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DEVELOPMENT OF ISOTHERMAL DETECTION METHODOLOGY FOR *PLASMOPARA VITICOLA* AND *PHYTOPHTHORA INFESTANS*

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DEVELOPMENT OF ISOTHERMAL DETECTION METHODOLOGY FOR *PLASMOPARA VITICOLA* AND *PHYTOPHTHORA INFESTANS*

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Thesis submitted to the Università degli Studi della Tuscia of Viterbo,
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To my parents

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Abstract

This research concerns the development and application of isothermal amplification detection techniques to two important plant pathogenic Oomycete: *Plasmopara viticola* causing grapevine downy mildew and *Phytophthora infestans*, the causal agent of potato late blight. Real-time Loop-Mediated Isothermal Amplification (LAMP) assay was applied for the early detection of *Plasmopara viticola* from infected grapevine plants. A rapid crude plant extract (CPE) preparation method from infected leaves was developed for on-site testing. The LAMP assay targeting the large ribosomal subunit gene (LSU) was specific to *P. viticola* and sensitive to 10 sporangia/ml. In the inoculation experiments of greenhouse plants and leaf discs samples, LSU primers detected downy mildew infections at a concentration of 10^3 sporangia/ml after 24h of inoculation. In addition, Real-time LAMP and Recombinase Polymerase Amplification (RPA) assays were developed targeting the ITS2 region of the ribosomal DNA of *P. infestans*. Both LAMP and RPA assays showed specificity to *P. infestans* and the closely related species *P. andina*, *P. mirabilis*, *P. phaseoli* and *P. ipomoeae*. No cross-reaction occurred with the potato pathogens tested. LAMP and RPA assays detected DNA at 50 fg/ul and showed to be insensitive to CPE inhibition. The isothermal assays were validated with inoculated potato plants using a portable Smart-DART device. The LAMP and RPA assays effectively detected *P. infestans* DNA in symptomless leaf tissue 24 h and 72 h post-inoculation, respectively. Finally, the suitability of the latter LAMP assay was tested for a rapid detection and quantification of airborne inoculum of *P. infestans*. Standard curves of *P. infestans* sporangia and ITS copy were constructed. The quantitative LAMP (qLAMP) assay was validated in the laboratory with silicon-coated rods containing a known number of sporangia. The analysis was performed using a regression procedure. A linear relationship between the number of sporangia deposited onto the rods estimated with microscopy and the number of sporangia estimated with the qLAMP assay was obtained. A rapid and accurate on-site detection of *P. infestans* and *P. viticola* in plant material and spore samplers will contribute to improved disease diagnosis, early detection of first infections and facilitate prompt management decisions.

Keywords: Plant pathology, Grapevine downy mildew, Potato late blight, Loop-Mediated Isothermal Amplification, Recombinase Polymerase Amplification, real-time detection, crude plant extract, on-site diagnostics.

Riassunto

La ricerca svolta riguarda lo sviluppo e l'applicazione di tecniche di amplificazione isotermica per il rilevamento di due importanti oomyceti fitopatogeni: *Plasmopara viticola*, agente causale della peronospora della vite e *Phytophthora infestans*, agente causale della peronospora della patata. Per il rilevamento precoce di *P. viticola* in piante di vite infette, é stata utilizzata la tecnica Real-time Loop-Mediated Isothermal Amplification (LAMP). Per il saggio in campo, é stato sviluppato un metodo di preparazione rapida di un estratto di pianta crudo (CPE) a partire da foglie infette. Il saggio LAMP é stato specifico per il gene per la sub-unità ribosomiale (LSU) di *P. viticola*, e sensibile a 10 sporangi/ml. Negli esperimenti di inoculazione su piante in serra e su dischi fogliari campionati, i primers LSU sono stati in grado di rilevare infezioni di peronospora a concentrazioni di 10^3 sporangi/ml dopo 24 ore dall'inoculazione. Inoltre, saggi di Real-time LAMP e Recombinase Polymerase Amplification (RPA) sono stati sviluppati per il rilevamento della regione ITS2 del DNA ribosomiale di *P. infestans*. Entrambi i saggi LAMP e RPA hanno dimostrato specificità per *P. infestans* e per le specie strettamente correlate *P. andina*, *P. mirabilis*, *P. phaseoli* e *P. ipomoeae*. Non sono state rilevate reazioni incrociate con gli altri patogeni della patata saggiati. I saggi LAMP e RPA hanno rilevato DNA a 50 fg/ul e non hanno mostrato alcuna inibizione al CPE. I saggi isotermici sono stati validati con piante di patate inoculate utilizzando uno strumento portatile Smart-DART. I saggi LAMP e RPA hanno rilevato DNA di *P. infestans* nei tessuti fogliari di piante asintomatiche a 24 e 72 ore dall'inoculazione, rispettivamente. In fine, l'utilizzo del saggio LAMP è stato testato per il rapido rilevamento e quantificazione dell'inoculo airborne di *P. infestans*. Sono state ottenute le curve standard degli sporangi e del numero di copie ITS2 di *P. infestans*. Il saggio LAMP quantitativo (qLAMP) è stato validato in laboratorio con barrette rivestite di silicone contenenti un numero noto di sporangi. L'analisi è stata effettuata tramite regressione lineare, con cui è stata ottenuta una relazione lineare tra il numero di sporangi presenti sulle barrette, valutato mediante conteggi al microscopio, e il numero di sporangi stimato dal saggio qLAMP. Un rapido e accurato rilevamento in campo di *P. infestans* e *P. viticola* contribuirà a migliorare

la diagnosi delle malattie, il rilevamento precoce delle prime infezioni e a facilitare le decisioni per una gestione tempestiva.

Parole chiave: Patologia delle piante, peronospora della vite, peronospora della patata, Loop-Mediated Isothermal Amplification, Recombinase Polymerase Amplification, rilevamento Real-time, estratto di piante crudo, diagnosi in campo.

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Chapter I

I GENERAL INTRODUCTION

This chapter relates the background and the main hypothesis of the study. As a first step, we will discuss the main concepts of integrated plant disease management. We will also define some aspects related to Oomycete plant pathogens, especially the pathogens studied: *Plasmopara viticola* and *Phytophthora infestans*. We will then address the contribution of molecular methods in the detection of plant pathogens and the recent advances in isothermal detection methods that account for this thesis. Finally, we will conclude this chapter by covering the objectives of this thesis.

Integrated disease management

Plant diseases cause enormous crop losses worldwide. Damages can occur in the field as early as seed sowing to harvesting, but also in storage. Major historical consequences of plant disease epidemics are the Irish great famine (1845) due to late blight of potato and Bengal famine (1943) due to brown spot of rice. Plant pathology addresses the cause, biology, epidemiology, subsequent losses and management of the plant diseases.

Foremost, a correct disease diagnostic is essential in order to identify the right causal agent. Disease management practices rely essentially on preventing the occurrence of disease, and target critical stages of the pathogen in the disease cycle. Integrated disease control strategy or Integrated Disease Management (IDM) is a resultant concept from the popular Integrated Pest Management (IPM) systems developed by entomologists for insect and mite control. IDM consists of disease and pathogen scouting with proper application of a combination of control strategies. This approach integrates the adaptation of cultural practices, the pathogen and disease survey, and monitoring the environmental factors. Based on established economic thresholds, fungicide treatments are usually applied with the support of disease forecasting models (Maloy 2005).

An integrated disease control program aims to (1) eradicate or reduce the initial inoculum, (2) reduce the effectiveness of initial inoculum, (3) increase the host

resistance, (4) delay the disease onset, and (5) slow the secondary cycles (Agrios 2005).

Oomycete plant pathogens

The oomycetes are a group of fungal-like organisms that represent some of the most destructive plant pathogens and considered as persistent threats in agriculture. Oomycetes are different from true fungi and more related to diatoms and seaweeds (Thines 2014). The main features distinguishing oomycetes from fungi are: their cell wall contains cellulose and have glucans instead of chitin derivatives, their mycelium is coenocytic (non-septate), and many species produce wall-less, biflagellate swimming zoospores (Bartnicki-Garcia 1968; Burki *et al.* 2007). They cause important diseases that include seedling blights, damping-off, root rots, foliar blights and downy mildews (Fry and Grünwald 2010), such as, late blight of potato, sudden oak death, downy mildew of grape vine, root and stem rot of soybean, and root rot and gummosis of citrus (Kamoun *et al.* 2015).

Plasmopara viticola

Plasmopara viticola (Berk et Curt.) Berlese et de Toni is the causal agent of grapevine downy mildew, an important disease in all grape-growing areas with frequent rains (Lafon and Clerjeau 1988). This pathogen has been the subject of study since its introduction into European vineyards in 1878 (Gessler *et al.* 2011). *P. viticola* is a heterothallic diploid oomycete and obligate biotrophic parasite native to North America (Dick 2002; Wong *et al.* 2001), and consequently more aggressive on European *Vitis vinifera* than the American *Vitis* varieties (Agrios 2005).

P. viticola overwinters in fallen leaves as sexual dormant structures called oospores or as mycelium in dormant twigs (Fig.I-1). The primary infections usually start when the oospores germinate producing sporangia that release zoospores.

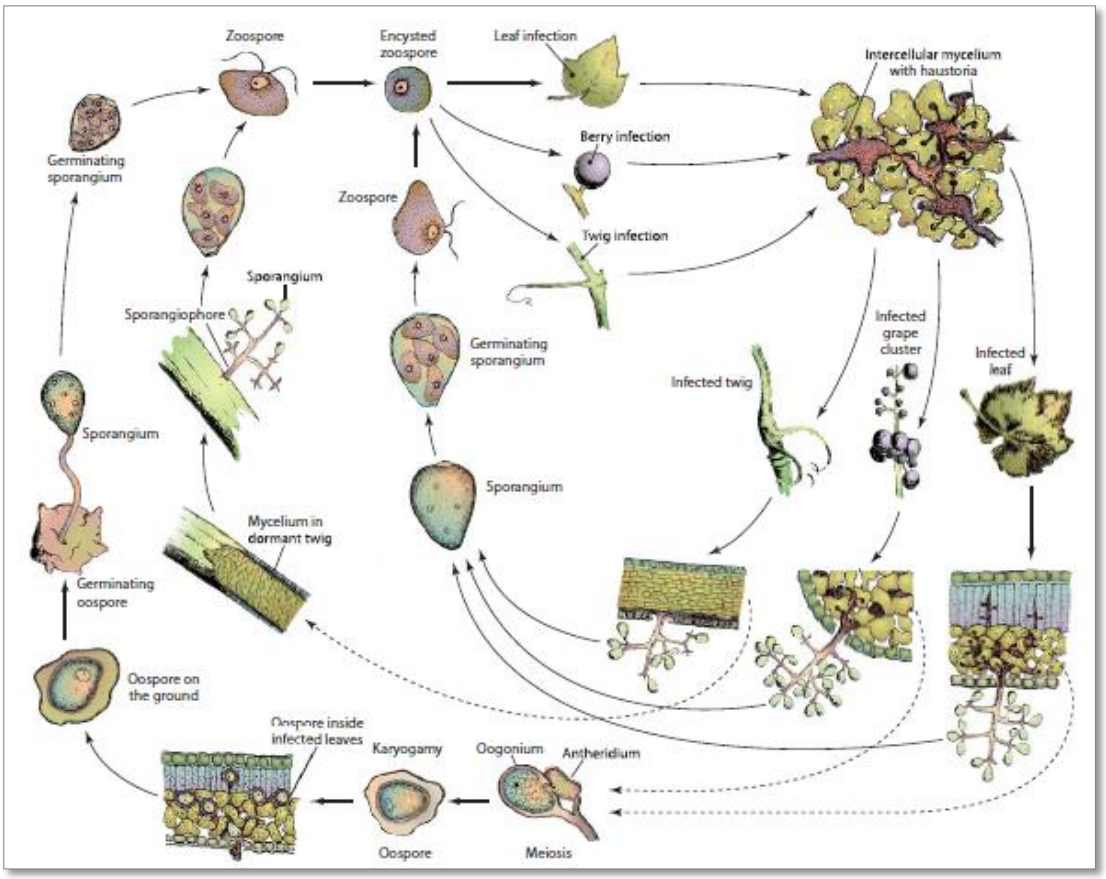

Figure I-1. Disease cycle of downy mildew of grapes caused by *Plasmopara viticola* (Agrios, 2005).

The sporangia or their zoospores are transported by wind or water, and can penetrate through stomata in grapevine wet green tissues (Agrios 2005). Early infections are recognized as yellowish lesions on leaves upper surface, known as “oil spots”. After 5 to 10 days of infection, sporangiophores and sporangia are produced forming a white-cottony mildew in lower leaf surface (Caffi *et al.* 2012), hence the name of the disease. Sporangia become airborne and release clonal zoospores as secondary inoculum. A sexual secondary infection cycles occur usually in humid nights (Caffi *et al.* 2012).

The most effective fungicides for the control of downy mildew are copper-based products such as the Bordeaux mixture and some broad-spectrum protective fungicides usually applied in combination with several systemic fungicides (Agrios 2005). Downy mildew control has been thoroughly reviewed by Gessler *et al.* (2011). Currently, the disease can be controlled with properly timed fungicide applications

that aim to control primary infections in spring and to limit the spread of secondary inoculum during the summer. In Italy for instance, during the growing season 2009-2010, an average of 12.3 treatment per hectare of area treated were used to protect vineyards (Istat 2010). Across the entire Piedmont region, in particular, the annual cost for downy mildew control typically ranges from 8 to 16 million Euros, depending on weather conditions (Salinari *et al.* 2006). Moreover, downy mildew control remains difficult as the disease cycle, including the production of primary inoculum, occurs throughout the growing season (Gessler *et al.* 2011). In addition to seasonal weather variations that can lead to unexpected disease outbreaks.

The relationship between the environmental factors, host susceptibility, and *P. viticola* has been established and numerous disease prediction models for downy mildew have been developed following different modelling approaches (Rossi *et al.* 2013). Those models are mostly weather-driven and aim to improve the fungicide schedule application while reducing the number of applications and associated costs. Two Decision Support Systems, namely "Vitebio.net™" for organic growers (Caffi *et al.* 2011) and "Vite.net™" for growers following IPM guidelines (Rossi *et al.* 2014) are currently used in several areas of Italy.

Phytophthora infestans

Phytophthora infestans (Mont.) de Bary. is the causal agent of late blight, the most devastating disease of potato (*Solanum tuberosum*). It had been introduced into Europe and North America from the Toluca Valley in central Mexico (Grunwald and Flier 2005), which triggered disastrous blight epidemics in the 1840s in Europe. Besides potato, the blight pathogen is also destructive to tomatoes (*S. lycopersicum*), and affects other hosts including hairy nightshade (*S. sarrachoides*), petunia (*Petunia hybrida*) and bittersweet nightshade (*S. dulcamara*) (Dorrance and Inglis 1997; Knapova and Gisi 2002; Platt 1999). *P. infestans* is considered a heterothallic oomycete, although the existence of self-fertile isolates has been reported (Prakob and Judelson 2007; Zhu *et al.* 2016). These genetic recombination lead to the emergence of new clonal lineages that bear different aggressiveness and fungicide resistance (Cooke *et al.* 2012; Fry *et al.* 2015), thus causing severe outbreaks worldwide (Chowdappa *et al.* 2015; Fry *et al.* 2013). Late blight pathogen receives, to this day, a constant attention among oomycete plat pathogens (Kamoun *et al.* 2015).

P. infestans is a hemibiotroph organism that exhibits distinct phases of its life cycle: an early asymptomatic biotrophic phase and a late necrotrophic stage that is characterized by tissue degradation and disease symptoms (Lee and Rose 2010). The oospores of *P. infestans* may survive in the soil for 3 to 4 years. They germinate by means of a germ tube that produces a sporangium (Fig.I-2). In the absence of a sexual stage, sporangia can also be produced from infected tubers or infected shoots. When mature, the sporangia are dispersed by air or by rain and cause new infections either by direct germination or indirectly by the release of zoospores (Agrios 2005). Foliar blight symptoms appear at first as water-soaked spots that turn into black/brown lesions, and expand rapidly to become necrotic. In moist weather, *P. infestans* produces sporangia and sporangiophores on the surface of infected tissue. This sporulation appears as a white mildew at the edge of the lesions on the lower leaf surface (Schumann and D'Arcy 2000). In favorable conditions, foliar epidemics may cause total destruction of all plants in a field within a week or two (Agrios 2005). On the other hand, tuber blight infections happen in the field, during rainy weather, when sporangia are washed from the leaves into the soil. The released zoospores germinate and penetrate the tubers through lenticels or through wounds (Agrios 2005). Infected tuber tissues are copper brown, reddish or purplish in color. Most of the infected tubers rot in the ground or during storage (Schumann and D'Arcy 2000).

The first step in integrated control of late blight is reducing the primary source of inoculum by the elimination of infected seed tubers and volunteer plants, but also cull piles in potato fields should be destroyed.

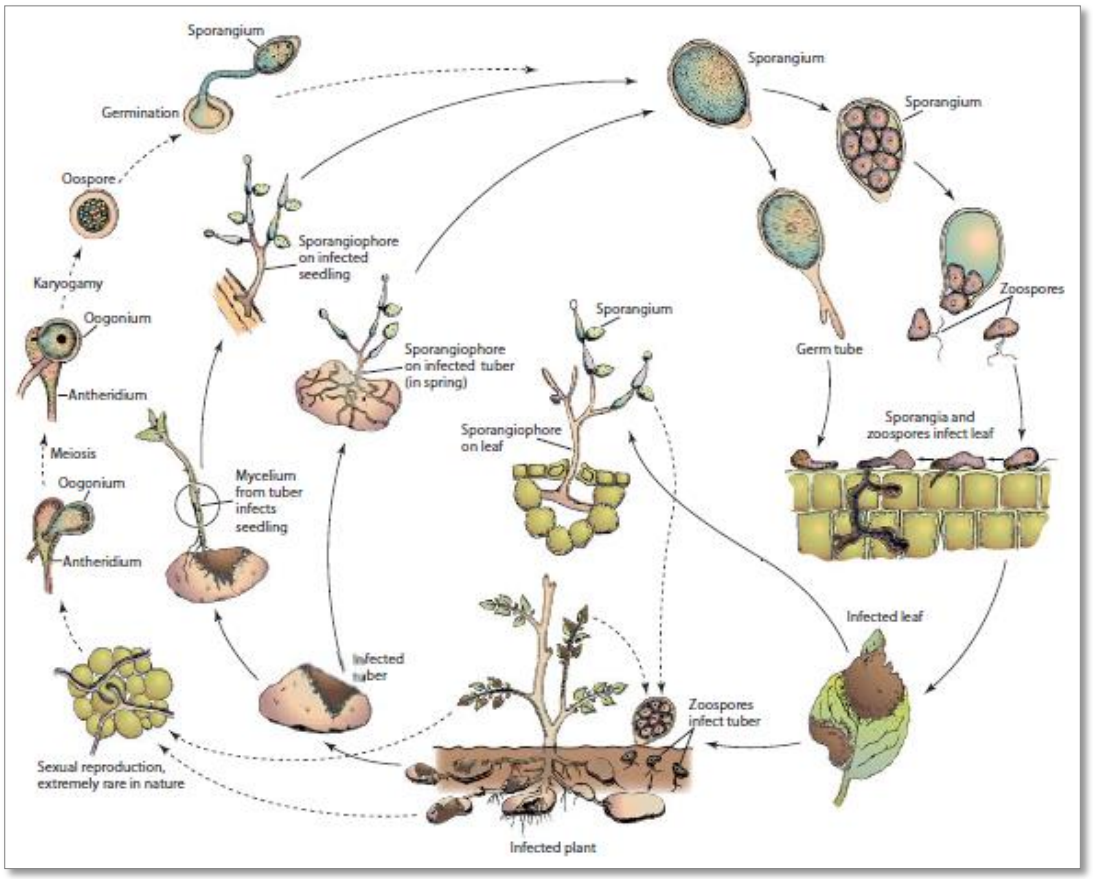

Figure I-2 Disease cycle of late blight of potato caused by *Phytophthora infestans* (Agrios, 2005).

