12 days, pea seedlings were washed with tap water to remove vermiculite and placed on a paper towel side by side. Each soil bioassay consisted of five replicates of 4 seedlings placed side by side on a paper towel for a total of 20 seedlings per soil. A non-infested soil was included as a control. The plants were visually screened for the presence of symptoms (water-soaked lesions and honey brown discolouration) every week and the final evaluation was on day 21. Plants were scored for severity of symptoms on a scale of 0-5 (Vandemark and Grünwald, 2005) as shown in Table 3.2. Plants scoring 3 or above were rated as diseased plants. Total percentage of the diseased plants was calculated by counting all plants scoring 3 or above and dividing by the total number of plants per soil.

Table 3. 2. Disease severity scale of 0-5 and their corresponding symptoms by (Vandemark and Grünwald, 2005).

Disease severity scale	Symptoms
0	no visible symptoms
1	a few small discoloured lesions on the entire root system
2	minor discolouration covering of root system
3	brown discolouration on entire root system, but no symptoms on epicotyl or hypocotyl
4	brown discolouration on entire root system, and shrivelled and brown epicotyl or hypocotyls
5	dead plant

3.2.6. Statistical analysis

A non-parametric Kruskal-Wallis test was performed on the number of oospores added per g of soil to the corresponding RT-iPCR Ct values (R studio, Inc. version 3.4). All comparisons were made through pairwise Wilcoxon rank sum tests at a family wise significance level of $p = 0.05$ using a Benjamini-Hochberg correction for comparing RT-iPCR Ct values at each concentration (oospores/g soil) and for the RT-iPCR Ct for field soil samples. These tests were conducted to determine the sensitivity of the RT-iPCR assay to different oospore concentrations.

3.3. Results

3.3.1. Extraction of oospores from artificially spiked soil samples

The Kruskal-Wallis test showed a significant difference for the concentrations of oospores and the corresponding RT-iPCR Ct values. Paired Wilcoxon rank sum tests showed significant differences ($p<0.05$) for some concentrations. For the clay loam soil, there was no significant differences in Ct values between 1 and 10 and between 10 and 100 oospores added per g of soil but Ct values were significantly different for 100 - 10,000 oospores added ($p<0.05$) (Table 3.3). Statistical analysis showed that the negative control (no oospore) was significantly different from all spiked concentrations ($p<0.05$), a pattern not observed in the sandy loam. For the sandy loam soil, no significant difference was observed between 0 and 100 oospores. However, data varied significantly for values greater than 100 oospores (added to the soil) ($p<0.05$). The boxplot for both soils (Figure 3.2) shows that the variation in RT-iPCR Cts was highest at lower numbers of oospores (i.e. 10 and 100 oospores per g of dry soil), and lower at higher oospore concentrations, (1000-10,000 oospores per g of dry soil).

Table 3. 3. RT-iPCR Ct mean values for clay loam and sandy loam soils. Letters A, B, C, D and E represent significant differences within soil types (family wise $p<0.05$).