The use late blight resistant varieties has been widely investigated in order to reduce fungicide application rates, or extend intervals between applications (Bain *et al.* 2009; Fry 1978; Nærstad *et al.* 2007; Nielsen and Bødker 2001). Conversely, these cultivars are not grown on a large scale as they do not possess the commercially important characteristics such as quality, yield and earliness (Cooke *et al.* 2011). Chemical control of late light includes the use of broad-spectrum and systemic fungicides, preferably applied with a previous knowledge of the prevailing pathogen population and fungicide resistance trait (Saville *et al.* 2015). Various late blight forecasting systems are used for well-timed chemical sprays. These Decision Supports Systems (DSSs) are regularly updated with weather information and late blight scouting inputs. Several DSSs have been developed and validated in a number of European countries (Hansen *et al.* 2002). The so-called EuroBlight network has made available a free online platform that provides an overview of the existing and new European DSSs

(http://euroblight.net). The Euroblight tool allows testing and comparing the weather based late blight sub-models, from different European regions, in order to improve their quality (Hansen *et al.* 2010). Likewise, in the United States, DSS BlightPro is a web platform that integrates considerable information useful to the management of late blight (http://usablight.org). In addition to site-specific weather data and weather forecasts, it incorporates disease reports containing information about the pathogen genotype, fungicide resistance, the host, and management technologies (Small *et al.* 2015).

Molecular diagnostics of plant pathogens

It is crucial to accurately detect and identify pathogens to initiate preventive disease control measures. An essential key in disease management is the early detection of pathogens, particularly in seeds, mother plants and propagative plant material but also in the early stages of the infection to avoid the introduction and further dispersal of the inoculum (Narayanasamy 2011). Continuous advances in DNA-based detection methods have provided fast, sensitive and reliable detection and quantification of fungal pathogens, when compared to culture-based identification methods (Capote *et al.* 2012). Most of these techniques rely on polymerase chain reaction (PCR) and real-time PCR assays and have been extensively applied to plant pathology from soil, water, air samples and plant material (Böhm *et al.* 1999; Carisse *et al.* 2009; Lievens *et al.* 2006; Schaad and Frederick 2002; Schena *et al.* 2013; van de Graaf *et al.* 2003; West *et al.* 2008). Moreover, PCR-based techniques provided highly specific assays that can discriminate between species isolates and genotypes (Abbott *et al.* 2010; Kroon *et al.* 2004).

In the aim for on-site testing of plant pathogens, the equipment for such systems is quite expensive and is not mobile to achieve testing closer to the point of sampling. Research efforts have been made to move real-time PCR technology from the laboratory to the field using portable thermocycler (Almassian *et al.* 2013; De Boer and Lopez 2012; Mavrodieva *et al.* 2004; Schaad *et al.* 2002). Despite some successful applications, these technologies have not been widely adopted as the portable thermocyclers are expensive, but predominantly, the assays require laborious modifications of DNA extraction protocols in order to adapt to field conditions (Hughes *et al.* 2006; Tomlinson *et al.* 2005). Recently, insulated isothermal PCR

(iiPCR) method, has been described for sensitive and specific detection of both DNA and RNA (Tsai *et al.* 2012). In addition, iiPCR can be performed in relatively simple and inexpensive device when compared thermocyclers (Lin *et al.* 2016).

Isothermal amplification detection methods

Isothermal amplification detection methods have been developed to overcome the use of PCR thermocyclers for a possible on-site testing. As the name suggests, isothermal amplification of DNA (or RNA) occurs at a constant temperature, which confers to some of these methods the potential to be used in the field using portable instruments (Chang *et al.* 2012). A number of reviews have fully described these methods for isothermal amplification (Gill and Ghaemi 2008; Li and Macdonald 2015; Niessen 2014; Yan *et al.* 2014).

Isothermal amplification methods rely on several approaches to generate single-stranded primer binding sites, facilitate primer annealing and template replication using DNA polymerase (or RNA polymerase) without thermal cycling. These methods can be based on:

- Transcription of RNA, such as nucleic acid sequence-based amplification (NASBA) and signal-mediated amplification of RNA technology (SMART) methods (Compton 1991; Wharam *et al.* 2001);
- Additional enzymes for the separation of the target nucleic acid and primer annealing, such as, helicase-dependent amplification (HDA) and recombinase polymerase amplification (RPA) methods (Piepenburg *et al.* 2006; Vincent *et al.* 2004);
- Strand displacement DNA polymerase for the replication of linear or circular targets such as, rolling circle amplification (RCA) and loop mediated isothermal amplification (LAMP) methods (Fire and Xu 1995; Notomi *et al.* 2000);
- Restriction enzyme nicking of primer extension products and strand displacement DNA polymerase, namely, strand displacement amplification (SDA) method (Walker *et al.* 1992).

Loop Mediated Isothermal Amplification

Loop-mediated isothermal amplification (LAMP) is an isothermal nucleic acid amplification method offering rapid, accurate, and cost-effective diagnosis of diseases. It has been described as a novel method by Notomi *et al.* (2000), and has been applied to produce highly specific and sensitive amplification of DNA or RNA. The addition of reverse transcriptase makes it possible to amplify DNA from RNA sequences (RT-LAMP) (Curtis *et al.* 2008). LAMP method has attracted much attention, in fact, numerous reports have been recorded to evaluate its efficiency in recognizing bacterial, viral, fungal and parasitic diseases worldwide (Damhorst *et al.* 2015; Fu *et al.* 2011; Kurosaki *et al.* 2016; Parida *et al.* 2008; Temple and Johnson 2011). In the initial phase of development, LAMP has been applied to many kinds of pathogens causing food-borne diseases, such as many LAMP kits for detecting *Salmonella*, *Legionella*, *Listeria*, verotoxin-producing *Escherichia coli*, and *Campylobacter* have been commercialized (Mori and Notomi 2009). Recently, a growing interest in this method has been also observed in the detection of plant pathogenic agents, especially with the possible on-field application through portable devices (Harper *et al.* 2010; Keremane *et al.* 2015; Moradi *et al.* 2012; Thiessen *et al.* 2016; Tomlinson *et al.* 2010a; Villari *et al.* 2016).

The mechanism of the LAMP reaction can be explained through two steps: i) a starting structure producing step, i.e.: the double stem-loop (Fig.I-3b) and ii) a cycling amplification step (Fig.I-3c). LAMP does not require initial template denaturation (Nagamine *et al.* 2001) and employs a DNA polymerase with strand-displacement activity, along with two forward and backward inner primers (FIP, BIP) and outer primers (F3, B3) which recognize six separate regions (Fig.I-3a) within a target DNA (Tomita *et al.* 2008).

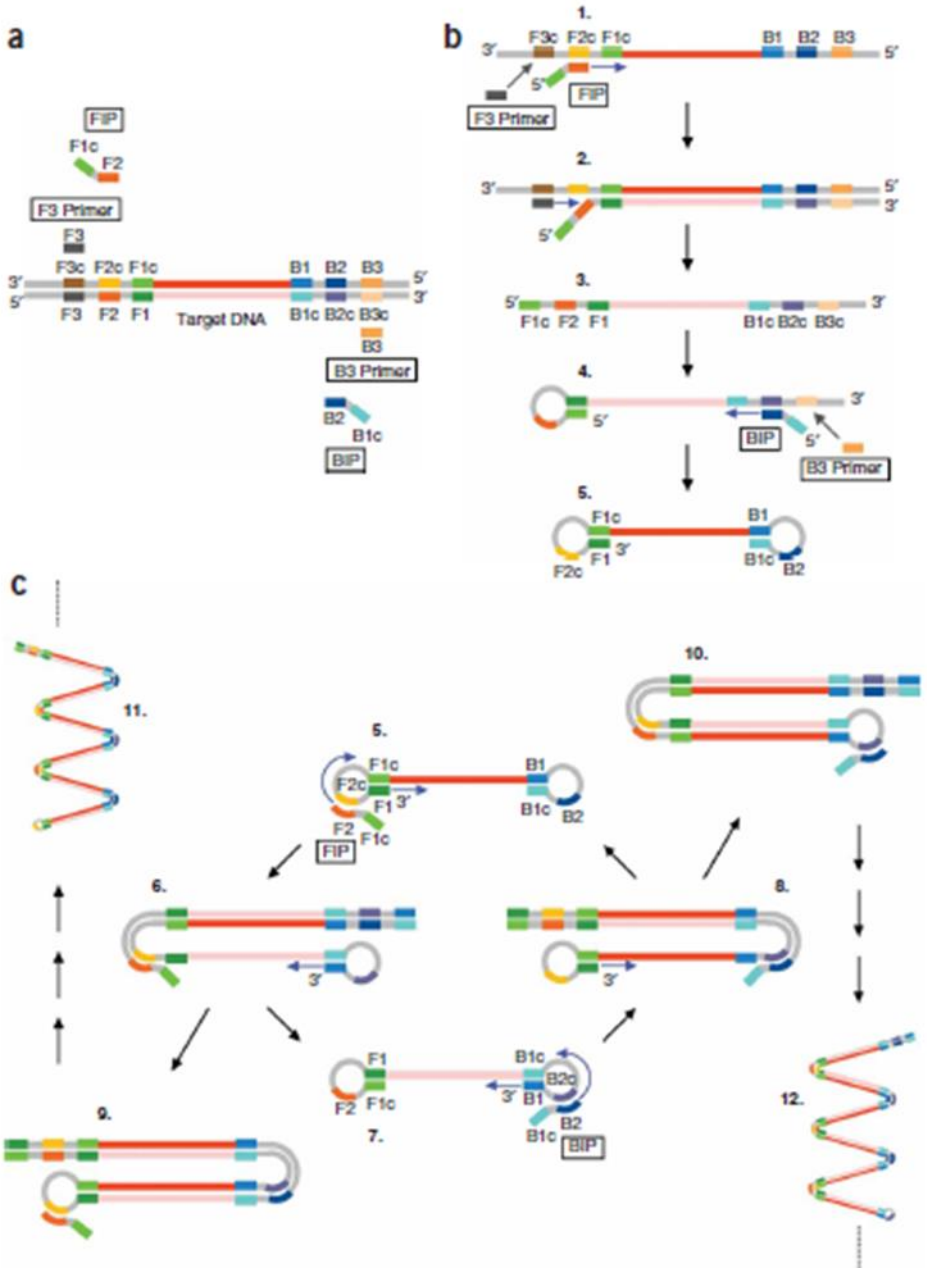

Figure I-3 Principle of loop-mediated isothermal amplification (LAMP) method.

(a) LAMP outer primers (F3 and B3) and inner primers (FIP and BIP) targeting six distinct regions within a target DNA; **(b)** Starting structure producing step: FIP primer anneals to the targeted sequence and initiates DNA synthesis. F3 primer carries out the strand-displacement DNA synthesis and produces a single stranded DNA that forms a DNA loop structure at 5' end. This structure works as a template for B3 and BIP primers that anneal to the 3' end. A double stem-loop structure is formed; **(c)** Cycling amplification step: FIP and BIP primers bind to the loops and synthesize new DNA strands. The extension of each primer opens the loop end and elongated structures are continuously produced. Source: Tomita *et al.* 2008.

The loop primers (LF, LB) are additional primers designed to anneal at the loop structure in LAMP amplicons, can accelerate and enhance the sensitivity of the LAMP reaction (Nagamine *et al.* 2002). An animation of the amplification process is available on the Eiken Chemical website for better understanding of the LAMP principle (EikenChemical).

The LAMP assay is highly specific as the amplification reaction occurs only when the primers correctly recognize all six regions, within a target DNA. The procedure is rapid and is able to amplify from a single copy to 10^9 in one hour at constant temperature; typically in the range of 60–70°C (Notomi *et al.* 2000). Moreover, it was reported that adding in the loop primers (LF, LB) can reduce the amplification time from the previous one hour to less than 30 minutes (Ushikubo 2004). It is robust, with reagents stable at ambient temperature for up to 2 hours (Thekisoe *et al.* 2009), and consistently insensitive to extraneous nucleic acids or interference from sample or media components that are problematic for other detection methods (Kaneko *et al.* 2007; Niessen 2014). However, the main limitation of the LAMP method is the primers design that is complicated and different from the usual PCR primers design, making it difficult for beginners. Even though, software for LAMP primer design are available (e.g. Primer Explorer and LAMP Designer), optimal primers performance is not certain and many primers set candidates should be evaluated when developing an assay.

Several methods have been reported to detect LAMP products: the existence of ladder-like bands on agarose gel, the use of lateral-flow-devices, visual observation of turbidity derived from magnesium pyrophosphate formation, real-time measurement of turbidity, as well as colorimetric LAMP assays using DNA or metallic ions binding dyes (Parida *et al.* 2008; Tomita *et al.* 2008; Tomlinson *et al.* 2010b, a). The peculiarity of the LAMP reaction is that a large amount of magnesium pyrophosphate is precipitated as a by-product of the reaction that enables visual assessment of amplification (Mori *et al.* 2001). Moreover, these methods generally use *Bst* DNA polymerase (*Bacillus stearothermophilus*) (Hafner *et al.* 2001) and different dyes as fluorescent indicators for positive or negative results. These dyes can be added after the reaction is complete, for example, SYBR Green I as DNA binding dye (Notomi *et*

al. 2000), or pre-added in the reaction mix, such as calcein and hydroxynaphthol blue as metallic ions binding dyes (Wastling *et al.* 2010).

Since LAMP reaction generates tremendous amount of self-replicating amplicons, it is often strongly advised not to open completed LAMP reaction tubes to detect LAMP products (Keremane *et al.* 2015; Tomita *et al.* 2008). Therefore, monitoring the isothermal LAMP reaction in real-time has been made available using a LAMP master mix that contains an engineered GspSSD LF DNA Polymerase with strand displacement and reverse transcriptase activities and a double-strand DNA binding dye (OptiGeneLimited). This reagent contains also a pyrophosphatase that hydrolyses magnesium pyrophosphate and therefore does not allow visual observation of the LAMP amplification. Moreover, a fluorescence resonance energy transfer (FRET)-based probes technology was described by Kubota *et al.* (2011), namely, assimilating probes. These probes are designed in a particular architecture, where a quenching strand is displaced from a partially complementary fluorescent strand, during the process of DNA synthesis (Fig.I-4). Assimilating probes have been used for sequence-specific detection of the LAMP amplicon, but also for quantification and multiplexing purposes (Kubota and Jenkins 2015; Tanner and Evans 2014; Villari *et al.* 2016).

Recombinase Polymerase Amplification

Piepenburg *et al.* (2006) first introduced recombinase polymerase amplification (RPA) as another isothermal technique, similar to HDA. The RPA process employs three enzymes – a recombinase, a single-stranded DNA-binding protein (SSB) and strand-displacing polymerase. The RPA reaction initiates with the combination of the recombinase with each of the two primers prior to their annealing to specific sequences in the target (Fig.I-5). After the primer annealing, the recombinase detaches from the primers making their 3' end accessible to the *Sau* polymerase (*Staphylococcus aureus*). This form a D-loop structure, which is stabilized by the SSB protein to keep the DNA open as the DNA polymerase carries on the amplification.

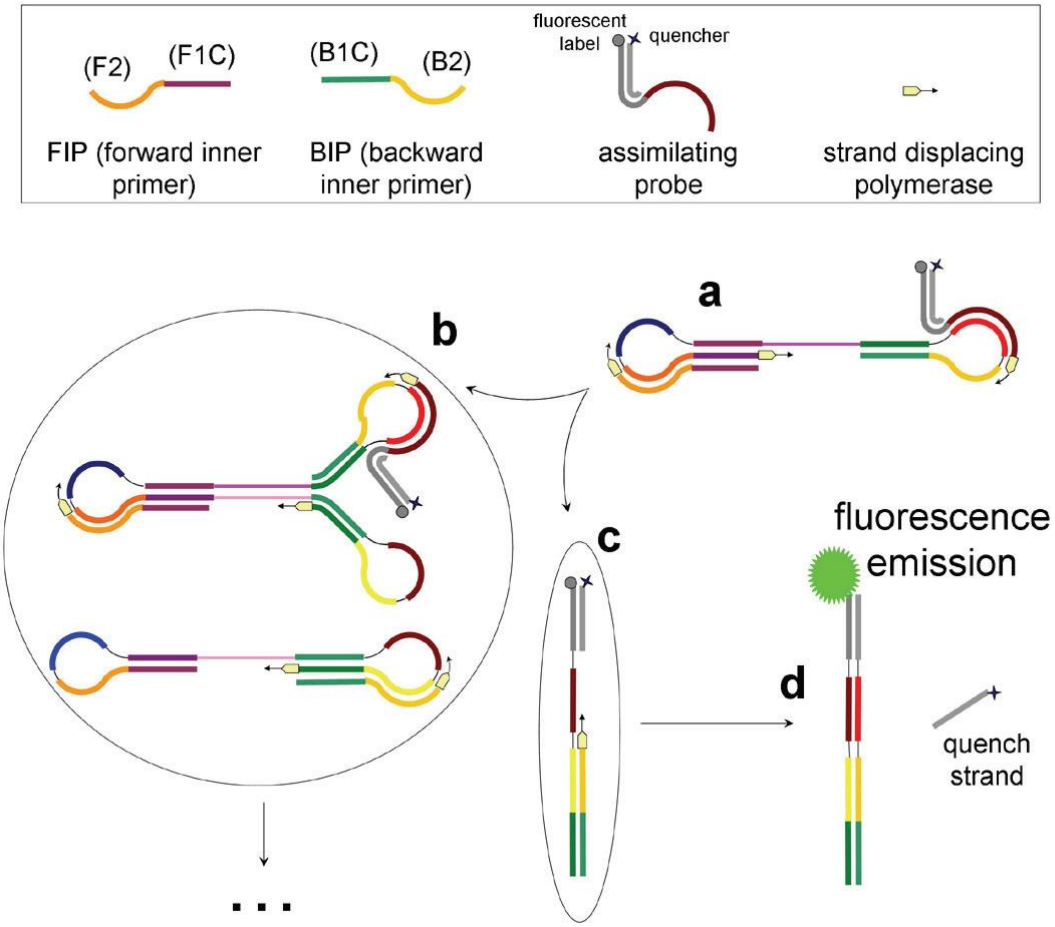

Figure I-4 Principle of assimilating probe with LAMP amplification process.

(a) the inner primer and assimilating probe anneal to the double stem-loop structure and new DNA strands are synthetized; **(b)** secondary products from self-primed double stem-loop, extension of inner primer and assimilating probe; **(c)** assimilating probe extended sequence separated from the double stem-loop and annealed to inner primer BIP; and **(d)** quench strand displaced by synthesis of new strand from BIP, resulting in fluorescence emission. Source: Kubota *et al.* 2011.

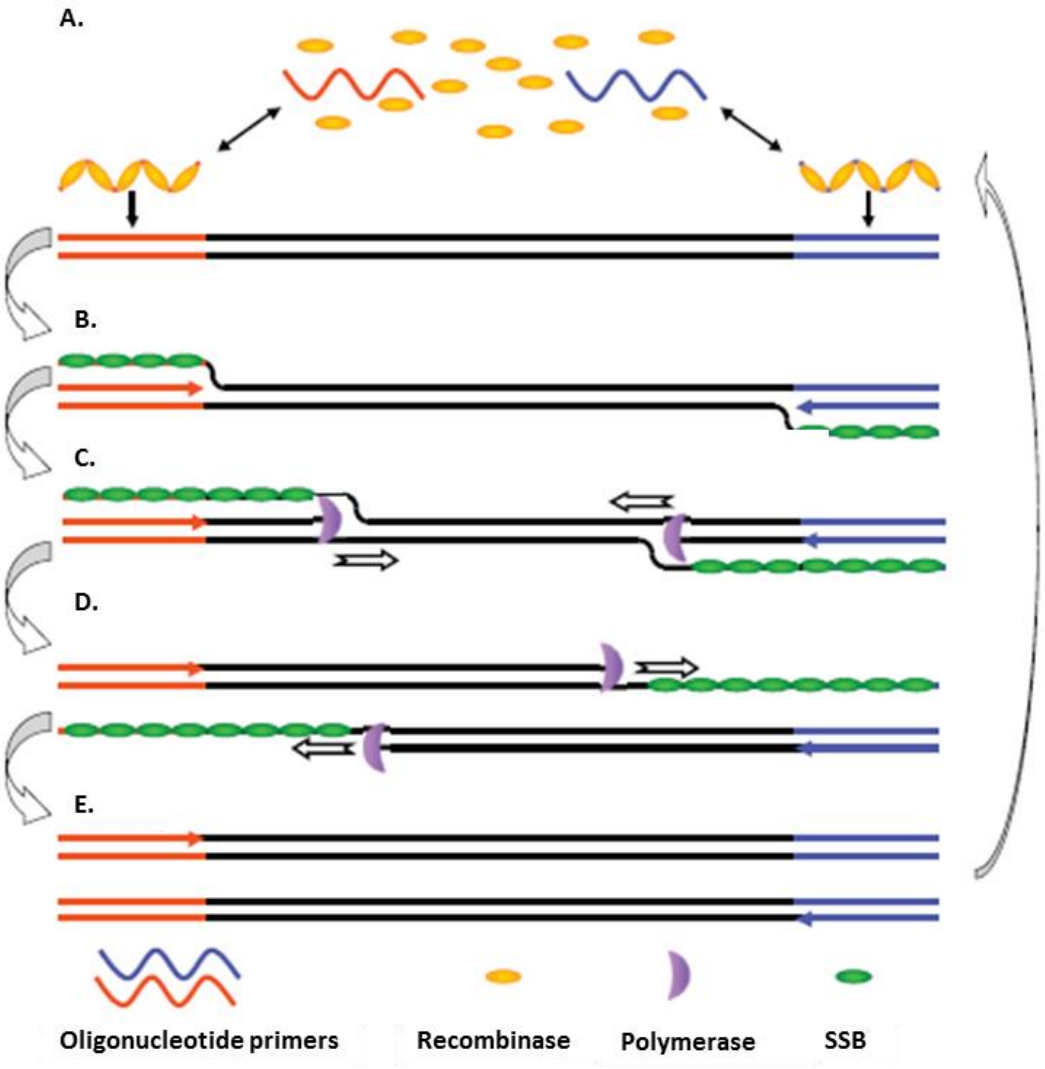

Figure I-5 Schematic outline of the recombinase polymerase amplification (RPA)

(A) Recombinase integrates with primers to form recombinase-primer complexes and target specific DNA sequences. **(B)** Strand exchange occurs and single stranded binding proteins (SSB) bind to the DNA to form a D-loop. **(C)** DNA polymerase initiates DNA amplification. **(D)** Displaced D-loop stabilized by SSB as amplification continues. **(E)** Two dsDNA molecules form and the entire cycle starts again. Source: TwistDx-Limited 2013.

RPA is very efficient, as billions of DNA copies can be generated within 40 to 60 minutes at incubation temperatures between 37 °C and 42 °C (Piepenburg *et al.* 2006). Since primers form a complex with the recombinase to target the homologous sequences, RPA primer design is simple without consideration of annealing temperature. However, the primers required for RPA are longer than PCR primers (30 to 35 nucleotides), and are often reported to generate high background noise in low temperature RPA reactions (Yan *et al.* 2014). Methods that are used to detect PCR or HDA products can be applied to detect RPA products. In order to improve amplification and detection efficiency, the use of lateral flow devices and real-time RPA detection are made possible through the design of unique probes: specifically, TwistAmp® LF probe for lateral flow detection, and TwistAmp® fpg probe and TwistAmp® exo probe for real-time detection (TwistDx-Limited 2016). Exo probes carry internal fluorophore and quencher linked to thymine bases and separated by an abasic site mimic (tetrahydrofuran or THF), that is localized approximately 15 nucleotides upstream from the 3' end of the probe (45 to 55 nucleotides). In addition, a suitable 3' modification group blocks probes from polymerase extension (Fig.I-6). The THF residue presents a substrate for Exonuclease III present in the TwistAmp® exo kit, which will cleave the exo probe at the THF position, thereby separating the fluorophore and the quencher and generating a fluorescent signal for real-time detection (TwistDx-Limited 2013).

RPA method has been extensively reported in clinical applications (Abd El Wahed *et al.* 2015; Abd El Wahed *et al.* 2016; Boyle *et al.* 2014; Daher *et al.* 2016; Euler *et al.* 2013; Hill-Cawthorne *et al.* 2014). Besides, a number of RPA assays have been described to detect plant pathogens. Reverse transcriptase RPA (RT-RPA) has been applied for the detection of important plant viruses, such as plum pox virus, little cherry virus, and tomato yellow leaf curl virus, and has proven to be more cost effective and sensitive than RT-PCR and ELISA (Londoño *et al.* 2016; Mekuria *et al.* 2014; Silva *et al.* 2015; Zhang *et al.* 2014).

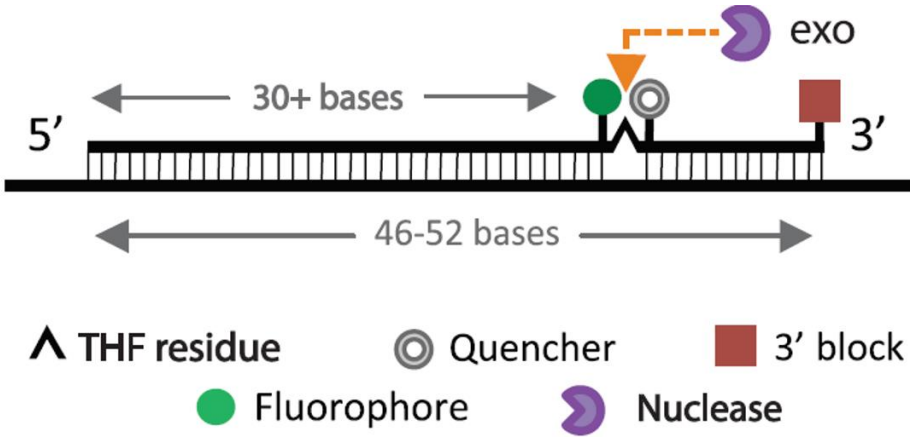

Figure I-6 Schematic outline of the TwistAmp® exo probe for RPA real-time detection. Exonuclease cuts the THF residue during RPA amplification. Source: TwistDx-Limited 2013

Miles *et al.* (2015) recently used RPA approaches to develop a genus-specific assay for detection of *Phytophthora* spp., and other assays for *P. ramorum* and *P. kernoviae* species detection from crude plant extract.

On-site detection of plant pathogens

Early detection of plant pathogens is crucial for timely disease management. On-site nucleic acid-based methods that can be performed with minimal equipment, rapidly and at low cost are meeting a growing interest in plant pathology. Isothermal amplification detection methods have a greater portability potential over PCR-based methods (LaBarre *et al.* 2011). For instance, LAMP method combined with lateral flow strips or portable fluorometers have been developed to enable field detection of plant pathogens (Bühlmann *et al.* 2013; Keremane *et al.* 2015; Mekuria *et al.* 2014; Rigano *et al.* 2014; Thiessen *et al.* 2016). More recent handheld instruments are available for real-time isothermal detection, such as: Genie III (Optigene Ltd., Horsham, UK), T8 isothermal device (TwistDx Ltd., Cambridge, UK), and Smart-DART V3.0 device (Diagenetix Inc., Honolulu, HI, USA). Most of these instruments consist of simple heating block, with a testing capacity of eight standard 0.2 ml tubes, and usually include dual channel fluorescence measurement to allow the use of internal controls and multiplexed assays. Some of them also provide positional information through GPS in addition to wireless connectivity via Bluetooth and Wi-Fi.

Surely, phytopathology can benefit from the use of affordable and robust on-site assays as plantations can be distant from diagnostic laboratories, in particular the interval of time between sampling and diagnosis, and in some cases it would be highly recommended to perform testing in the sampling site (e.g. quarantine plant pathogens). On-site molecular testing requires not only a portable platform and suitable assay but also a simple and robust alternative DNA extraction method, which can be performed in the field. Preparation of a sample has traditionally been difficult and time consuming. It is particularly known that plant tissue samples require DNA extraction methods that are able to efficiently wash away any chemical compound that can inhibit the DNA amplification reaction (Porebski *et al.* 1997). However, isothermal assays showed to efficiently detect plant pathogenic DNA from crude extract samples (Fukuta *et al.* 2004; Keremane *et al.* 2015; Londoño *et al.* 2016; Miles *et al.* 2015; Tomlinson *et al.* 2005), following simple and short sample preparation methods.

Thesis objectives

The aim of this research project was to investigate the use of isothermal amplification detection methods for the detection of Oomycete plant pathogens, by developing assays potentially suitable for on-site deployment.

Specific goals were:

1- to develop a real-time LAMP assay for the early detection of *Plasmopara viticola* from infected grapevine plants (Chapter II);

2- to develop real-time LAMP and RPA assays for on-site detection of *Phytophthora infestans* in potato leaves (Chapter III);

3- to investigate the application of the real-time LAMP assay for the detection and quantification of airborne inoculum of *Phytophthora infestans* (Chapter IV).

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Chapter II

II REAL-TIME LAMP FOR THE EARLY DETECTION OF *PLASMOPARA VITICOLA* FROM INFECTED PLANTS

The experiments described in this chapter were carried out during a research internship in the Edmund Mach Foundation in San Michele all'Adige, Department of Plant Protection, Trento (Italy) under the supervision of Dr. Ilaria Pertot. The LAMP trials were inserted as a tool for early detection of *P. viticola* infection in grapevine plant tissue.

Introduction

The European Union (EU) is the world's leading producer of wine. In 2015, Italy (29.4 %), France (26.3 %) and Spain (23.6 %) were the EU countries producing most grapes for wine use, making up 79.3 % of total production (EurostatReport 2016). Fungal diseases represent a constant threat to grapevine production causing significant economic losses. In areas with frequent rains, grapevine downy mildew, caused by *Plasmopara viticola* (Berk et Curt.) Berlese et de Toni, is an important disease in all vineyards planted with susceptible cultivars of *Vitis vinifera* (Lafon and Clerjeau 1988). Despite the progresses in disease management strategies and new breeding programs, chemical control remains the most important measure to manage grapevine diseases. In Italy for instance, during the growing season 2009-2010, an average of 12.3 treatments per hectare of area treated were carried out to protect vineyards, which corresponds to 26.6 kilograms of plant production products used per hectare (Istat 2010). Particularly for downy mildew control, the annual cost for the disease management typically ranges from 8 to 16 million Euros, across the entire Piedmont region depending on weather conditions (Salinari *et al.* 2006).

In the perspective of a sustainable use of pesticides (EU Directive 2009/128/EC), alternative approaches to downy mildew control has been considered, including biocontrol agents, natural substances, and physical methods. However, they are not capable to prevent the disease, especially under high disease pressure (Dagostin *et al.* 2011; La Torre *et al.* 2005). It is even more difficult to control the disease since the life cycle of *P. viticola*, including the production of primary inoculum, occurs throughout the growing season (Gessler *et al.* 2011), not counting seasonal weather

variations that can lead to unexpected disease outbreaks. In contrast to the use of calendar sprayings, disease prediction models have contributed to the improvement of downy mildew management through timely fungicide applications (Kuflik *et al.* 2009; Rossi *et al.* 2013). However, these models are weather-based and might warn for treatments in the absence of infection. Rapid tools for monitoring the presence of the infection in the vineyard, before the symptoms appear, would help growers to intervene with more accuracy and at a higher efficiency. Several methods have been described to provide a rapid detection and quantification of *P. viticola* during early infections of grapevine leaves, such as direct Polymerase Chain Reaction (PCR), Real-time PCR, and chlorophyll fluorescence imaging (Cséfalvay *et al.* 2009; Gindro *et al.* 2014; Valsesia *et al.* 2005).

Loop-mediated isothermal Amplification (LAMP) is a molecular detection technique known to be rapid, accurate and run at a constant temperature, usually 65°C (Notomi *et al.* 2000). The main advantage of the LAMP method is the possible on-field using portable isothermal instruments (Chang *et al.* 2012). The amplification requires a strand displacing polymerase to amplify DNA, and relies on a set of six primers, which can increase the specificity, and the accuracy of the reaction. Plant tissues are known to contain potential inhibitory substances, such as polysaccharides and polyphenols, including secondary metabolites as phytoalexins and lignin (Taiz and Zeiger 2015). However, LAMP reaction is stable and insensitive to inhibitors from sample components which may cause problems in other PCR-based detection methods (Kaneko *et al.* 2007; Kogovšek *et al.* 2015; Tomlinson *et al.* 2007), making purification steps of nucleic acids more simple.

In this study, we describe the development of a real-time LAMP assay for the detection of *P. viticola* from infected grapevine leaves, with a rapid crude plant extract (CPE) preparation method. The potential of this assay is the early detection of *P. viticola* in grapevine leaves in the field as a contribution to an efficient, precise and sustainable disease management strategy.

Materials and methods

II.1.1 Design of LAMP primers

Two LAMP primer sets were designed targeting different genes specific to *P. viticola* : i) ß-tubulin gene (DQ361159), and ii) large ribosomal subunit (LSU) gene (AY250173.1). The LAMP primers, including Loop primers (Table II-1), were designed using PrimerExplorerV4 software (Eiken Chemicals, Tokyo, Japan).

II.1.2 Real-time LAMP reaction

The real-time LAMP assays for *P. viticola* detection were performed in 25 µL reaction mixtures using the Isothermal Master Mix with dsDNA intercalating dye (ISO001, Optigene, Inc., Horsham, UK), containing the GspSSD DNA polymerase. The optimized reaction mixtures contained 1 µM of the inner primers FIP and BIP, 0.1 µM of the outer primers F3 and B3, 0.5 µM of the loop primers LF and LB, and 5 µl template DNA. LAMP amplifications were carried out at 65°C for a period of 30 minutes, and real-time fluorescence value was measured every 1 min. using a real-time PCR instrument (LightCycler® 480 Instrument, Roche Diagnostics, Indianapolis, IN, USA). LAMP reaction time cut-off (min.), to determine whether samples were positive or negative, was defined based on the results of the sensitivity tests and inoculation experiments.

II.1.3 Crude plant extract preparation

A rapid crude plant extract (CPE) preparation method, with reduced laboratory equipment, was developed for LAMP testing from grapevine leaves. Briefly, leaf discs were cut using a cork borer (18 mm) and placed in a 2 ml assay tube containing 500 µl extraction buffer: 20 mM Tris-HCl; pH 8.0, 2 mM EDTA and 1% TritonX100 (Keremane *et al.* 2015). For tissue disruption, 50 mg of acid washed glass beads ≤160 µm (Sigma-Aldrich S.r.l. Milan, Italy) and 2 stainless steel grinding balls (5 mm) were added in each tube, and the samples were placed in a FastPrep instrument for 90 seconds at maximum speed.

Table II-1 LAMP primers used in this study for *Plasmopara viticola* isothermal detection

	Primer Sequence (5'-3')	Length (bp)[a]
ß tubulin LAMP primers		
Btub F3	TGCACCTACTCTGTTTGCC	19
Btub B3	AGTGATTCAAGTCGCCGTAC	20
Btub FIP[b]	CTGGTGCACCGACAACGTGG**TTTT**GTCGCCCAAGGTTTCTGAC	43
Btub BIP[c]	AATGCTGACGAGGTCATGTGCC**TTTT**GTGGGAGTGGTGAGTTTCAG	46
Btub LF	TGTAGGGCTCGACGACAGT	19
Btub LB	TGGACAATGAAGCCTTGTACG	21
LSU LAMP primers		
LSU F3	AGTGTATGCGTGCGTGTG	18
LSU B3	AGGCACCTCAGTCCCAAC	18
LSU FIP[b]	TGACGTCCCACCACAGCACA**TTTT**GTTGCAGTGGCCTTTTTGG	43
LSU BIP[c]	CTGCGGGAAATGGCCACTGA**TTTT**CGACTACTCGTCCACCAAGA	44
LSU LF	GGCACCAGCAAGCACAC	17
LSU LB	GTAGGGCTTACGCTTGCGTTTG	22

[a] bp, base pair

[b] Forward internal primer, FIP consists of two fragments, F1c and F2, separated by a TTTT spacer in bold. They are in reverse (F1c) and forward (F2) orientations.

[c] Backward internal primer, BIP consists of two fragments, B1c and B2, separated by a TTTT spacer in bold. They are in forward (B1c) and reverse (B2) orientations.

After an incubation in a water bath at 90°C for 10 minutes, the samples were centrifuged for few seconds and the lysate was diluted 1:10 in sterile Milli-Q water. The obtained CPE samples were used for the LAMP reactions promptly after preparation. Healthy CPE samples were prepared from non-infected grapevine leaf discs and used in LAMP assays as negative control.

II.1.4 Inoculum preparation

Sporangial suspensions of *P. viticola* were prepared as inoculum for the infection experiments and for LAMP sensitivity tests. Grapevine leaves, bearing 7 days-old sporulating lesions of downy mildew, were collected from infected greenhouse plants (Fig.II-1). Fresh sporangia were detached from the lesions using a brush and suspended in cold distilled water (4°C). The suspension was filtered through a cheesecloth, and examined under a light microscope for quantification. *P. viticola* sporangia decimal dilutions were prepared, placed on ice and used promptly.

Figure II-1 Sporangia-bearing grapevine leaves used for the preparation of *Plasmopara viticola* inoculum

For the LAMP specificity tests, *Botrytis cinerea* and *Phytophthora infestans* isolates were cultured on potato dextrose agar (PDA) and pea agar medium, respectively, at 20°C and 12h photoperiod for 7 days. The respective conidia and sporangia were scraped off from the culture surface, suspended in sterile distilled water and filtered through a cheesecloth. The suspensions were examined under a light microscope for quantification and the concentration was adjusted to 10^4 sporangia or conidia/ml.

II.1.5 Specificity of the LAMP assays

The specificity of the LAMP assays to *P. viticola* was tested against *B. cinerea* and *Erysiphe necator* isolates, the other most important grapevine pathogens, as well as *P. infestans* (Oomycete). In order to mimic natural conditions, grapevine leaf discs were sprayed with *B. cinerea* and *P. infestans* spore suspension (10^4 spores/ml). In addition, leaf discs were sampled from grapevine greenhouse plants infected with *E. necator*. In total, two infected leaf discs *i.e.* biological replicates, from each fungal species were collected and extracted, following the CPE preparation method previously described. Each biological replicate used in the LAMP specificity test consisted of three technical replicates. Positive control samples were prepared from grapevine leaf discs infected with *P. viticola*. Healthy CPE samples were used as negative control and sterile Milli-Q water was used as no-template control in the LAMP reactions.