Oospores added	Cts (clay loam soil)	Cts (sandy loam soil)
0	24.62[A]	24.42[A]
1	22.68[B]	24.78[A]
10	22.18[BC]	23.36[AB]
100	21.15[C]	22.82[B]
1000	18.55[D]	17.57[C]
10,000	14.95[E]	16.00[D]
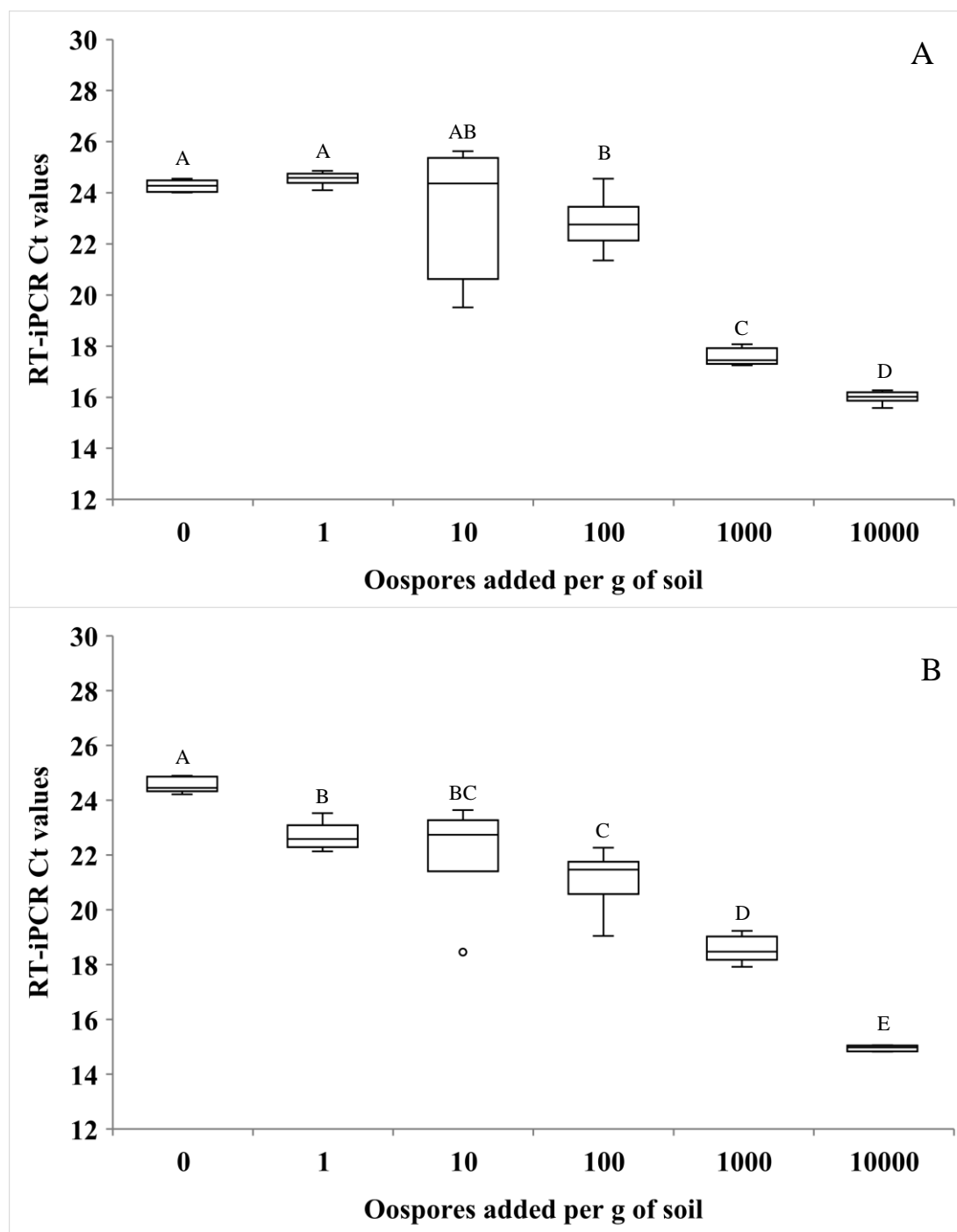
Figure 3. 2. Relationship between the number of *A. euteiches* oospores added per g soil and corresponding RT-iPCR Ct values in the sandy loam (A) and clay loam (B) soils. The shape, distribution and variability of RT-iPCR Cts across oospore concentration shown from five number summary (minimum and maximum denoted by the whiskers, first quartile, median and third quartile from the box). The RT-iPCR Ct values are inversely proportional to the oospore concentration.

3.3.2. Rolled towel bioassay/RT-iPCR

Field soils were deemed positive for the presence of *A. euteiches* when the RT-iPCR Ct value for the soil extracts was below the (negative control - 3*SD) or 21.3. Results for the determination of *A. euteiches* in soils (RT-iPCR, rolled towel bioassay) and plant tissue (root PCR) are presented in Table 3.4. Field soils collected in 2018 (soils 1011, 809, 203, 803, 415) were deemed positive for *A. euteiches* by all detection methods. However, discrepancies among the results for each assay were observed: bioassays of the soil samples resulted in a percentage of infected plants that varied from 15 to 100%, and the average Ct values for the same soil samples following RT-iPCR ranged between 15.2 and 19.8 (Table 3.4).

For survey soils collected in 2018 (soils 8115, 8122, 8614, 8609, 8610), three were positive for *A. euteiches* by the bioassay (soils 8614, 8609, 8610, with 75%, 90% and 100% infected plants respectively; Table 3.4). The severity of infection of pea plants grown in soil 8115 fell under 2 (low end of the severity scale) so were not considered positive, while a few plants grown in soil 8122 were symptomatic (Figure 3.3). Soils 8609 and 8610 were positive by both bioassay and RT-iPCR methods, and had tested positive in previous root PCR (Table 3.4) (Chatterton *et al.*, 2019) whereas soils 8115 and 8122 tested negative by both methods. Soil 8614 was negative by RT-iPCR but previously positive by root PCR (Chatterton *et al.*, 2019) and the bioassay.

Soils collected in 2019 were analyzed for the presence of *A. euteiches* by RT-iPCR and root PCR (unpublished data, Chatterton's lab, 2019) only (Table 3.5). These soils were all positive, irrespective of the method used.

Table 3. 4. *Aphanomyces euteiches* detection by RT-iPCR (average Ct values), root PCR and rolled towel bioassay. Symbols indicate disease presence (+) or absence (-), respectively. The pairwise Wilcoxon rank sum test indicates a significant differences (p<0.05) amongst the field trial soils (415, 203, 803, 1011 and 809) as indicated by letters a and b. Similarly, significant differences (p<0.05) observed amongst survey soils (8610, 8609, 8614, 8115 and 8122) are indicated by letter x, y and z. Letters in common are not significantly different. Symbol * shows the significant difference (p<0.05) between survey and field trial soils which have similar percentage of infected plants (90-100%) in rolled towel bioassay.

Soil	Soil type	RT-iPCR (Ct values)	RT-iPCR	Root PCR (Willsey *et al.*, 2018; Chatterton *et al.*, 2019)	Rolled towel bioassay (% of plants infected)
415	loam	17.9^{a*}	+	+	100
203	loam	17.5^{a}	+	+	65
803	loam	16.4^{b}	+	+	40
1011	loam	18.0^{ab}	+	+	35
809	loam	16.4^{b}	+	+	15
8610	heavy clay	20.3^{y*}	+	+	100
8609	sandy loam	14.1^{z*}	+	+	90
8614	loam	22.3^{x}	-	+	75
8115	clay loam	22.2^{x}	-	-	0
8122	loam	22.6^{x}	-	-	10

Table 3. 5. *Aphanomyces euteiches* detection by RT-iPCR (average Ct values), root PCR for 2019 soils. The pairwise Wilcoxon rank sum test shows a significant difference (p<0.05) for the location sites (Lacombe, Lomond, Drumheller, Saskatchewan and Taber) indicated by letters a and b). Letters in common are not significantly different. Symbols + or - indicate disease presence or absence respectively.