II.1.6 Sensitivity of the LAMP assays

The limit of detection of the LAMP assays was determined through testing *P. viticola* sporangial decimal dilutions, as described above, either in pure suspension or with plant extract. *P. viticola* sporangial decimal dilutions were prepared, ranging from 10^4 to 1 sporangia/ml and kept in ice. Each of the sporangial dilution (200 µl) was extracted using 300 µl extraction buffer and following the above-mentioned CPE preparation method. In parallel, to determine the inhibition effect of the plant extract on the LAMP assays, a healthy grapevine leaf disc was added to the sporangial suspension before the extraction. The obtained extracts were used in the LAMP sensitivity tests in triplicates. Healthy CPE samples were used as negative control and sterile Milli-Q water was used as no-template control in the LAMP reactions.

II.1.7 Plants inoculation and experimental design

The main purpose of this experiment was to define the LAMP detection limit in inoculated grape plants, in terms of minimum time of detection (hours post inoculation - hpi) and the minimum concentration of inoculum (sporangia/ml).

In order to determine how early and how sensitive LAMP detection could be, *P. viticola* sporangial suspensions were sprayed on leaf discs, greenhouse plants (Var. Pinot nero) and field plants (Var. Pinot Grigio). Serial dilutions of the sporangial suspension were prepared, as described above, and used as spray inoculum. Each

treatment was applied in three biological replicates and water was used for non-inoculated plants (negative control). For each biological replicate, two technical replicates were used for the LAMP testing. The inoculation experiments were repeated two times to confirm the reproducibility of the assay. The experimental design is described below in Table II-2.

Leaf discs: The leaf discs were cut using a cork borer (18 mm), from non-infected greenhouse plants (Var. Pinot Nero), and placed abaxial side up on damp paper tissue in plastic boxes. The leaf discs were sprayed with sporangial suspensions of *P. viticola*, ranging from 10^5 to 10^2 sporangia/ml (Table II-2). The plastic boxes were sealed, to keep high humidity conditions, and placed in the greenhouse at 25°C for 7 days. At each time point, 3 leaf discs were collected and abundantly rinsed with sterile Milli-Q water before CPE extraction for LAMP reaction. Each leaf disc was tested by LAMP in duplicates. To determine whether the infection was successful, 2 leaf discs were placed on a damp paper tissue in Petri dishes to assess the sporulation of *P. viticola* after 7 days of inoculation.

Greenhouse plants: Three greenhouse plants (4 weeks-old) were sprayed with sporangial suspensions of *P. viticola* (10^5 sporangia/ml), and maintained at 25°C and 80% relative humidity conditions for 7 days. At each time point, 3 leaf discs (18 mm) were carefully cut from the inoculated leaves and abundantly rinsed with sterile Milli-Q water before PCE extraction for LAMP reaction. Each leaf disc was tested by LAMP in duplicates. The remaining inoculated leaves were left to assess for *P. viticola* sporulation after 7 days of inoculation.

LAMP for early detection of *Plasmopara viticola*.

Table II-2 Experimental design of *Plasmopara viticola* inoculation on leaf discs, greenhouse plants and field plants.

<table>
<tr><th colspan="2" rowspan="2">Sample</th><th rowspan="2">Biological replicates</th><th rowspan="2">*P. viticola* concentration
Sporangia/ml</th><th colspan="6">Sampling - Hours post-inoculation (hpi)</th></tr>
<tr></tr>
<tr><td rowspan="6">Leaf discs</td><td>1</td><td rowspan="4">5 discs</td><td>10^5</td><td rowspan="10">15 hpi</td><td rowspan="5">21 hpi</td><td rowspan="10">24 hpi</td><td rowspan="10">39 hpi</td><td rowspan="5">45 hpi</td><td rowspan="10">48 hpi</td></tr>
<tr><td>2</td><td>10^4</td></tr>
<tr><td>3</td><td>10^3</td></tr>
<tr><td>4</td><td>10^2</td></tr>
<tr><td>5</td><td>2 discs</td><td>water</td></tr>
<tr><td rowspan="2">Greenhouse plants</td><td>A</td><td>3 plants</td><td>10^5</td></tr>
<tr><td>B</td><td>1 plant</td><td>water</td></tr>
<tr><td rowspan="3">Field plants</td><td>a</td><td rowspan="2">3 shoots</td><td>10^5</td><td rowspan="3">Not performed</td><td rowspan="3">Not performed</td></tr>
<tr><td>b</td><td>10^3</td></tr>
<tr><td>c</td><td>1 shoot</td><td>water</td></tr>
</table>

Field plants: Grapevine plants (Var. Pinot Grigio) were inoculated with sporangia suspensions of *P. viticola* of 10^5 and 10^3 sporangia/ml. Three shoots per concentration were sprayed and covered with plastic bags, for 12h after the inoculation, to maintain high humidity conditions and protect from light. At each time point, 3 leaf discs were carefully cut from the inoculated leaves and abundantly rinsed with sterile Milli-Q water before PCE extraction for LAMP reaction. Each leaf disc was tested by LAMP in duplicates. After 7 days of inoculation, the remaining inoculated leaves were collected, and placed in humid chambers to assess for *P. viticola* sporulation.

Results

II.1.8 Specificity of the LAMP assays

The results from the specificity experiments showed that the LAMP assays did not amplify DNA of *B. cinerea*, *P. infestans* and *E. necator* from leaf discs' samples, within 20 minutes of LAMP reaction time (Rt). These results were consistent in both the β tubulin and LSU LAMP assays, indicating that the designed primers were specific to *P. viticola.*

II.1.9 Sensitivity of the LAMP assays

The analytical sensitivity of the LAMP assays was determined using *P. viticola* sporangial decimal dilutions ranging from 10^4 to 1 sporangia/ml. In pure suspension, the limit of detection of the β tubulin and LSU LAMP assays was 10^4 and 10 sporangia/ml, respectively. On the other hand, the presence of plant extract slightly delayed the reaction time (2-3 minutes) of the LSU LAMP assay (Fig.II-2), and had a strong inhibition effect on the β tubulin LAMP assay, as no consistent amplification was obtained from the replicates. In a reaction of 30 minutes, a cut-off of Rt value was fixed at 20 minutes, as a non-specific background amplification occurred from the negative control samples after 20 minutes reaction.

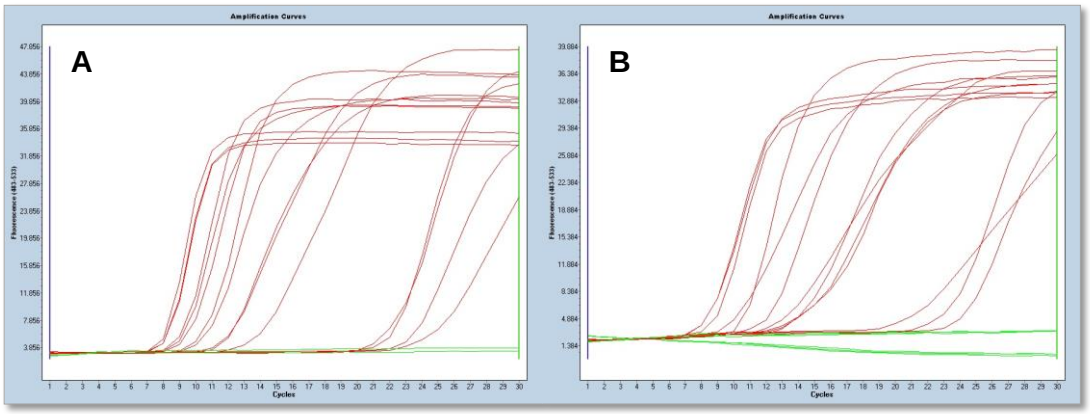

Figure II-2 Sensitivity of the LSU LAMP assay. Real time amplification curve generated in the LAMP assay using 10 fold serial dilutions of *P. viticola* A) pure sporangia suspension ranging from 10^4 to 1 sporangia/ml and B) sporangia suspension serial dilutions extracted with healthy grape leaf disc.

II.1.10 Inoculation experiments

The suitability of the LAMP assays for the early detection of *P. viticola* infections was determined by inoculating grape leaf discs, greenhouse and field plants. Leaf disc samples were collected after 15h, 21h, 24h, 39h, 45h, and 48h of inoculation. In all the inoculation experiments, the infection was successful, which was observed through the sporulation of *P. viticola* (Fig.II-3).

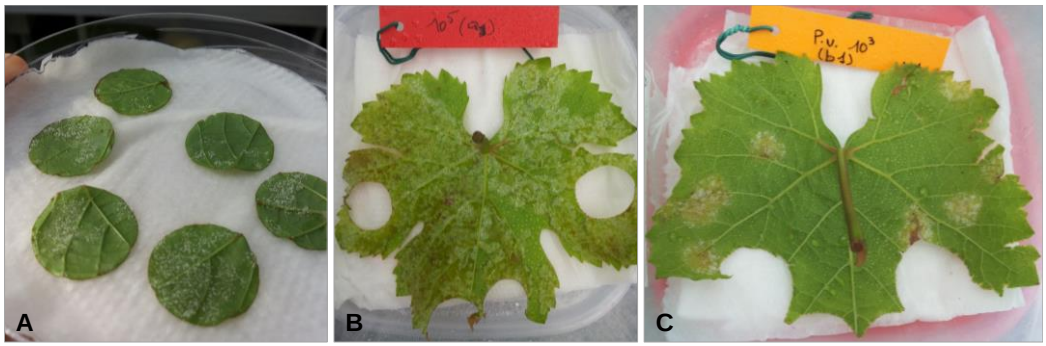

Figure II-3 *Plasmopara viticola* sporulation on the inoculated A) leaf discs, B) Greenhouse, and C) field plants.

In the LAMP assays, only the amplifications obtained in all the biological (3) and technical (2) replicates were considered as positive samples (Table II-3).

Table II-3 Results of the inoculation experiments of *Plasmopara viticola*.

Inoculation Experiments	*P.viticola* Sporangia/ml	LAMP Btubulin Hours post-inoculation (hpi)						LAMP LSU Hours post-inoculation (hpi)					
Samples		15h	21h	24h	39h	45h	48h	15h	21h	24h	39h	45h	48h
Leaf discs	10^5	Negative	Negative	Negative	Negative	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Positive
	10^4	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Positive	Positive	Positive	Positive
	10^3	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Positive	Positive	Positive
	10^2	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
	water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Greenhouse	10^5	Negative	Negative	Negative	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
	water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Field	10^5	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Not performed	Positive	Positive	Not performed	Positive
	10^3	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Not performed	Negative	Negative	Not performed	Negative
	water	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Not performed	Negative	Negative	Not performed	Negative

Positive
Negative
Not performed

N.B. Only consistent LAMP detection in biological (x3) and technical (x2) replicates were taken into account in each experiment.

The β-tubulin LAMP assay, consistently detected *P. viticola* infections in greenhouse plants and leaf discs samples, inoculated with 10^5 sporangia/ml, 45 and 48 hours post-inoculation (hpi), respectively. The assay did not detect *P. viticola* infections in the inoculated field plants and no amplification occurred in non-inoculated samples (negative control).

The LSU LAMP assay instead, consistently detected *P. viticola* infections in leaf discs inoculated with 10^5, 10^4, and 10^3 sporangia/ml, 15, 21 and 24 hpi, respectively. In greenhouse and field plants, the LSU LAMP assay, detected *P. viticola* infections using suspensions of 10^5 sporangia/ml, in 15 hpi. No amplification occurred in non-inoculated greenhouse and field plants.

Discussion

The aim of this study was to develop real-time LAMP assays for the early detection of *P. viticola*, and to determine the limit of detection of the LAMP assays on infected grapevine leaves, following a rapid crude plant extract (CPE) preparation method.

Two LAMP assays were designed to target the ß-tubulin gene and the large ribosomal subunit (LSU) gene of *P. viticola*. The assays showed to be specific to *P. viticola* when tested with *B. cinerea*, *P. infestans* and *E. necator*. However, a non-specific background amplification occurred from the negative control samples after 20 minutes reaction. Therefore, a LAMP reaction time (Rt) cut-off was fixed at 20 minutes to determine positive and negative results. The implementation of a cut-off is frequently described in real-time PCR assays, in terms of threshold cycle (Ct) value, for diagnostic purposes in order to minimize the probability of either false-positive or false-negative results (Caraguel *et al.* 2011). The real-time LAMP reactions were carried out using the Isothermal Master Mix containing a dsDNA intercalating dye, resulting to an aspecific amplification of dsDNA, especially since the LAMP amplicon is known to be extremely efficient at self-replication (Kubota *et al.* 2011). Real-time LAMP detection can be improved by a sequence-specific monitoring of the LAMP reaction, using the so-called Assimilating probes (Kubota and Jenkins 2015).

When compared, the LSU LAMP primer set showed a higher sensitivity than β-tubulin LAMP assay. The latter failed in the detection of sporangial dilutions of *P. viticola* lower than 10^5 sporangia/ml, whereas LSU LAMP primers amplified low

concentrations up to 10 sporangia/ml. The sensitivity of the LSU LAMP assay was not affected by the presence of plant extract in the CPE template, despite a short delay in the Rt values. The limit of detection of the LAMP assays was further investigated in the inoculation experiments of grapevine leaves. The LAMP results were analyzed in a qualitative approach taking into account the amplified/not-amplified samples, within 20 minutes of LAMP reaction. Both LAMP assays detected *P. viticola* infections before the appearance of symptoms. The β-tubulin LAMP assay amplified only high inoculum concentrations after 2 days of inoculation. LSU LAMP assay instead, allowed the detection of *P. viticola* infections from leaf discs, greenhouse and field trials after 15h inoculation, when using a high inoculum concentration (10^5 sporangia/ml), and after 24 h inoculation of leaf discs with a low inoculum (10^3 sporangia/ml). Despite the effective inoculation trials, observed through the sporulation of *P. viticola* in the leaf samples, the spray inoculation method used was a limiting factor for the inter-assay reproducibility. Indeed, the spray application of the inoculum lead to a non-homogenous distribution of infection site on the leaf. This resulted in a non-consistent LAMP detection from randomly sampled leaf discs. Considering biological and technical replicates have reduced this variability in the LAMP assays.

Those results compare positively with previously described DNA-based methods. A direct PCR assay with a rapid DNA extraction, and a quantitative real-time PCR assay were described to detect downy mildew infections in asymptomatic leaf samples (Gindro *et al.* 2014; Valsesia *et al.* 2005). However, the portability of the PCR-based assays is limited with the use of thermocyclers. Unlike other molecular methods, isothermal amplification detection methods are more suitable for local on-site detection using handheld instruments as simple heat block, or portable devices for real-time detection, such as: Genie III (Optigene Ltd., Horsham, UK), T8 isothermal device (TwistDx Ltd., Cambridge, UK), and Smart-DART V3.0 device (Diagenetix Inc., Honolulu, HI, USA). Kong *et al.* (2016) developed a colorimetric LAMP assay, on the basis of the ITS sequence of *P. viticola*, and concluded that LAMP is more sensitive than conventional PCR for the detection of the latent infection of *P. viticola* in grape leaves.

Several practical solutions and alternatives are now available for the grapevine growers to apply in order to prevent the negative impact of synthetic chemical pesticides (Pertot *et al.* 2016). Among these tools, disease monitoring represents a crucial component for IPM implementation in vineyards (Oliva *et al.* 1999). Being able to detect early disease on-set in the field supports in the decision of treatment application only when necessary, before extensive damage is caused, and before the spread of pathogen secondary inoculum. However, visual observation of first downy mildew symptoms in the vineyard can be difficult. An on-site real-time LAMP assay can be used for the early detection of *P. viticola* infection. This early evaluation needs to be improved by further validation with field samples using proper sampling design (Seem *et al.* 1985). More attention can be given to the lower canopy part of the vine, having the highest risk of initial downy mildew lesions resulting from rain splashes (Rossi and Caffi 2012). This tool would be helpful in decision-making regarding the control of grape downy mildew.

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Chapter III

III DEVELOPMENT OF REAL-TIME ISOTHERMAL AMPLIFICATION ASSAYS FOR ON-SITE DETECTION OF *PHYTOPHTHORA INFESTANS* IN POTATO LEAVES

The research project described in this chapter was set up under the supervision of Dr. Odile Carisse, Research and Development Centre Agriculture and Agri-Food Canada, Saint-Jean-sur-Richelieu, QC (Canada), and the collaboration of Dr. Guillaume Bilodeau, Canadian Food Inspection Agency, Ottawa, ON (Canada). The experiments were carried out in both institutions in the context of a research internship abroad as intended in the doctoral program.

The goal of the study was to develop isothermal detection assays (LAMP and RPA) for a species-specific identification of *P. infestans* and a reliable early on-site diagnosis of the disease as a contribution to late blight management.

The outcome of this research has been accepted for publication in Plant Disease journal:

Si Ammour, M., Bilodeau, G. J., Tremblay, D. M., Van der Heyden, H., Yaseen, T., Varvaro, L., and Carisse, O. 2016. Development of real-time isothermal amplification assays for on-site detection of *Phytophthora infestans* in potato leaves. Plant Dis. Accepted on Jan. 2017.

Introduction

Late blight, caused by the oomycete *Phytophthora infestans* (Mont.) de Bary, has historically been an important disease in solanaceous plants and remains a major constraint to the production of potato and tomato worldwide (Fry *et al.* 2015; Kamoun *et al.* 2015). Potato late blight infections are considered a perpetual threat to global food security causing considerable economical losses estimated at billions of US Dollars yearly (Cooke *et al.* 2012; Haverkort *et al.* 2009). Recently, the appearance of highly aggressive genotypes of *P. infestans*, which are resistant to the most widely used phenylamide fungicides, and the spread of A1 and A2 mating type isolates, resulting in sexual recombination, have caused severe outbreaks (Chowdappa *et al.* 2015; Cooke *et al.* 2012; Danies *et al.* 2014; Fry and Goodwin 1997; Fry *et al.* 2013; Gisi *et al.* 2011; Peters *et al.* 2014). Potato late blight is a polycyclic disease with under favorable condition multiple short infection-sporulation cycles. Consequently, disease progress is influenced by the amount of initial inoculum and weather

conditions favoring the completion of sporulation-infection cycles. Late blight has the potential to progress rapidly which challenges disease management and explains why several potato growers are risk averse, and the large adoption of scheduled preventive fungicide. However, in practice, time to disease onset varies significantly from one year to another (Fall *et al.* 2015b). Regardless of the late blight management strategy; conventional, integrated or organic, being able to detect early infections is crucial for disease containment. Determining the occurrence or risk of initial infection is achieved using weather-based forecasting systems (Arora *et al.* 2014; Cooke *et al.* 2011; Small *et al.* 2015) or airborne monitoring devices (Fall *et al.* 2015b). Being a destructive disease, late blight tolerance level is very low (Stein and Kirk 2002), hence early detection methods could be helpful to accurately and rapidly confirm the presence of *P. infestans* and to trigger management actions.

Over the past decades, advances in DNA-based molecular diagnostics and DNA sequencing have enabled accurate detection and characterization of the *Phytophthora* genus (Bilodeau *et al.* 2014; Cooke *et al.* 2007; Martin *et al.* 2012), including *P. infestans* (Haas *et al.* 2009; Hussain *et al.* 2014). Several molecular tools, such as Polymerase Chain Reaction (PCR) and real-time PCR, offered not only better comprehension of pathogen–host interaction, pathogenicity (Avrova *et al.* 2003; Khavkin 2015) and *P. infestans* population genetics (Cooke and Lees 2004; Gagnon *et al.* 2016; Li *et al.* 2012), but also reliable detection and/or quantification of airborne sporangia (Fall *et al.* 2015b), and pathogen inoculum from infected plants and soil (Böhm *et al.* 1999; Fry 2016; Judelson and Tooley 2000; Lees *et al.* 2012; Llorente *et al.* 2010; Trout *et al.* 1997).