Soil	Soil type	RT-iPCR (Ct values)	RT-iPCR	Root PCR (unpublished data, Chatterton's lab, 2019)
Lacombe	sandy loam	18.2[b]	+	+
Lomond	loam	19.5[a]	+	+
Drumheller	clay loam	19.5[a]	+	+
Saskatchewan	sandy loam	19.5[a]	+	+
Taber	clay loam	20.0[a]	+	+
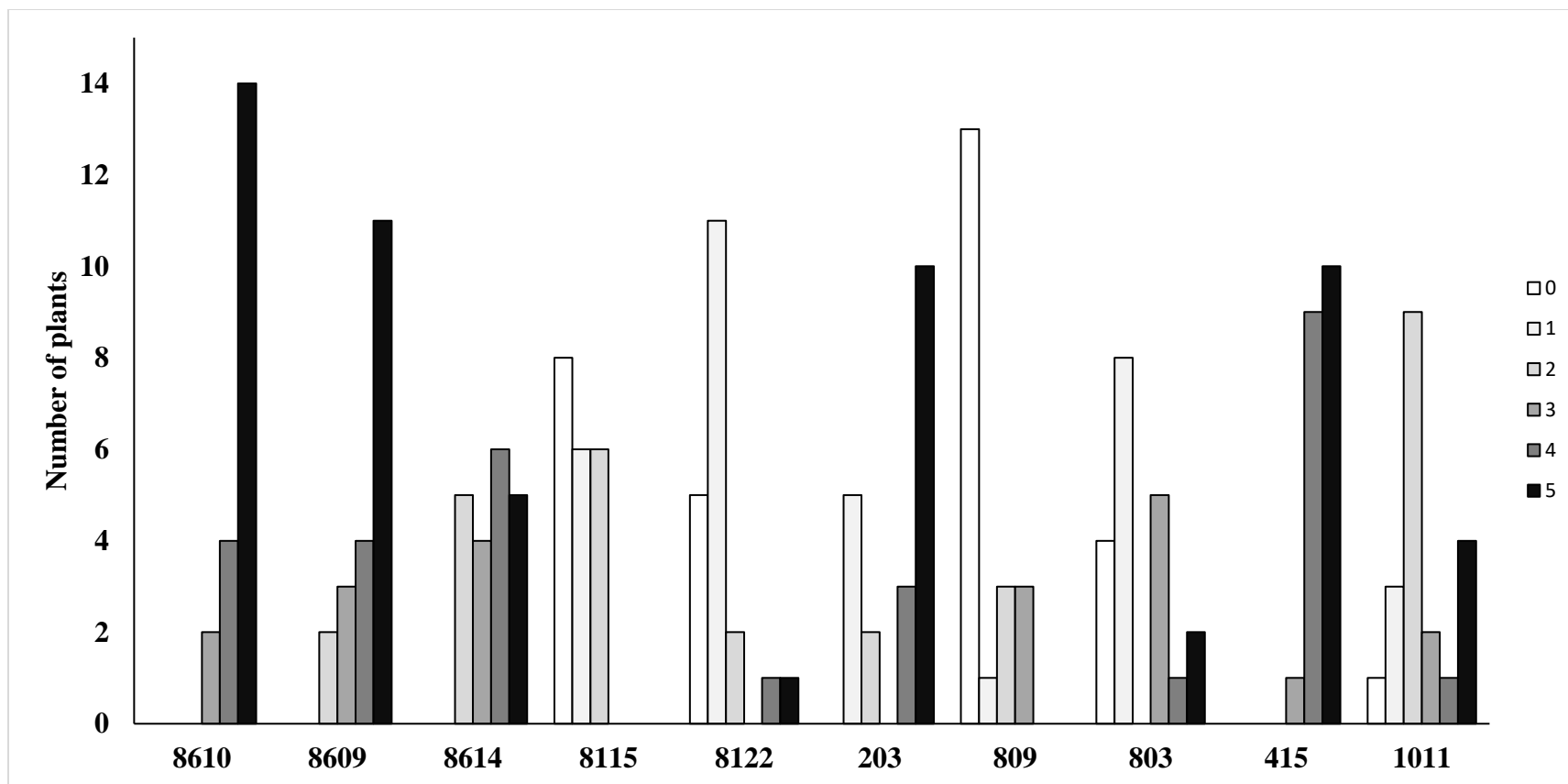
Figure 3. 3. Disease severity rating on a scale of 0 to 5 (Vandemark and Grünwald, 2005) for surveyed soils collected in 2018 (8610, 8609, 8614, 8115 and 8122) and field trial soils 203, 809, 803, 415 and 1011, rolled towel assay. Symptomatic plants which scored above 2 according to the severity scale were considered "diseased".

Although soils were tested for *A. euteiches* by the rolled towel assay and root PCR, mixed results were observed according to soil type. For example, results with loam soils (415, 203, 803, 809, 1011) were inconsistent (Figures 3.4). Pairwise Wilcoxon rank sum test indicated a significant difference ($p<0.05$) in RT-iPCR Ct between site 415 with 803, 809 and between 203 and 803 despite being collected from the same field (Table 3.4). Significant difference ($p<0.05$) in RT-iPCR Ct values were observed for soils 415 and 8610 despite their similar percentage of infected plants (100%): Soil 415 (loam) amplified early (17.9 Ct) while soil 8610 (heavy clay) amplified later (20.3 Ct). Another soil (8609, sandy loam) resulted in 90% infection (rolled towel bioassay), and amplified early (14.1 Ct value) (Figures 3.4). That soil also displayed a low variability by RT-iPCR compared to the other soil types (loam and heavy clay, Figure 3.4). Soil 8614 (loam) resulted in 75% infection while being negative by RT-iPCR (22.3 Ct value). This soil also demonstrated the lowest variability among all loam soils (Figures 3.4). For location based sites, Lacombe was significantly different in RT-iPCR Ct with remaining sites while others were similar to each other (Table 3.5). Due to their mixed results, field soils were categorized as positive (presence) or negative (absence) for *A. euteiches*. All three methods resulted in identical results (positive or negative) for all soils except one (8614).
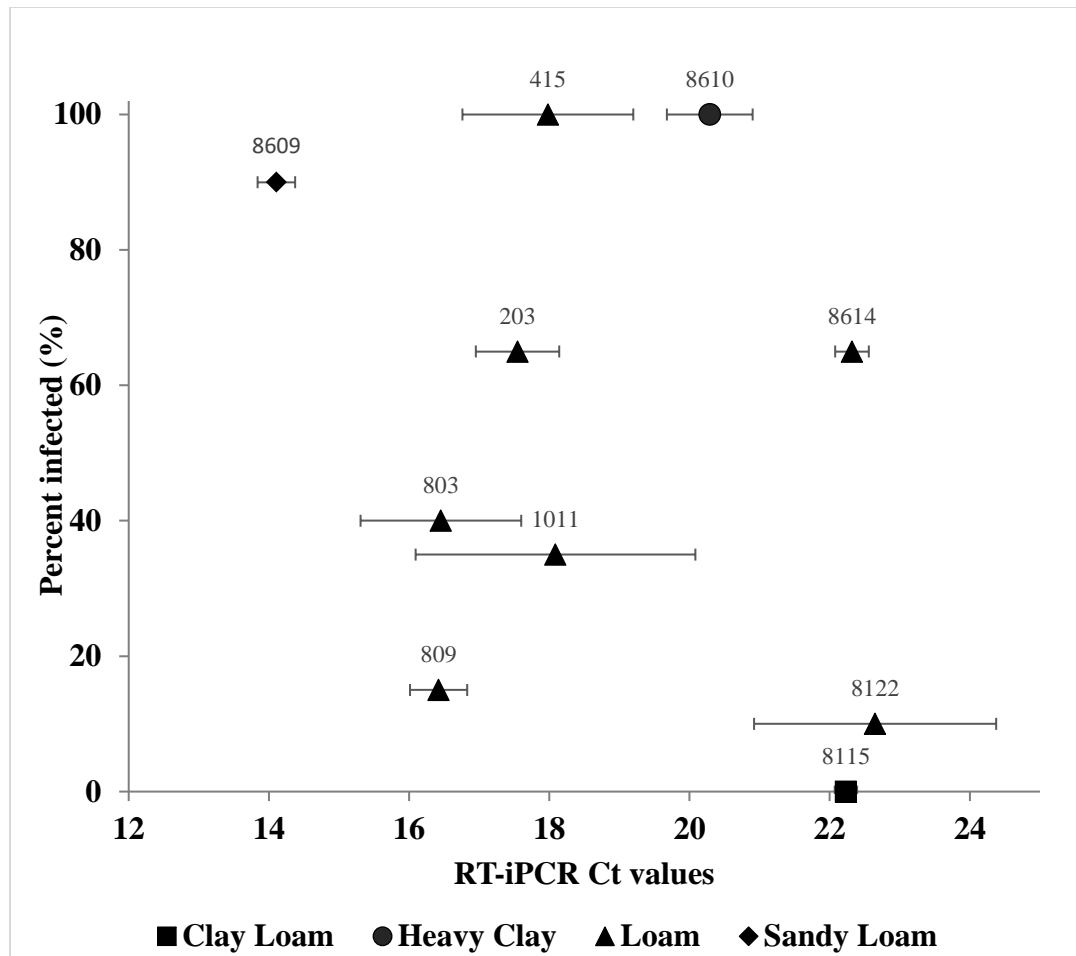
Figure 3. 4. Percentage of infected plants (rolled towel bioassay) as a function of RT-iPCR Ct values. Soil types are indicated with symbols (see legend above). The error bar shows the variation in RT-iPCR Ct for the field samples.