Isothermal nucleic acid amplification technologies have a significant advantage over PCR-based methods, as they can be implemented in a single step process at a constant temperature (Li and Macdonald 2015). Removing the need for thermal cycling allows for on-site diagnostics to be carried out using small and handy instruments (Chang *et al.* 2012). The most commonly used isothermal technique is Loop-Mediated Isothermal Amplification (LAMP), known to be rapid, accurate and requiring the use of a strand displacing polymerase to amplify DNA, typically *Bst* polymerase (Notomi *et al.* 2000). The amplification relies on a set of four to six primers, specially designed to recognize six to eight distinct regions of a target gene, resulting in high efficiency and specificity (Tomita *et al.* 2008). Moreover, LAMP has been shown to be tolerant to inhibitory substances present in biological samples, hence, simple and rapid sample preparation methods, without DNA purification steps, are sufficient for LAMP

assays (Kaneko *et al.* 2007; Niessen 2014). Recently, several *Phytophthora* species-specific assays have been developed using different methods to detect LAMP products (Chen *et al.* 2013; Dai *et al.* 2012; Dong *et al.* 2015; Tomlinson *et al.* 2010). Hansen *et al.* (2016) developed colorimetric LAMP assays for the detection of *P. infestans* using hydroxynaphthol blue in a closed-tube reaction. Since LAMP reaction generates up to 10^9 self-replicating amplicons within a 1 hour reaction (Notomi *et al.* 2000), it is often strongly advised not to open completed LAMP reaction tubes to detect LAMP products. Therefore, monitoring the isothermal LAMP reaction in real-time has been described using double-strand DNA binding dye (Keremane *et al.* 2015), or FRET-based assimilating probes technology as previously described by Kubota *et al.* (2011) for a sequence-specific detection (Kubota and Jenkins 2015; Tanner and Evans 2014). Moreover, the use of portable devices for real-time monitoring of the LAMP reaction allows performing on-site diagnostics and point-of-care testing.

Another relatively new and promising isothermal technique that can be implemented for on-site diagnostic is Recombinase Polymerase Amplification (RPA). RPA uses recombinase and co-enzymes, which form complexes with the primers, to facilitate the annealing of primers into a double stranded template and initiate the amplification (Piepenburg *et al.* 2006). Several types of RPA kits (TwistDx Ltd., Cambridge, UK) are available to develop specific assays through the design of a primer pair (30-35 bp), and a fluorescent probe with unique structure (46-52 bp), namely TwistAmp™ exo probe, for real-time detection (TwistDx-Limited 2016). Recently, Miles *et al.* (2015) used RPA approaches to develop a genus-specific assay for detection of *Phytophthora* spp., and other assays for *P. ramorum* and *P. kernoviae* species detection.

These methods can be used for rapid and accurate on-site detection of *P. infestans* in plant material and improve diagnosis of the disease, especially when infected plants are symptomless or late blight lesions can be atypical or similar to symptoms caused by other pathogens (Judelson and Tooley 2000). Stein and Kirk (2002) suggested an action threshold for fungicide application of 1% diseased leaf area, supporting a need for improved late blight field diagnostic.

The objectives of this study were to (i) develop real-time LAMP and RPA assays specific to *P. infestans*, (ii) develop a rapid crude plant extract (CPE) preparation method for on-site diagnostic using a Smart-DART platform, and (iii) establish the sensitivity of the assays on infected plant material.

Materials and methods

III.1.1 Fungal isolates.

DNA of the different fungal isolates and *P. infestans* cultures were provided from different sources listed in Table III-1. Cultures of *P. infestans* isolates were maintained on Rye B Agar medium (Caten and Jinks 1968) and kept at 20°C and 12h photoperiod.

About 2 mg of fresh mycelium was collected from the surface a 10 days old culture, using a sterile scalpel, and DNA was extracted using DNeasy plant mini kit (QIAGEN, Hilden, Germany) folowing the manufacturer's instructions. DNA of the different fungal isolates and of *P. infestans* was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and kept at -20 ºC until use in the sensitivity and specificity tests.

III.1.2 Design of LAMP primers and assimilating probe.

Sequences of the ITS region of *Phytophthora* and *Pythium* species (Coffey *et al.* 2009) were compared to identify potential regions specific to *P. infestans*. The LAMP target sequence was selected in the ITS2 region and includes the *P. infestans* specific region previously described by Fall *et al.* (2015b). Three LAMP set candidates were designed using the online software Primer ExplorerV4 (Eiken Chemicals, Tokyo, Japan), and their analytical sensitivity was compared. The LAMP primers of the best performing LAMP set are listed in Table III-2. Additionally, the designed Loop Backward (LB) primer was modified by adding assimilating probes sequences, as developed by Kubota *et al.* (2011), to detect only LAMP amplicons of *P. infestans* in real time. The LB primer sequence was attached to a supplementary sequence bearing a fluorescent label (6-carboxyfluorescein) at the 5' end. The latter sequence is complementary to a Quencher strand (Qstrand) carrying a Black Hole Quencher-1 (BHQ) at the 3' end (Table III-2). The LAMP primers and assimilating probe were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

III.1.3 Design of RPA primers and Exo-probe.

The RPA assay was developed to target the selected ITS2 region of *P. infestans* as described above. Forward and reverse RPA primers and Exo-probe (Table III-2) were designed manually following the instructions provided by the TwistAmp exo kit (TwistDx Ltd., Cambridge, UK). RPA oligonucleotides and the Exo-probe were synthesized by Biosearch Technologies, Inc. (Petaluma, CA, USA).

Table III-1 Isolates of *Phytophthora* species and other fungal species used for sensitivity and specificity tests of the isothermal assays and tests on infected plant material. The LAMP and RPA specificty test results are indicated as amplified (+) and not-amplified (-).

Genus	species	Isolate number	Host species	Country	Source	LAMP reaction[a]	RPA reaction[a]
Alternaria	*alternata*	U429	*Brassica oleracea*	Canada	Phytodata[b]	-	-
Alternaria	*alternata*	U436A	*Brassica oleracea*	Canada	Phytodata	-	-
Colletotrichum	*coccodes*	CC	*Solanum tuberosum*	Canada	Phytodata	-	-
Fusarium	*oxysporum*	U90	*Lactuca sativa*	Canada	Phytodata	-	-
Fusarium	*oxysporum*	U99	*Lactuca sativa*	Canada	Phytodata	-	-
Fusarium	*solani*	U454	*Daucus carota subsp. sativus*	Canada	Phytodata	-	-
Fusarium	*solani*	U460	*Daucus carota subsp. sativus*	Canada	Phytodata	-	-
Phytophthora	*andina*	P13365	*Solanum brevifolium*	Ecuador	AAFC[c]	+	+
Phytophthora	*cactorum*	P0714	*Syringa vulgaris*	The Netherlands	AAFC	-	-
Phytophthora	*cactorum*	DOAM 234594	*Panax quinquefolius*	Canada	CFIA[d]	-	-
Phytophthora	*capsici*	Phyto 19235	-	Canada	Phytodata	-	-
Phytophthora	*capsici*	Phyto 19236	-	Canada	Phytodata	-	-
Phytophthora	*clandestina*	P3942	*Trifolium subterraneum*	Australia	AAFC	-	-
Phytophthora	*erythroseptica*	P1699	*Solanum tuberosum*	USA	AAFC	-	-
Phytophthora	*erythroseptica*	BR 664	*Solanum tuberosum*	Canada	CFIA	-	-
Phytophthora	*erythroseptica*	DAOM 233917	*Solanum tuberosum*	Canada	CFIA	-	-
Phytophthora	*fragariae*	P1435	*Fragaria x ananassa*	England	AAFC	-	-
Phytophthora	*fragariae*	DAOM 229204	*Fragaria x ananassa cv. Cavendish*	Canada	CFIA	-	-
Phytophthora	*fragariae var.fragariae*	BR 1057	*Rubus idaeus*	Canada	CFIA	-	-
Phytophthora	*hedraiandra*	P11061	*Viburnum tinus*	Balearic Islands, Spain	AAFC	-	-
Phytophthora	*idaei*	P6767 (T)	*Rubus idaeus*	UK	AAFC	-	-
Phytophthora	*ipomoeae*	P10225 (T)	*Ipomoea longipedunculata*	Mexico	AAFC	+	+

(Continued)

Table III. 1. Continued

Genus	species	Isolate number	Host species	Country	Source	LAMP reaction[a]	RPA reaction[a]
Phytophthora	*idaei*	P6767 (T)	*Rubus idaeus*	UK	AAFC	-	-
Phytophthora	*ipomoeae*	P10225 (T)	*Ipomoea longipedunculata*	Mexico	AAFC	+	+
Phytophthora	*iranica*	P3882 (T)	*Solanum melongena*	Iran	AAFC	-	-
Phytophthora	*mirabilis*	P3008 (T)	*Mirabilis jalapa*	Mexico	AAFC	+	+
Phytophthora	*nicotianae*	P0991	*Citrus sp.*	USA	AAFC	-	-
Phytophthora	*nicotianae*	BR 255	*Gloxinia sp.*	Canada	CFIA	-	-
Phytophthora	*phaseoli*	P6609	*Phaseolus lunatus*	USA	AAFC	+	+
Phytophthora	*pseudotsugae*	P10339 (T)	*Psendotsuga menziesii*	USA	AAFC	-	-
Phytophthora	*ramorum*	P10303	*Viburnum sp.*	Netherlands	AAFC	-	-
Phytophthora	*ramorum*	15-0076	*Rhododendron sp.*	Canada	CFIA	-	-
Phytophthora	*ramorum*	14-0075	*Viburnum sp.*	Canada	CFIA	-	-
Phytophthora	*tentaculata*	P8497	*Chrysanthemum leucanthemum*	Germany	AAFC	-	-
Pythium	*ultimum*	U201	*Lactuca sativa*	Canada	Phytodata	-	-
Pythium	*ultimum*	U307	*Lactuca sativa*	Canada	Phytodata	-	-
Pythium	*ultimum*	U528	*Daucus carota subsp. sativus*	Canada	Phytodata	-	-
Pythium	*irregulare*	R-I-NW-5a	*Rhododendron sp.*	Canada	CFIA	-	-
Rhizoctonia	*solani*	R.sol 19466	*Daucus carota subsp. sativus*	Canada	Phytodata	-	-
Rhizoctonia	*solani*	U133	*Lactuca sativa*	Canada	Phytodata	-	-
Rhizoctonia	*solani*	U238	*Daucus carota subsp. sativus*	Canada	Phytodata	-	-
Verticillium	*dahliae*	Veda 5-3303	*Fragaria ananassa*	Canada	Phytodata	-	-
Phytophthora	*infestans* US11	Pi 09-30-COI	*Solanum tuberosum*	Canada	AAFC	+	+
Phytophthora	*infestans* US22	Pi-rusinek	*Solanum tuberosum*	USA	AAFC	+	+
Phytophthora	*infestans US23*	Pi US23 NIS	*Solanum tuberosum*	Canada	AAFC	+	+
Phytophthora	*infestans US24*	Pi US24MAN	*Solanum tuberosum*	Canada	AAFC	+	+
Phytophthora	*infestans US6*	Pi 09-30-DOI	*Solanum tuberosum*	Canada	AAFC	+	+

(Continued**)**

Table III. 1. Continued

Genus	species	Isolate number	Host species	Country	Source	LAMP reaction[a]	RPA reaction[a]
Phytophthora	*infestans* US8	Pi 281-P3C10	*Solanum tuberosum*	Canada	AAFC	+	+
**Phytophthora*	*infestans* US-11	*Pi LA 1145 A1b	*Solanum tuberosum*	Canada	AAFC	+	+
**Phytophthora*	*infestans* US-24	*Pi 2011-072NB	*Solanum tuberosum*	Canada	AAFC	+	+
***Phytophthora*	*infestans* US-23	**Pi 2011-056NB	*Solanum tuberosum*	Canada	AAFC	+	+

[a] a reaction time (Rt) cut-off of 25 minutes for RPA and LAMP assays was used to determine positive and negative samples.

[b]Compagnie de recherche Phytodata inc., Sherrington (QC), [c]AAFC: Agriculture and Agri-Food Canada, St-Jean-sur-Richelieu (QC), [d]CFIA: Canadian Food Inspection Agency, Ottawa (ON).
Isolates of *P. infestans* in culture used for the *sensitivity tests and ** inoculum preparation.

III.1.4 Design of RPA primers and Exo-probe.

The RPA assay was developed to target the selected ITS2 region of *P. infestans* as described above. Forward and reverse RPA primers and Exo-probe (Table III-2) were designed manually following the instructions provided by the TwistAmp exo kit (TwistDx Ltd., Cambridge, UK). RPA oligonucleotides and the Exo-probe were synthesized by Biosearch Technologies, Inc. (Petaluma, CA, USA).

III.1.5 LAMP reaction.

The LAMP assays for *P. infestans* detection were performed in 25 µL reaction mixtures using the Isothermal Master Mix without intercalating dye (Catalog No. ISO001-nd, Optigene, Inc., Horsham, UK), containing the GspSSD DNA polymerase.

The optimized reaction mixtures contained 1.6 µM FIP and BIP, 0.2 µM of the F3 and B3 primers, 0.8 µM of the loop primer LF, 0.08 µM of the assimilating probe FLB strand, 0.12 µM of the assimilating probe Q-strand, and 3µl template DNA. LAMP amplifications were carried out at 65°C for a period of 30 minutes, and real-time fluorescence values of the assimilating probe were measured every 30 seconds either using a real-time PCR instrument (Mx3005P QPCR System, Agilent Technologies, Santa Clara, CA, USA) for standard curve analysis, or a Smart-DART instrument (Diagenetix Inc., Honolulu, HI, USA) for CPE preparation and real-time isothermal detection.

III.1.6 RPA reaction.

The RPA assay was performed in a 50 µl reaction volume using a TwistAmp exo kit (TwistDx Ltd., Cambridge, UK), 0.42 µM of each of the primers F2 and R2, and 0.12 µM of the exo-probe P2, 14 µM magnesium acetate and 29.5 µl TwistAmp rehydration buffer. A mastermix was prepared and distributed into 200 µl reaction tubes, each containing lyophilized enzymes of the TwistAmp exo kit to which 3 µl of template DNA was added. Finally, magnesium acetate (2.5 µl) was pipetted into the tube lids before closing. To initiate the reactions, closed tubes were mixed by inversion then centrifuged briefly and immediately placed into the real-time PCR instrument for standard curve analysis, or the Smart-DART instrument for CPE preparation and real-time isothermal detection. RPA amplifications were carried out at 39°C for a period of 30 minutes and real-time fluorescence values of the exo-probe were measured every 30 seconds.

Table III-2 LAMP and RPA primers and probes used in this study for *Phytophthora infestans* isothermal detection[a]

	Primer Sequence (5'-3')	Length (bp)[b]
LAMP primers		
F3	GGCATTGCTGGTTGTGGA	18
B3	CAACATTTCCCAAATGGATC	20
FIP[c]	CATTGTTCAGCCGAAGCCAA**TTTT**GCTGCGGCGTTAATGGAG	38
BIP[d]	CGCTTATTGGGTGATTTTCCTG**TTTT**CAAAGCCGATTCAAATGCCA	42
LF	CCATACCACGAATCGAGCA	19
LB	CGTGATGGACTGGTGAACCATG	22
LAMP Assimilating Probe		
FLB strand[e,f,g]	5'FAM-*ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGA*CGTGATGGACTGGTGAACCATG	62
Q-strand[h]	TCGGCATCCGCATCCGCATTCGCATCCGGGTCCTCAGCGT—3'BHQ	40
RPA primers		
2-F	GCTGAACAATGCGCTTATTGGGTGATTTTC	30
2-R	GATGCATACCGAAGTACACAACATTTCCCAAATG	34
RPA exo Probe[i]		
2-P	CATGGCTCTTTAGCTTGGCATTTGAATCGGCT[T(FAM)]T[dSpacer]C[T(BHQ-1)]GTTGCGAAGT-(3'-SPACER C3)	47

[a] LAMP primers and assimilating probe were synthetized by Integrated DNA Technologies and RPA primers and exo-probe were synthetised by Biosearch Technologies.

[b] bp, base pair

[c] Forward internal primer, FIP consists of two fragments, F1c and F2, separated by a TTTT spacer in bold. They are in reverse (F1c) and forward (F2) orientations.

[d] Backward internal primer, BIP consists of two fragments, B1c and B2, separated by a TTTT spacer in bold. They are in forward (B1c) and reverse (B2) orientations.

[e] Underlined text represents LB primer sequence used in Assimilating Probe

[f] text in italics represents Q-strand complementary sequence

[g] FAM: 6-carboxyfluorescein

[h] BHQ: Black Hole Quencher

[I] exo-probe; T(FAM): thymidine nucleotide carrying Fluorescein, dSpacer: tetrahydrofuran (THF) spacer, T(BHQ-1): thymidine nucleotide carrying Blackhole quencher1,

III.1.7 Specificity of the isothermal assays.

The specificity of the LAMP and RPA assays was tested on several isolates of 24 fungal species of *Phytophthora* and other potato fungal pathogens, including five different *P. infestans* genotypes (Table III-1), and one no template control consisting of nuclease-free water (Integrated DNA Technologies, Inc. Coralville, IA, USA). Before use, DNA concentration of the different isolates was diluted to 20 pg/µl.

III.1.8 Sensitivity of the isothermal assays.

The limit of detection of LAMP and RPA assays was determined using serial dilutions of the above-mentioned pure genomic DNA obtained from *P. infestans* cultures and DNA serial dilutions incorporated into non-diseased CPE samples, for monitoring of plant extract inhibition within the assays. DNA decimal dilutions ranging from 0.5 ng/µl to 5 fg/µl were prepared in both TE buffer pH.8 (Integrated DNA Technologies, Inc. Coralville, IA, USA) and in healthy CPE samples, similarly. Each sample from pure DNA dilutions and from DNA dilutions added to healthy CPE samples was replicated three times. Standard curves were constructed by plotting DNA serial dilutions with the correspondent Ct values with a fluorescence threshold set manually. Additionally, the LAMP and RPA amplifications were scored based on the Reaction time (Rt), in order to record any delay in the reaction due to plant extract inhibition. The use of a real time PCR instrument to carry out isothermal reactions requires the conversion of the qPCR instrument output Cycle threshold (Ct) to Rt (min.) through the application of a time multiplier. Since for both real-time LAMP and real-time RPA reactions fluorescence was measured every 30 sec., we considered Rt values equal to two times Ct values, *i.e.* Rt = 2Ct (min.). A cut-off of Rt value, to determine whether samples were positive or negative, was defined based on the results of the detection limit and tests with infected plant materials.

III.1.9 Crude plant extract preparation method.

Crude plant extract (CPE) preparation was carried out as an alternative DNA extraction method that could be used in the field with minimal laboratory equipment. Briefly, a single potato leaf disc (about 13 mg plant material) was cut using a handheld paper punch (0.6 mm), placed in a 200 µl PCR strip-tubes containing 150 µl of extraction buffer: 20 mM Tris-HCl; pH 8.0, 2 mM EDTA and 1% TritonX100 (Keremane *et al.* 2015), and heated in the Smart-DART device for 10 min at 90°C. The samples were centrifuged for few seconds in a micro-

centrifuge and the lysate was diluted 1/10 in TE buffer pH 8.0. The obtained CPE samples were used promptly, within an hour after preparation, for the LAMP and RPA reactions. For later use, the freshly cut leaf disc was placed in a 2 ml screw cap tube containing 150 µl of isopropanol 100 % and stored at – 20°C. The latter leaf disc was left to dry on a filter paper for 5 minutes before proceeding to the CPE preparation. Healthy CPE samples were prepared from non-infected potato leaf discs and used in LAMP and RPA assays as negative control.

III.1.10 Inoculum preparation.

In order to promote sporulation, *P. infestans* isolates (clonal lineage US-23) were grown on Rye B agar (Caten and Jinks 1968) at 20°C and 12h photoperiod. After 7 to 10 days of incubation, sterile distilled water was poured over the petri dish, the mycelium was gently scraped off and the suspension was filtered through a cheese cloth. Sporangia suspension was observed under a light microscope for quantification and the concentration was adjusted to 15 sporangia/µl to be used as inoculum.

III.1.11 Plants inoculation and sampling.

Leaflets from 3-week-old greenhouse-grown potato plants cv. Russet Burbank (second leaves counting from the top) were inoculated by placing a 20 µl droplet of *P. infestans* inoculum on the upper surface of each leaflet. Five plants were inoculated and placed in closed plastic cages in the bottom of a growth chamber (PGC20 growth chamber; Conviron, Winnipeg, MB, Canada) with incubation conditions of 18°C and 95% relative humidity. A first cycle of 24h of darkness was applied to promote infection followed by a photoperiod of 10h for 6 days. Sampling was performed before the inoculation (T0), at 1 day post-inoculation (dpi), 2 dpi, 3 dpi and 6 dpi. At each time point, a single leaf disc was excised from the inoculated area using a handheld paper punch (0.6 mm) and washed with distilled water, in order to remove any inoculum residues from the leaf disc before the CPE preparation and the isothermal assays. The handheld paper punch was cleaned with ethanol 70% between each sample. Two inoculated leaflets per plant were not sampled and kept for symptoms observation. Similarly, non-inoculated leaf discs were collected from 3 potato plants, on which droplets of distilled water were placed, and maintained in a separate growth chamber under the same incubation conditions. The inoculation experiment was conducted twice. LAMP and RPA assays were conducted and their sensitivity (early detection) was determined. Healthy CPE samples were included as negative control and positive control samples were prepared by adding *P. infestans* pur DNA (50 pg/ µl) into healthy CPE.

III.1.12 Field samples.

Potato late blight infections were monitored from July to September 2016. A total of 24 potato leaf samples were collected from fields with and without late blight infections observed, in New-Brunswick and Quebec provinces (Canada), respectively. The collected samples from fields with late blight infections were classified as true positives. Based on visual inspections, samples from late blight-free potato fields showing symptoms similar to P. infestans lesions were collected (Fig.III-1) and classified as true negatives. Leaf discs were excised from the edge of the lesions and were processed following the above-mentioned CPE preparation method. LAMP and RPA assays were conducted using the Smart-DART instrument and the true positive, true negative, false positive, and false negative proportions were defined and used to determine the reliability of the assays. For the LAMP and RPA reactions, healthy CPE samples were included as reaction negative control and positive control samples were prepared by adding P. infestans pur DNA (50 pg/ µl) into healthy CPE.

III.1.13 Isothermal assays using the Smart-DART instrument.

The Smart-DART platform (Diagenetix Inc., Honolulu, HI, USA) was used in our experiments as a heat block for the CPE preparation, as well as for the detection of the LAMP and RPA reactions in real-time. The platform includes a portable device able to analyze eight samples simultaneously and periodically measuring fluorescence for real-time detection of the isothermal amplification. The Smart- DART device is connected via Bluetooth to an Android device using the application provided by the manufacturer, which allows the user to control the reaction settings and view the real-time data graphically. Fluorescence readings were recorded using the channel optimized for fluorescein (FAM) every 1 minute. LAMP and RPA amplifications were carried out at 65°C and 39°C respectively, for a period of 30 min.

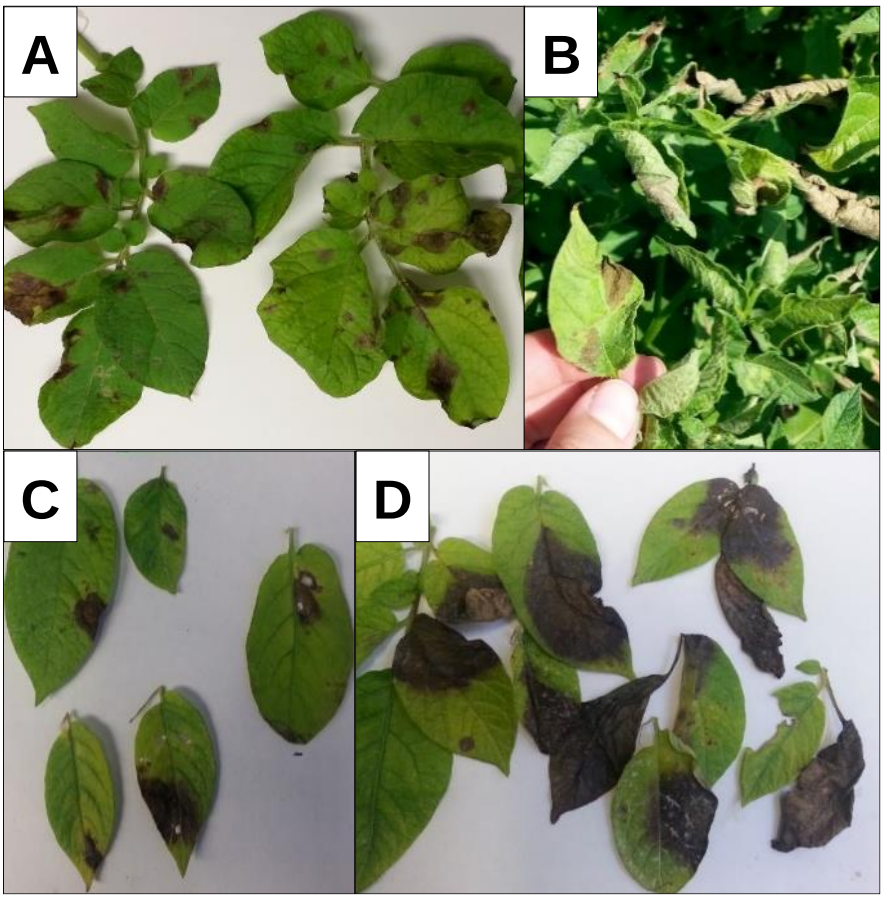

Figure III-1 Potato leaf samples collected for the reliability test of the LAMP and RPA assays. Samples were collected from **A**, **B)** potato fields without late blight infections showing symptoms similar to *P. infestans* lesions, and from **C**, **D)** potato fields with late blight infections.

Results

III.1.14 Specificity of the LAMP and RPA assays.

When tested for *P. infestans* specificity, LAMP and RPA assays presented a cross-reaction with the taxonomically closely related species *i.e. P. andina*, *P. mirabilis*, *P. phaseoli* and *P. ipomoeae*. Moreover, no cross-reaction occurred with the potato fungal pathogens tested including *P. erythroseptica* and *P. nicotiana* (Table III. 1).

III.1.15 Sensitivity of LAMP and RPA assays.

The sensitivity of the optimized isothermal assays was determined using *P. infestans* pure DNA serial dilutions ranging from 0.5 ng/µl to 5 fg/µl. The limit of detection of both LAMP and RPA assays was 50 fg/µl. Similarly, plant extract had limited effect on amplification of pure DNA that was added into healthy CPE samples. *P. infestans* detection occurred at all the concentrations up to 50 fg/µl, in both LAMP (Fig.III-2A and 2B) and RPA assays (Fig.III-3A and 3B). However, a slight delay of Rt values of 1 to 2 minutes for LAMP and 30 sec to 1 min for RPA assays was noted in samples containing plant extract.

Considering the obtained limit of detection, the reaction time of the LAMP and RPA assays was 20 minutes and 10 minutes, respectively. The obtained standard curves, plotting DNA serial dilutions with the correspondent Ct values, presented a linear correlation with a regression coefficient (R^2) of 0.980 for LAMP and 0.963 for RPA, when using *P. infestans* pure DNA serial dilutions (Fig.III-2C and Fig.III-3C). Moreover, when pure DNA was added into healthy CPE, R^2 values were 0.972 and 0.943 for LAMP and RPA, respectively (Fig.III-2D and Fig.III-3D).

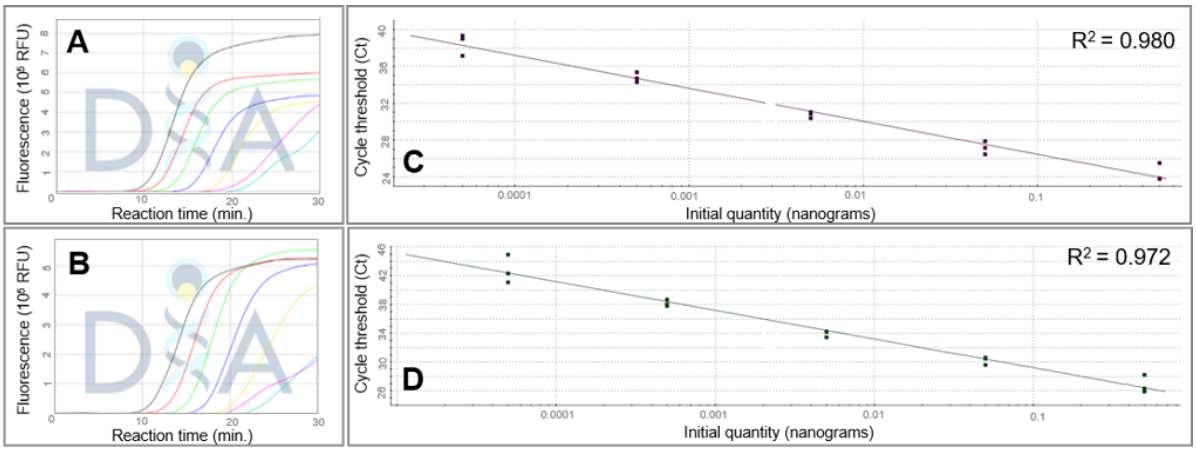

Figure III-2 Sensitivity of LAMP assay. Smart- DART amplification curve generated in the LAMP assay using 10 fold serial dilutions of *P. infestans* **A)** pure DNA ranging from 0.5ng/ul to 5fg/ul and **B)** DNA serial dilutions incorporated into healthy crude plant extract. Standard curve obtained in a real-time machine by plotting **C)** *P. infestans* pure DNA concentration, and **D)** DNA serial dilutions incorporated into healthy crude plant extract against LAMP Ct values. R^2 values of standard curve obtained from LAMP assay are indicated.

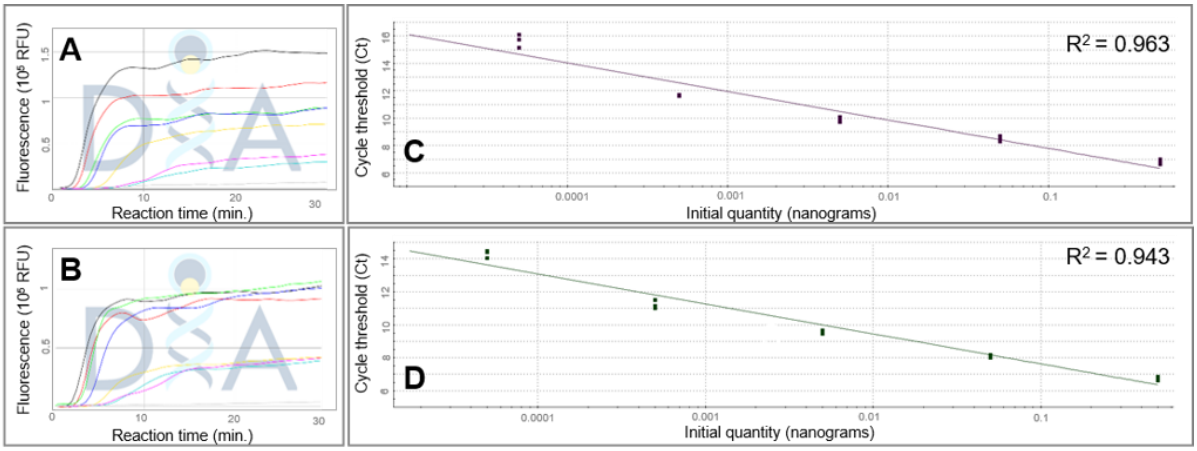

Figure III-3 Sensitivity of RPA assay. Smart- DART amplification curve generated in the RPA assay using 10 fold serial dilutions of *P. infestans* **A)** pure DNA ranging from 0.5ng/ul to 5fg/ul and **B)** DNA serial dilutions incorporated into healthy crude plant extract. Standard curve obtained in a real-time machine by plotting **C)** *P. infestans* pure DNA concentration, and **D)** DNA serial dilutions incorporated into healthy crude plant extract against RPA Ct values. R^2 values of standard curve obtained from RPA assay are indicated.

III.1.16 Testing on inoculated plants.

In both inoculation experiments, LAMP assay successfully detected *P. infestans* infections as soon as 1 dpi and consistently until 6 dpi from symptomatic and asymptomatic leaves (Fig.III-4). RPA assay detected *P. infestans* infections starting from 3 dpi, which corresponds to the observation of the first lesions, and consistently until 6 dpi (Table III-3). No amplification occurred from samples at T0 and non-inoculated plants with both LAMP and RPA.

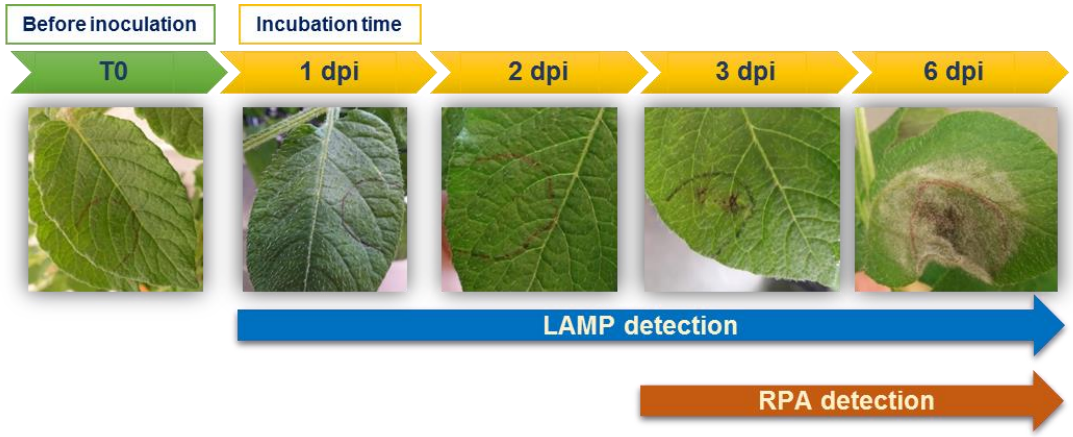

Figure III-4 Lesions progression of *Phytophthora infestans* on inoculated potato plants and LAMP and RPA detection over incubation time.

dpi: days post-inoculation

Table III-3 Results of LAMP and RPA detection of *Phytophthora infestans* infections on inoculated potato plants

Samples			Time, day post-inoculation (dpi)									
			T0		1 dpi		2 dpi		3 dpi		6 dpi	
			LAMP[a]	RPA[a]	LAMP	RPA	LAMP	RPA	LAMP	RPA	LAMP	RPA
Experiment 1	Inoculated plants	1	-	-	+	-	+	-	+	+	+	+
		2	-	-	+	-	+	-	+	+	+	+
		3	-	-	+	-	+	-	+	+	+	+
		4	-	-	+	-	+	-	+	+	+	+
		5	-	-	+	-	+	-	+	+	+	+
	Non-inoculated plants	1	-	-	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	-	-	-	-
		3	-	-	-	-	-	-	-	-	-	-
Experiment 2	Inoculated plants	1	-	-	+	-	+	-	+	+	+	+
		2	-	-	+	-	+	-	+	+	+	+
		3	-	-	+	-	+	-	+	+	+	+
		4	-	-	+	-	+	-	+	+	+	+
		5	-	-	+	-	+	-	+	+	+	+
	Non-inoculated plants	1	-	-	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	-	-	-	-
		3	-	-	-	-	-	-	-	-	-	-
Symptoms observation[b]			-		-		+		++		+++	

[a]LAMP and RPA results: + Positive; - Negative
[b]Symptoms observation: - Asymptomatic; + Small lesions; ++ Medium lesions; +++ Large lesions.

III.1.17 Field samples.

The reliability of the developed assays was determined on a total of 24 potato leaf samples collected from fields with and without visible late blight symptoms (Table III. 4). A single leaf disc was tested per sample. The LAMP assay reported 11 out of 12 true positives and 11 out of 12 true negatives, and thus an overall accuracy of 91.7%. Despite a high value of true negatives (12/12), the RPA assay presented a low proportion of true positive samples (4/12).

Discussion

The objectives of this study were to develop real-time LAMP and RPA assays specific to *P. infestans* and to evaluate the sensitivity of the isothermal assays on infected plant material. We also developed a rapid crude plant extract (CPE) preparation method from potato leaves for late blight on-site diagnostic and early detection.

When tested for their specificity to P. infestans, the real-time isothermal assays presented a limited cross-reaction with its closely related species. The real-time LAMP and RPA assays, designed to target *P. infestans* ITS2 region, also amplified DNA from *P. mirabilis*, *P. phaseoli*, *P. ipomoeae* and *P. andina*. This is due to the high similarity (99.9%) of the ribosomal DNA internal transcribed spacer regions among *Phytophthora* species (Blair *et al.* 2012; Raffaele *et al.* 2010). Similar results are often reported in molecular assays developed for the detection and quantification of *P. infestans* (Fall *et al.* 2015b; Judelson and Tooley 2000; Lees *et al.* 2012; Tooley *et al.* 1997) and are considered of minor consequence as these species do not infect potato or tomato (Goss *et al.* 2011; Lees *et al.* 2012). Notably, the LAMP and RPA assays did not cross-react with *P. nicotianae* and *P. erythroseptica,* nor with the other known potato infecting fungal species.

The targeted ITS region is known to be in high copy number, thus improving the sensitivity of detection (Bilodeau *et al.* 2014). Moreover, LAMP and RPA isothermal technologies are efficiently able to generate billions of DNA copies within 40 to 60 minutes (Li and Macdonald 2015; Notomi *et al.* 2000).

Table III-4 Contingency table of the LAMP and RPA testing on potato leaf samples collected from fields with and without late blight (LB) infections.

Potato fields	LAMP			
	Positive	**Negative**	Total
With LB	11	1	12
Without LB	1	11	12
Total	12	12	24

	RPA		
	Positive	**Negative**	Total
With LB	4	8	12
Without LB	0	12	12
Total	4	20	24

The two developed isothermal assays showed their ability to detect very low quantities of *P. infestans* DNA, *i.e.* 50 fg/µl, and proved to be insignificantly affected by plant extract inhibition when DNA was spiked into healthy CPE samples. The sensitivity of LAMP and RPA assays compares favorably with previously described *P. infestans* qPCR and LAMP assays, such as: 2 pg (Llorente *et al.* 2010), 1 pg (Böhm *et al.* 1999), 100 fg (Lees *et al.* 2012), 2 pg for LAMP ITSII and 200 pg for LAMP Rgn86_2 assays (Hansen *et al.* 2016). Within the obtained limit of detection, the reaction time (Rt) of the LAMP and RPA assays was 20 minutes and 10 minutes, respectively. However, after 25 minutes LAMP reaction, we observed a DNA amplification background from the lowest *P. infestans* DNA dilution (5 fg/µl) and the healthy CPE samples. Based on these results, a cut-off value of 25 minutes for the LAMP assays was used and any amplification that occurred beyond this limit (*i.e.* Rt> 25 min.) was considered negative.