3.4. Discussion

3.4.1. RT-iPCR detection of *A. euteiches* oospores in artificially spiked soils

RT-iPCR could accurately detect oospores at a concentration of 100 oospores per g of soil and above. Soils spiked with oospores demonstrated greater variability in RT-iPCR Ct values at low concentrations (1-100 oospore per g of soil) irrespective of the soil type. This variability could be a reflection of the clumpy nature of *A. euteiches* oospores, resulting also in inconsistent detection by the polyclonal antibody. In addition, the inconsistency could be from pipetting out a subsample (30 µl) from a large volume of solution. At lower oospore concentrations, the extract subsample (30 µl) used to coat wells in the RT-iPCR assay may not have been representative of the entire sample. In addition, the number of oospores added to soil was diluted by a factor of 4 through addition of buffers. As a result, a 30 µl aliquot theoretically represented 0.0075, 0.075, 0.75, 7.5, and 75 oospores, for 1, 10, 100, 1000 and 10000 oospores added per g of soil respectively. Differences in RT-iPCR Ct were observed starting at 100 oospores per ml (0.75 oospores in 30µl aliquot), indicating the potential for this method to determine the presence of a single oospore in 30 µl. However, the detection observed at low level of oospore concentrations should be further explored due to the variability in the oospore suspension counts. It should be noted that because the RT-iPCR assay is quantifying epitopes on the surface of *A. euteiches* oospores, it is also possible that detection of free epitopes (in solution) results in low level signals at theoretical concentrations below 1 oospore/ml.

Differences in detection levels observed with soil types may have been influenced by spike concentrations (described above) as well as soil properties. Almquist *et al.* (2016)

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found that soil type influenced the extraction of *A. cochlioides* DNA from soil, with higher

limits of detection for sand. Similarly, this study observed differences in detection of

oospores extracted from sandy loam and clay loams. Nonetheless, this is the first report of

an extraction method to isolate *A. euteiches* oospores from soils combined with RT-iPCR

analysis. While a significant variability in results was observed, overall this method was

able to quantify oospores at concentrations of 100 oospores/g of soil and above which

means that it could be a potential alternative to quantify/detect oospores from soil. When

the RT-iPCR sensitivity was compared to that of other quantitative methods for *A.*

euteiches such as qPCR (Sauvage *et al.*, 2007; Gangneux *et al.*, 2014), equal sensitivity