The CPE preparation method was considered as an alternative DNA extraction for field use with minimal equipment. A simple and rapid tissue maceration step, *i.e.* incubation of a leaf disc at 90°C for 10 minutes, was sufficient to obtain an amplification from template DNA in LAMP and RPA assays. These reactions were successfully carried out in real-time detection using the portable Smart-DART instrument. Interestingly, the latter device can also be used as a heat block for the preparation of CPE samples. This emphasizes the potential for on-site application of these real-time isothermal assays through commercially available fluorescence based instruments, such as: Genie III (Optigene Ltd., Horsham, UK) and Twista real-time fluorometer (TwistDx Ltd., Cambridge, UK) for a rapid and accurate on-site detection of *P. infestans* in plant material.

In the inoculation experiments we performed, the LAMP assay detected *P. infestans* infections from asymptomatic leaves as soon as 1 day after the inoculation, whereas the RPA assay detected the infections 3 days after the inoculation, which corresponds to the appearance of the first small lesions on the leaves. These results highlight the sensitivity and the potential of *P. infestans* early detection of the developed LAMP assays. Moreover, the tests carried out on field samples provided information on the reliability of the assays, highlighting the better suitability of the LAMP for on-site early detection of *P. infestans* infections. However, the obtained results do not permit to consider the developed RPA assay as an accurate diagnostic tool.

In commercial potato fields, late blight is difficult to manage mostly because of the potentially high rate of disease development and consequent losses despite the available toolbox of detection methods and disease monitoring tools. In several potato production areas, potato cultivars are selected based on tuber shape, starch contents or other characteristic related to processing, transformation and commercialization, regardless of the susceptibility to *P. infestans*, and yet the suggested intervention threshold is as low as 1% of diseased leaf area (Stein and Kirk 2002). Therefore, in conventional, IPM or organic production systems, timing of management action is crucial for controlling late blight. Various disease decision support systems (DDSS) have been developed to time fungicide applications including initiation and interval between applications (UC-IPM 2016).

To a large extent, these systems improved disease management. However, because they are weather-based, risk estimation could be improved by adding field observations. Recently, Fall *et al.* (2015a) studied the spatial distribution of *P. infestans* airborne sporangia and showed that risk estimation from DDSS could be greatly improved by adding information on airborne inoculum and disease onset. These authors reported that the first field observations of late blight symptoms occurred about a week after the first significant peak of airborne sporangia concentration. However, *P. infestans* overwinters in stored tubers (seed potatoes) and in stored infected potatoes discarded in cull piles, and in infected volunteer potato plants. Consequently, it is expected that initial disease will appear as foci and that detection of the first infections might be difficult, requiring that a large number of plants are observed and that proper symptoms identification performed in a timely fashion.

The real-time isothermal tool developed in this study could be used for training scouts for early detection of late blight symptoms, and for *in situ* confirmation of any doubtful symptoms, especially in the early stages of the disease, providing pathogen-based information for proper fungicide application. This method is an additional tool in late blight management strategy that requires further validation and implementation, notably for the results interpretation depending on the management strategy as well as an adaptation to test infected seed tubers in storage. Besides, a large scale sampling methodology should be defined to have better information on the spatial distribution of the first infections in the field, for example: a bulked samples approach as described by Villari *et al.* (2016) could be useful in a large scale plant-screening plan with a limited number of samples to be tested.

Furthermore, it would be of a great interest to investigate the potential use of isothermal technologies for airborne inoculum detection and quantification assays when coupled with a spore trap system as recently described in a turbidity-based LAMP assay for *Erysiphe necator* (Thiessen *et al.* 2016), and a quantitative real-time LAMP assay for *Magnaporthe oryzae* (Villari *et al.* 2016) . Additionally, for the specific identification of the different *P. infestans* genotypes that bear different aggressiveness and sensitivity to fungicide (Gagnon *et al.* 2016; Saville *et al.* 2015). Indeed, as more tools become available more informed late blight management decisions could be taken.

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Chapter IV

IV TOWARDS THE APPLICATION OF A REAL-TIME QUANTITATIVE LAMP ASSAY FOR THE DETECTION OF *PHYTOPHTHORA INFESTANS* AIRBORNE INOCULUM.

This chapter relates the preliminary results of the evaluation and the application of the LAMP assay for the quantification of *P. infestans* airborne inoculum. The experiments were carried out in the Research and Development Centre Agriculture and Agri-Food Canada, Saint-Jean-sur-Richelieu, QC (Canada) under the supervision of Dr. Odile Carisse, in the context of a research internship abroad as intended in the doctoral program.

Introduction

Many fungal plant diseases are initiated by airborne spores. Disease develops when infectious pathogen spores land on susceptible hosts under favorable environmental conditions. Potato late blight is a polycyclic disease with multiple short infection-sporulation cycles of the pathogen, *Phytophthora infestans* (Mont.) de Bary. The disease spreads rapidly under cool, humid conditions when sporangia are produced on infected leaves and further splashed or carried by the wind onto adjacent plants, resulting into foliar epidemics that may cause total destruction of all plants in a field within a week or two (Agrios 2005; Blair *et al.* 2012). Therefore, the timing of sporangia release and late blight progress is influenced by the amount of initial inoculum and weather conditions, favoring the completion of infection-sporulation cycles. Potato growers are unwilling to take risks, which establishes the large adoption of scheduled preventive fungicide. Moreover, several late blight Decision Supports Systems (DSSs) are used for well-timed chemical sprays (Hansen *et al.* 2010; Small *et al.* 2015). These DSSs are regularly updated with weather information and late blight monitoring, but most of them assume that inoculum is constantly present in the area. However, in reality, time to disease onset vary significantly from one year to another (Fall *et al.* 2015a). Subsequently, the effectiveness of the DSSs varies, as well, from year to year and spray recommendations could underestimate or overestimate the late blight risk (Fall *et al.* 2015a; Taylor *et al.* 2003), which challenges late blight management.

Considered as a key factor in late blight epidemic development, the inoculum quantity and dispersal has been well documented (Aylor *et al.* 2001; Cooke *et al.* 2006; Fall *et al.* 2015a;

Skelsey *et al.* 2009). Moreover, the use of spore samplers to measure amounts of airborne pathogen inoculum can provide more accurate spore-based disease forecasts of the risks of severe epidemics (Jackson and Bayliss 2011; Mahaffee 2014; Manzano *et al.* 2015; West and Kimber 2015). Microscopy is generally used to monitor airborne inoculum, a rather time-consuming technique that requires experienced personal to make accurate identification of target spores and precise counts. DNA-based methods, such as Polymerase Chain Reaction (PCR) and real-time PCR, have been extensively used for a rapid and reliable identification and quantification of airborne plant pathogens, and can help to determine when to prolong or decrease fungicide application intervals (Calderon *et al.* 2002; Cao *et al.* 2016; Carisse *et al.* 2014; Carisse *et al.* 2009; Falacy *et al.* 2007; Fall *et al.* 2015b; Klosterman *et al.* 2014; Meitz-Hopkins *et al.* 2014). However these techniques require analyses to be performed in well-equipped laboratories as they are not suitable for on-site testing. This can be an issue when transportation of collected samples to diagnostic laboratories delays the response time, thus reducing the usefulness of inoculum quantity information for rapid management decisions.

Several, isothermal nucleic acid amplification technologies have been developed and adapted for on-site use. These methods are carried out in a single step process at a constant temperature, using simple and handy instruments (Chang *et al.* 2012; Li and Macdonald 2015). Among these techniques, Loop-mediated isothermal Amplification (LAMP) is known to be rapid, sensitive and robust (Notomi *et al.* 2000). The LAMP amplification requires a strand displacing polymerase to amplify DNA, and relies on a set of six primers, which can increase the specificity, and the accuracy of the reaction (Notomi *et al.* 2000). Recently, LAMP reaction can be performed using the Assimilating probes technology described by Kubota *et al.* (2011). LAMP method have been successfully applied for the detection of several plant pathogens (Bühlmann *et al.* 2012; Keremane *et al.* 2015; Tomlinson *et al.* 2010a; Tomlinson *et al.* 2010b; Ward and Harper 2012; Zhang *et al.* 2016), and particularly for the detection and quantification of pathogen airborne inoculum when coupled with spore trap systems, (Mahaffee 2014; Thiessen *et al.* 2016; Villari *et al.* 2016).

In this study, we describe the application of a real-time LAMP assay for the detection and quantification of airborne inoculum of *P. infestans* (hereafter, qLAMP). We also describe the laboratory validation of the qLAMP assay for an absolute quantification approach (Fall *et al.* 2015b). These results are expected to highlight the quantification potential of the LAMP

method as a tool for an improved and simplified information derived from late blight spore-sampling network.

Materials and Methods

IV.1.1 Real-time LAMP assay

The LAMP primers and assimilating probes design for the detection of *P. infestans*, as well as the specificity tests, are described in Chapter III. The real-time LAMP reaction was performed in a volume of 25 µL reaction mixtures using the Isothermal Master Mix without intercalating dye (ISO001-nd, Optigene, Inc., Horsham, UK), containing the GspSSD DNA polymerase. The optimized reaction mixtures contained 1.6 µM FIP and BIP, 0.2 µM of the F3 and B3 primers, 0.8 µM of the loop primer LF, 0.08 µM of the assimilating probe FLB strand, 0.12 µM of the assimilating probe Q-strand (Table IV. 1), and 3µl template DNA. LAMP amplifications were carried out at 65°C for a period of 30 minutes, and real-time fluorescence values of the assimilating probe were measured every 30 seconds using a real-time PCR instrument (Mx3005P QPCR System, Agilent Technologies, Santa Clara, CA, USA) and a Smart-DART instrument (Diagenetix Inc., Honolulu, HI, USA) for real-time isothermal detection and standard curve analysis.

IV.1.2 Preparation of DNA solutions for *P. infestans* ITS2 copy standard curve

DNA from *P. infestans* isolates (See Chapter III) was used for the amplification of a 1004 bp fragment of the internal transcribed spacer 2 (ITS2) region. The PCR reaction was performed in a 50 µL reaction mixture containing 6 ng of genomic DNA, 1X Taq PCR buffer, 4.0 mM of $MgCl_{2+}$, 0.03 U/µL of SurePRIME Taq polymerase (MP Biomedicals, Solon, OH, USA), 0.4 mM of dNTPs (New England Biolabs Inc., Ipswich, MA, USA), and 200 nM each of primers PITSstdF and PITSstdR (Table IV-1). The following PCR conditions were used: 95°C for 15 min, followed by 40 cycles at 94°C for 15 s, 58°C for 45 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR fragments were purified using the Nucleospin PCR clean-up kit (Macherey-Nagel GmbH & Co., Düren, Germany) according to the manufacturer's recommendations. The purified products were quantified using a 2100 Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA, USA). Finally, the DNA was converted into copy numbers, calculated as copy number = [(concentration of amplicon in g/µL)/(1004 bp × 660 g/mole) × (6.022×10^{23})]. This DNA was then used for generating the ITS2 copy number standard curve in the qLAMP assay.

LAMP for the quantification of *P. infestans* airborne inoculum.

Table IV-1 Primers and probes used in the PCR and qLAMP reactions

	Primer Sequence (5'-3')	Source
LAMP primers		This study (See Chapter III)
F3	GGCATTGCTGGTTGTGGA	
B3	CAACATTTCCCAAATGGATC	
FIP	CATTGTTCAGCCGAAGCCAATTTTGCTGCGGCGTTAATGGAG	
BIP	CGCTTATTGGGTGATTTTCCTGTTTTCAAAGCCGATTCAAATGCCA	
LF	CCATACCACGAATCGAGCA	
LB	CGTGATGGACTGGTGAACCATG	
LAMP Assimilating Probe		
FLB strand	5'FAM-ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGACGTGATGGACTGGTGAACCATG	
Q-strand	TCGGCATCCGCATCCGCATTCGCATCCGGGTCCTCAGCGT—3'BHQ	
PCR primers		Fall *et al.* 2015
PITSstdF	AACTAGATAGCAACTTTC	
PITSstdR	GTTTTCAGGTACTCTTTA	

The ITS2 copy standard curve was constructed from 10 fold serial dilutions ranging from 10^5 to 1 copy/µL, each dilution was performed in triplicates in the LAMP reaction using the real-time PCR instrument. Nuclease free water was used as no-template control in the LAMP reaction.

IV.1.3 Preparation of DNA solutions for *P. infestans* sporangia standard curve

Mature sporangia of *P. infestans* were harvested from sporulating potato leaves using an adapted 10 mL pipette connected to a vacuum pump. About 5 mL of isopropanol 100% was poured into the pipette to suspend the collected sporangia and filter the suspension through a cheese cloth. The sporangia suspension was observed under a light microscope to examine the status of the cells and for quantification. Suspensions of 3125 sporangia/µL were prepared in a final volume of 75 µL Isopropanol 100%, into 2 mL screw-cap tubes containing 100 mg of acid washed glass beads 425 to 600 µm. The tubes were stored at −20°C until DNA extraction.

The sporangia suspension were extracted using the DNA extraction as described below. However, during the first step of the procedure, a silicon coated polystyrene spore trap rod was added to the tubes to mimic same lysis conditions that field collected samples. The DNA was then used in the qLAMP assay for constructing the *P. infestans* sporangia standard curve.

For the real-time PCR instrument, the sporangia standard curve was constructed using five-fold serial dilutions from 3125 to 1 sporangia/ µL, each dilution was performed in triplicates in the LAMP reaction. Since the Smart-DART device allows the analysis of eight wells at a time, the sporangia standard curve was constructed using dilutions of 125, 50, 25, 10 and 1 sporangia/ µL, and the reaction was repeated three times. In both cases, a tube containing a rod without *P. infestans* sporangia was extracted and used as a negative control sample, and nuclease free water was used as no-template control in the LAMP reactions.

IV.1.4 Estimation of the number of sporangia from the number of ITS2 copies

The ITS2 copy standard curve was constructed using 10 fold serial dilutions of the ITS2 gene fragment of *P. infestans* (1004 bp), ranging from 10^5 to 1 copy/µL. Similarly, the sporangia standard curve was constructed using five-fold serial dilutions from 3125 to 1 sporangia/ µL. For both standard curves, the procedure was repeated three times. A three-step regression analysis was used to determine the number of copies of ITS2 per sporangia. First, regression analysis was used to establish the relationship between the quantification reaction time (Rt) value and the logarithm of the number of ITS2 copies. Second, regression analysis

was used to establish the relationship between the Rt value and the logarithm of the number of sporangia. Finally, the predicted number of sporangia was regressed against the predicted number of ITS2 copies, for Rt values interval corresponding to the limit of detection of ITS2 copy number serial dilutions. The slope of the regression line and the regression coefficient (R^2) were calculated using Microsoft Office Excel. The resulting equation was used to determine the number of sporangia from the number of ITS2 copies obtained from the qLAMP assay.

IV.1.5 DNA extraction procedure from spore trap rods

To maximize the quantity of extracted DNA of *Phytophthora infestans*, a lossless DNA extraction method without purification was used following Fall *et al.* 2015. In brief, lysis of sporangia and DNA recovery were carried out from a spore trap rod coated with silicon in a single extraction tube, containing 75 µl of Isopropanol 100% (Sigma-Aldrich Canada Ltd.) and 100 mg of acid washed glass beads 425 to 600 µm (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada). The tubes were placed in a Fast-Prep instrument (MP Biomedicals, Solon, Ohio) for 20 s at 4 m/s, and then centrifuged for 5 s at 10,000 x g. Isopropanol was evaporated using a Vacufuge instrument (Eppendorf Canada Ltd., Mississauga, Ontario, Canada) for 20 min at 60°C. The sporangial lysate was suspended in 300 µL of DNA extraction solution consisting of nuclease- free water, salmon sperm DNA at 10 ng/µL (Life Technologies Inc., Burlington, ON, Canada), 5% Chelex 100 (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada), and 100 ng/µL bovine serum albumin (New England Biolabs Inc.). Lastly, the tubes were heated at 105°C for 20 min in a dry bath, briefly agitated using a vortex, and centrifuged for 5 min. at 4°C and 15,000 × g. The obtained extracts were kept at 4°C for a maximum of 1 h to avoid DNA degradation, and used in the qLAMP assay.

LAMP for the quantification of *P. infestans* airborne inoculum.

IV.1.6 Laboratory validation of the qLAMP assay

Droplets (0.2 µL) of *P. infestans* sporangia solution in isopropanol 100% were deposited onto rotating-arm sampler rods, previously coated with silicon vacuum grease (Fig.IV-1). The number of spores per rod was determined under a light microscope. DNA was extracted following the DNA extraction procedure previously described, and the number of spores was estimated with the qLAMP assay and the constructed standard curves in the real-time PCR machine and the Smart-DART instrument. The relationship between the number of sporangia estimated by qLAMP and the number estimated by microscopy was established by regression analysis.

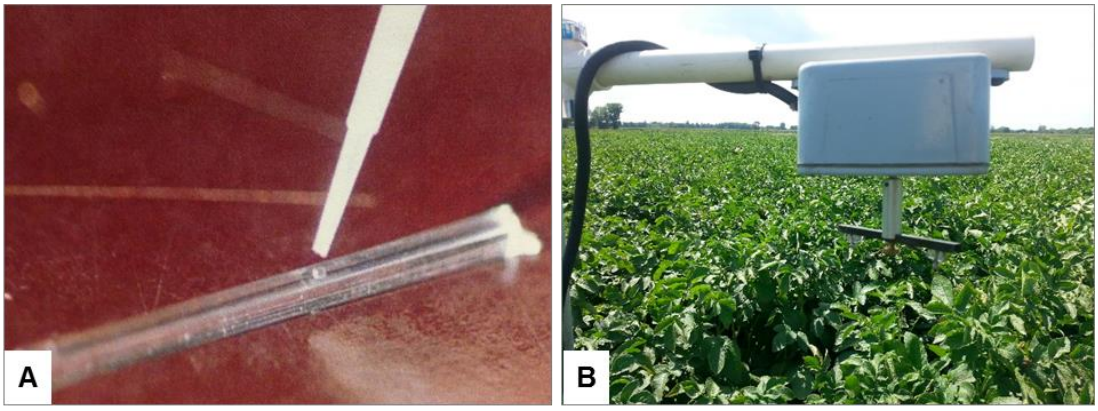

Figure IV-1 Preparation of spore trap rods for the laboratory validation of the qLAMP assay. A) Droplets of *P. infestans* sporangia solutions with variable concentration are deposited in the centre of the silicon-coated rod before microscope count and qLAMP assay (Photo M. Tremblay). **B)** Example of a rotating arm spore sampler placed in a potato field for *P. infestans* sporangia monitoring.