was achieved i.e. detection of 100 oospore/g of soil and above. When qPCR was performed

for field soil samples, inconsistency in amplification was observed in some soil extracts

demonstrating the challenges with qPCR (data not shown). Lack of amplification for some

soil extracts, decreased sensitivity due to the presence of humic and fulvic acids leading to

decreased sensitivity in qPCR are some ongoing constraints (S. Chatterton, personal

communication). These obstacles were partly eliminated using RT-iPCR. Oospores of *A.*

euteiches were directly detected by RT-iPCR without having to extract oospore DNA from

the soil extracts. Furthermore, the numerous washing steps in the RT-iPCR removed

inhibitors that could interfere via competition and mispriming resulting in lower DNA

yields when using PCR quantification (Malou and Raoult, 2011; Niemeyer *et al.*, 2005).

RT-iPCR is particularly useful when DNA extraction results in low yields solving the

problem of lower sensitivity and no amplification as faced currently in qPCR.

The successful development of an RT-iPCR assay rests on the specificity and

sensitivity of its antibodies. In our study, rabbits received several doses of an oospore

suspension that also contained mycelium matrix and dead oospores (and likely other debris). When counting oospores in suspensions by haemocytometer, only complete or intact cells were considered. However, since Ct values statistically different from those of the control ($p<0.05$) were observed at concentrations as low as 10 oospores per ml, it is possible that the polyclonal antibodies bind to cell debris and oospore surface proteins in solution. It is known that polyclonal antibodies recognize their specific epitopes on a variety of cell structures (Lipman *et al.*, 2005) and in solution. The fact that polyclonal antibodies can bind multiple binding sites, results in the high sensitivity of the RT-iPCR assay, but also may lead to undesirable cross-reactivity with other structures within the target species, although the latter has not been investigated in this study.

3.4.2. RT-iPCR detection of *A. euteiches* oospores in field soils

For all soils except 8614, the RT-iPCR results were consistent with those obtained via the rolled towel bioassay and the root PCR (Table 3.4). However, substantial variability within and between soil samples was observed. Soils collected in 2018 and 2019 included a diversity of soil types (heavy clay, clay loam, sandy loam and loam (Tables 3.3 and 3.4). Soils 8610, 415, and 8609 had high infection levels (100, 100, and 90% of plants infected in the rolled towel bioassay), and their textures were heavy clay, loam and sandy loam, respectively. While infection levels were similar, significant differences were detected in their Ct values (although all positive). A sigmoidal relationship was observed between number oospores per g of soil and IP (Gangneux *et al.*, 2014). When oospores reach a certain level in soil, the IP or the disease severity caused by the pathogen would remain the same despite increase in the number of oospores/g of soil. This could possibly explain the difference in Ct values for different

soils with the same disease infection. Significant differences for RT-iPCR Ct was observed for the field trail soil sample collected within a farmer's field. Chatterton *et al.* (2015a) highlighted the difficulty in recovering *A. euteiches* oospores from fields that surveyed positive and attributed this low recovery to the 'patchy distribution' of the pathogen. Moussart *et al.* (2009) conducted a study on the spatial distribution of *A. euteiches* in infested fields. Patches with different pathogen concentrations were observed within plots, further evidence of the clustering and aggregating nature of *A. euteiches* (Williams-Woodward *et al.*, 1998; Chan and Close, 2012; Gangneux *et al.*, 2014). Overall, the rolled towel bioassay developed for validating our developed method showed a good agreement with the RT-iPCR results and root PCR results with variability observed for some soil types.

The RT-iPCR assay was developed specifically to quantify *A. euteiches* oospore, However, the pathogen has multiple life stages in soil that will affect its quantification. It has both asexual (mycelium, zoospore) and sexual life stages (oospores) depending on the presence or absence of a host (Gaulin *et al.*, 2007). Thus, timing of soil collection during the growing season may affect the detection and quantification of oospores if soil is collected too early in the season before oospores are produced and returned to the soil.

The methods used for collecting soil samples can also be an important factor in recovering *A. euteiches* (Chatterton *et al.*, 2015a). The soil sampling strategy becomes important for pea root rot caused by *A. euteiches* due to its patchy distribution. The oospores of *A. euteiches* are clumped and aggregated in the soil which can lead to the inaccurate prediction (overestimation/underestimation) of the pathogen in the field if not sampled properly (Gangneux *et al.*, 2014). Other studies have observed genetic diversity

in *A. euteiches* across and even within fields (Malvick *et al.*, 2008; Grünwald and Hoheisel, 2006), which may also influence the detection of oospores. In this study, soil samples were collected from a range of geographical areas across the Canadian Prairies and therefore the local isolates may interact differently with the polyclonal antibodies.

3.5. Conclusions

When detecting *A. euteiches* oospores in soil samples, consideration should be given to using accurate standards so that the relationship between oospore concentration and RT-iPCR Ct can be utilized to quantify the pathogen from field samples. Influence of soil types on recoveries of soil-borne pathogen should be studied prior to detection, as natural field soil varies in type, texture and structure. Additionally, pathogen stage, behaviour, accurate sampling and ideal timing of oospore collection should also be considered as this information is critical for accurate and reliable estimation of the pathogen from soil. Due to the complex nature of polyclonal antibodies, interaction of antigen-antibody should be well studied and antibody development should be directed for specific antigen to be detected. An extraction method was developed to quantify *A. euteiches* oospores in soil by RT-iPCR and results were validated with a traditional method. As of now, our developed method showed a good agreement with validation methods.

CHAPTER 4: SUMMARY AND FUTURE DIRECTIONS

Many challenges need to be addressed before quantitative analysis of any soil borne pathogen. This project highlighted the need and importance of in-depth research of *A. euteiches* under field conditions. In the field, development of disease is governed by several factors such as soil type, environmental conditions and crop variety which needs extensive research. Pathogen population, sampling strategies for the accurate and reliable estimation of the pathogen need the immediate research.

Future work should be focussed on making a homogenous sample of *A. euteiches* oospores and creating effective methods for extracting oospores from soils since recovery rates could not be calculated. Adjustments to the ratio of liquid-to-soil and concentration of extract may be necessary to allow the 30 µl aliquot required for the microplate in RT-iPCR to be representative. In addition, the impact on oospore recovery of factors such as soil type, soil structure, pH and organic matter should be assessed. Genetic diversity of the pathogen populations from different geographic regions as well as within same region should be conducted. Further study into interaction with other species which may influence the detection of oospores should be carried out.

A better understanding of the anti-body antigen interactions is needed to determine if the mycelium growth and zoospore stages of the pathogen are detected or not. Specificity of the assay should be determined by testing the cross reactivity with related pathogens that are associated pea root rot and also to interacting pathogens from the field such as *Fusarium* spp.

The developed RT-iPCR method has the potential to be a promising method to detect *A. euteiches* in soil samples. It can detect the oospores or any pathogen structure without having to extract the DNA from the soil samples. In addition, it minimizes the challenges encountered with qPCR as numerous washing steps in the RT-iPCR remove inhibitors that may interfere via competition and mispriming with PCR quantification. RT-iPCR is particularly useful when DNA extraction results in low yields, thereby solving the problem of lower sensitivity and no amplification as often faced currently in qPCR.

The method developed for this project can be easily modified to detect any soil-borne pathogen by raising the polyclonal antibody against those species. The early, accurate and specific detection of the disease would help in understanding the concentration of this pathogen and risk to pea production. This allows farmers and producers to know beforehand to grow or avoid pea or any susceptible crops. Quantity or level of the pathogen can be monitored before planting season which would also assist in establishing the strategies to provide specific treatments and effective control management options. Although, more things need to be explored still, this project has provided a lot of preliminary information which can be useful for giant leap in accurate pathogen detection and quantification.

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