Results

IV.1.7 Estimation of the number of spores from the number of ITS2 copies

A linear regression line was obtained from the ITS2 copy standard curves generated by 10 fold serial dilutions in the qLAMP assay using a real-time PCR instrument; with an efficiency of 96.2%, a regression coefficient of 0.99, a slope of 3.42, and an intercept of 41.76. Similarly, the sporangia standard curves generated by five-fold serial dilutions of sporangia DNA extractions were also linear; with an efficiency of 98.7%, a regression coefficient of 0.99, a slope of 3.35, and an intercept of 35.16 (Fig.IV-2). Within an Rt interval of 11.5 to 18 minutes, a double linear regression, resulting from the two previous regression formulae, was used to convert the number ITS2 copies to the number of *P. infestans* sporangia as follows: Number of *P. infestans* sporangia = 0,0135 × number of ITS2 copies. In this equation, the intercept is 0 and the slope is 0.0135 (Fig.IV-3). Furthermore, when using the Smart-DART instrument, a linear regression line was obtained from the sporangia standard curves generated by the DNA dilutions of 125, 50, 25, 10 and 1 sporangia/ µL; with a regression coefficient of 0.98, a slope of 2.29, and an intercept of 20.97 (Fig.IV-4).

IV.1.8 Laboratory validation of the qLAMP assay

A linear relationship was obtained between the number of sporangia deposited onto the rods estimated by microscopy and the number of sporangia estimated with the qLAMP assay, either in the real-time PCR machine (R^2 = 0.96) (Fig.IV-5), or when using the Smart-DART instrument (R^2 = 0.86) (Fig.IV-6). Real time LAMP amplifications were obtained from all rods where at least one sporangia has been deposited, and no signals were detected from rods without sporangia (negative controls).

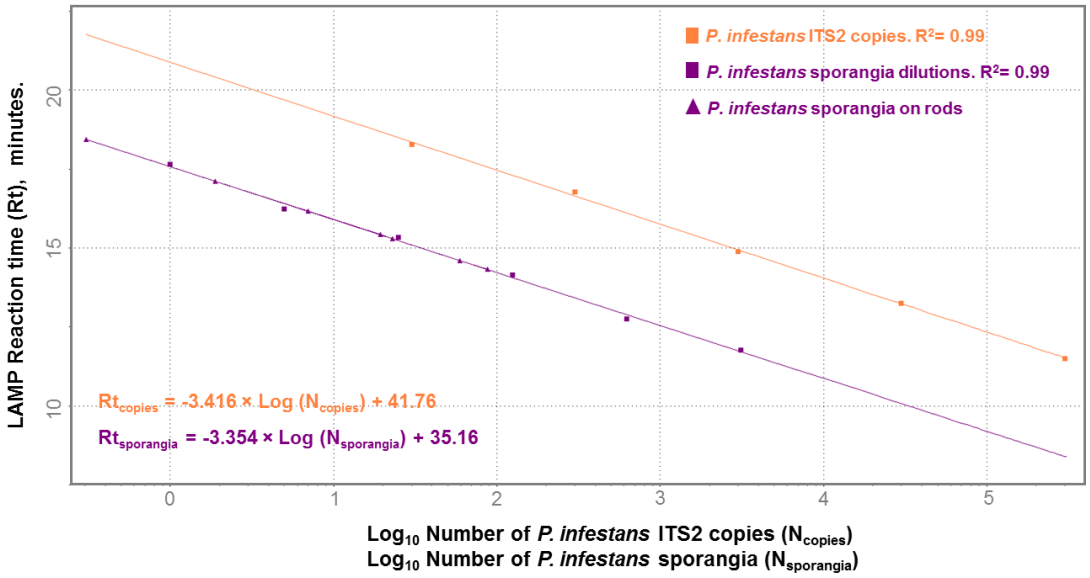

Figure IV-2 Real-time LAMP standard curves obtained in a real-time PCR machine by plotting the reaction time (Rt) value with the log number of *Phytophthora infestans* sporangia, and the Rt value with the log number of internal transcribed spacer 2 (ITS2) copies.

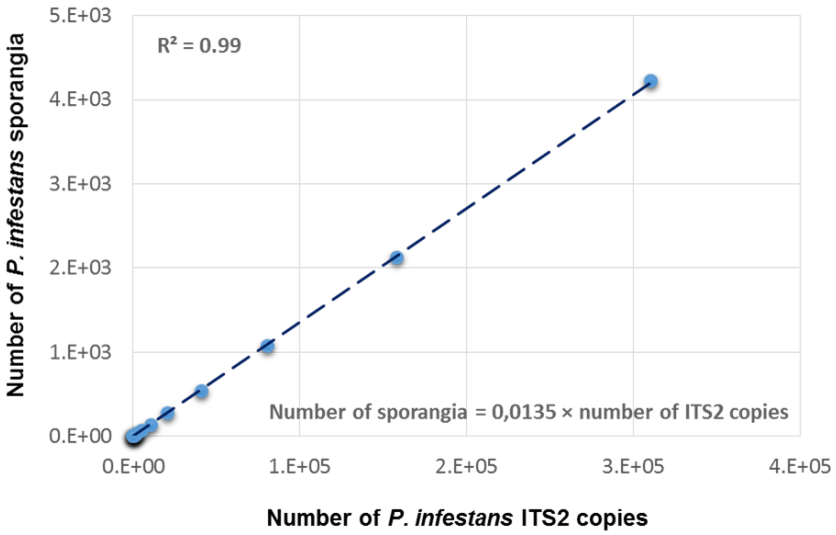

Figure IV-3 Double regression analysis of the qLAMP assay predictions of the number of *P. infestans* sporangia against the predicted number of ITS2 copies, based on Rt values interval of 11.5 to 18 minutes.

LAMP for the quantification of *P. infestans* airborne inoculum.

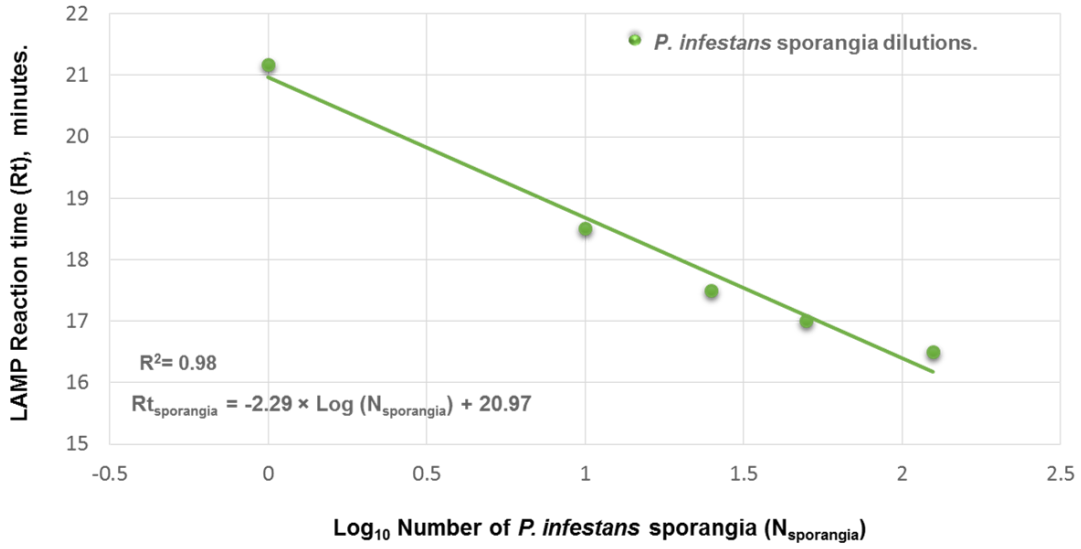

Figure IV-4 Real-time LAMP standard curves obtained in Smart-DART instrument by plotting the reaction time (Rt) value with the log number of *Phytophthora infestans* sporangia. The equation of the regression line and the regression coefficient (R^2) were calculated using Microsoft Office Excel.

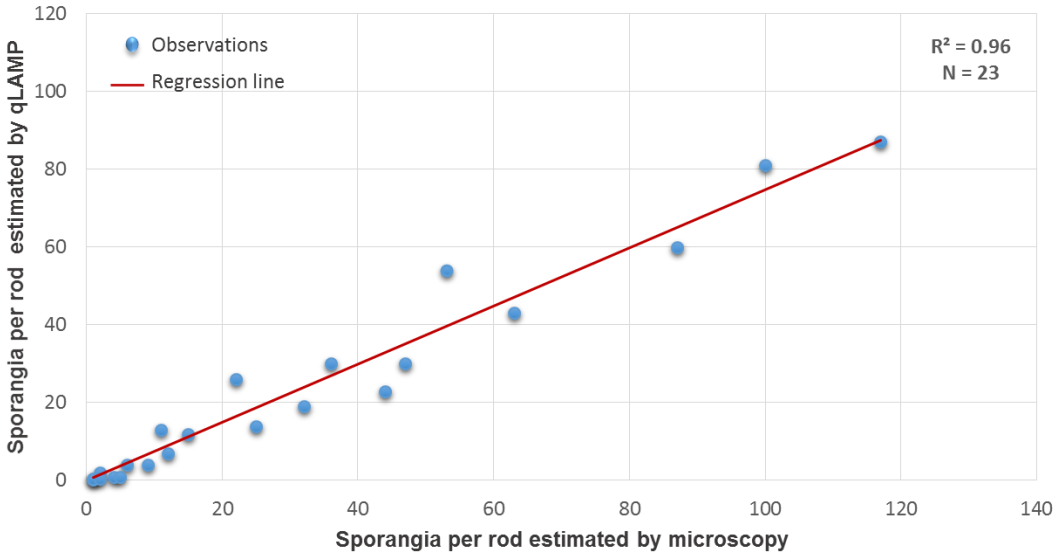

Figure IV-5 Relationship between estimates of the number of *Phytophthora infestans* sporangia deposited on silicon-greased rods, based on the *P. infestans* qLAMP assay in a real-time PCR machine and estimates based on microscope counts.

LAMP for the quantification of *P. infestans* airborne inoculum.

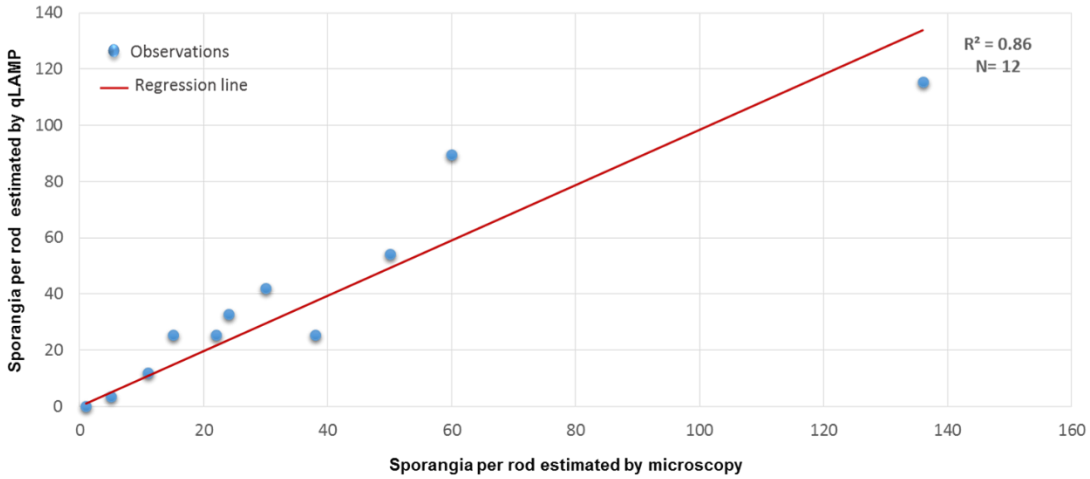

Figure IV-6 Relationship between estimates of the number of *Phytophthora infestans* sporangia deposited on silicon-greased rods, based on the *P. infestans* qLAMP assay in a Smart-DART instrument and estimates based on microscope counts.

Discussion

The suitability of the real-time LAMP assay developed for the detection of *Phytophthora infestans* was tested for a rapid detection and quantification of the pathogen's airborne inoculum. When the designed primers were tested for their specificity (See Chaper III), they cross-reacted with *P. mirabilis, P. phaseoli, P. ipomoeae* and *P. andina.* This was mainly due to the high similarity between these species, sharing about 99.9% of their internal transcribed spacer (ITS) region of the ribosomal DNA (Blair *et al.* 2012; Raffaele *et al.* 2010). These results are often observed in molecular assays developed for the detection and quantification of *P. infestans* (Fall *et al.* 2015b; Judelson and Tooley, 2000; Lees *et al.* 2012; Tooley *et al.* 1997). It is important to highlight that the aim of the present assay was to detect and quantify airborne sporangia from potato fields, and since none of these species is known to infect potato or tomato (Goss *et al.* 2011; Lees *et al.* 2012), in such environment, it can be expected that no other *Phytophthora* species or members of closely related taxa, such as *Pythium,* could interfere with the results obtained with the qLAMP assay. In addition, under a microscope, spores of *Pythium* would possibly be counted as *Phytophthora* due to their similar morphology. Since DNA-based methods can overcome the disadvantages of counting and identifying by microscopy, quantitative PCR assays were used as an appropriate tool for quantification, detection and identification of a number of airborne plant pathogens (Cao *et al.* 2016; Carisse *et al.* 2014; Carisse *et al.* 2009; Fall *et al.* 2015b; Klosterman *et al.* 2014; Meitz-Hopkins *et al.* 2014), in addition to LAMP assays when coupled with spore trap systems (Mahaffee 2014; Thiessen *et al.* 2016; Villari *et al.* 2016).

The analytical sensitivity evaluation and the laboratory validation of the qLAMP assay were performed following an absolute quantification approach (Bustin and Nolan 2004; Fall *et al.* 2015b). The qLAMP assay was sensitive to a single sporangium of *P. infestans.* Linear regression lines were obtained and standard curves of *P. infestans* sporangia and ITS2 copy were constructed. Finally, the qLAMP assay was validated in the laboratory with silicon-coated rods containing a known number of sporangia. A linear relationship between the number of sporangia deposited onto the rods estimated with microscopy and the number of sporangia estimated with the qLAMP assay ($R^2 = 0.96$) was obtained using a real-time PCR instrument. Additionally, no signals were detected from rods without sporangia, which is an indication of the reliability and the suitability of the assay for airborne inoculum quantification.

Furthermore, the Smart-DART instrument was used for the purpose of on-field use of the qLAMP assay. For potato growers, the precise quantification of airborne inoculum may not be the main importance. It is generally sufficient to know if disease is above or below an established action threshold. Therefore, *P. infestans* sporangia standard curves were generated (R^2 = 0.98) in accordance to the proposed threshold of 1 to 6 sporangia m^{-3} which corresponds to 5 to 30 sporangia/rod (Fall *et al.* 2015b). Similarly, a linear relationship between the number of sporangia deposited onto the rods estimated with microscopy and the number of sporangia estimated with the qLAMP assay (R^2 =0.86) was obtained using the Smart-DART instrument. Therefore, this qLAMP tool allows rapid and efficient count of sporangia.

Because of a lack of a gold standard method in the quantification of airborne inoculum, it is important to note that during the experiments, precise microscope rod counts for sporangia concentrations higher than 100 sporangia/rod were difficult to perform, thus making relatively approximate counts. However, equally to microscopy, the qLAMP assay does not distinguish between living and dead sporangia. Also, because the assay was not validated with field samples, further testing is required to more fully evaluate the potential utility of the LAMP assay for quantification of *P. infestans* airborne inoculum, as the data presented here represent the results of laboratory testing only. Due to the absence of potato late blight infections in Quebec province (Canada) during the summer 2016, the microscope counts of the rotating-arm sampler rods collected from the spore sampling network were not sufficient to perform a field validation of the assay (Data not shown).

On the basis of these results, however, some perspectives can be made regarding the potential for real-time LAMP assay to be used for on-site quantification of plant pathogens. Essentially, the DNA extraction method used was efficient in recovering low DNA quantity from the spore sampler rods. However, this method has been optimized for qPCR assays. The adaptation of an extraction method from various spore samplers with less laboratory equipment and possible on-site implementation would be useful. This will provide an added value to the LAMP assay to be performed on-field or/and in minimally equipped diagnostic laboratories. Additionally, it would be of a considerable interest to develop isothermal assays for the specific identification of the different *P. infestans* clonal lineages characterized by different aggressiveness and sensitivity to fungicide (Gagnon *et al.* 2016; Saville *et al.* 2015). The described qLAMP assay, when applied in the framework of a spore sampling network, could be used to advise farmers about *P. infestans* inoculum within an established disease action

threshold; *i.e*. its timing, concentration and proportion of the population of specific genotype. Prospects to develop accurate spore-based disease-forecasting systems to provide rapid, early, and precise warning of increases in airborne inoculum have the potential to greatly improve the control of late blight epidemics.

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Chapter V

V GENERAL DISCUSSION

The notion of on-site plant detection comprises situations ranging from in-field conditions, with rudimentary laboratory equipment available, to minimally equipped laboratories in contrast to conventional laboratory equipment for molecular diagnosis. Potentially, such on-site testing methods could be carried out by plant diagnosticians with adequate molecular biology background in limited resources laboratories, or by phytosanitary inspectors that may require to perform DNA-based testing on the sampling site. Defining the end-user to whom a diagnostic tool is being developed is the preliminary concern to be addressed, as the requirements of these users might be diverse. In this context, isothermal amplification detection assays have been developed along with adapted DNA extraction methods for the detection of *Plasmopara viticola* and *Phytophthora infestans* from plant material. Some aspects of the extraction method were mainly driven by the nature of the samples. For example, the grapevine samples required a mechanical cell lysis step in order to increase the detection sensitivity of the pathogen (Chapter II). Whereas it was possible to test infected potato leaves following a simple crude extract preparation method (Chapter III). Finally, in order to detect low levels of pathogen from spore trap rods additional steps may be required in the extraction method (Chapter IV).

In the development of a detection method, it is expected that this new method performs better than or at least as well as an existing test that is being used. The evaluation of the performance of the assays is usually compared with an already established "gold standard", preferably a DNA-based method, such as conventional or real-time PCR. However, for routine based disease monitoring, scouting for early symptoms appearance remains the most commonly used approach, for instance the visual observation grapevine downy mildew oil spots and sporulating lesions in vineyards, as well as microscopy for the quantification or airborne inoculum for spore trap samples (Chapters II and IV). In this circumstance, comparing the two approaches enables to confirm the results obtained from the developed assay and to draw the attention on the shortcomings of the standard method. Indeed, it is likely that the developed assays outperform the standard method. As it was possible with the LAMP assay to differentiate between actual lesions of *P. infestans* and lesions that are similar to late blight

symptoms, and consequently might be mistaken by an operator by visual observation (Chapter III). In addition to the early detection of *P. infestans* and *P. viticola* infections from asymptomatic leaves (Chapters II and III).

Most importantly, before the deployment of a detection assay, an accurate validation of the assay is essential for any testing method. Ideally, an adequate testing of the accuracy and reproducibility of the method is performed with a significant number of field samples from different locations and diverse growing seasons. It is true that an assay could display a high performance during the laboratory trials and, in contrast, present a reduced accuracy or even failure when validated with "real samples" (Chapter III). Besides, for on-site detection methods, it would be of great utility to truly take the test outdoor and perform on-field trials with different laboratory situations.

For the purpose to test in low-resource settings, portable instruments of isothermal amplification technologies possess the benefit to be affordable. Indeed, the cost of testing is likely to be a more important parameter than the speed of the results or the simplicity of the protocol. Small, handy instruments such as the Smart-DART are potentially accessible, and provide the benefit of real-time detection and thus the reduced contamination risk associated with post-amplification detection methods, as it is often strongly advised not to open completed LAMP reaction tubes to detect LAMP products.

The goals of developing on-site detection methods are to increase efficiency of testing and to broaden the range of pathogen detection abilities. On-site detection of plant pathogens supports decisions to be taken more rapidly in the field and also has the potential to allow a preliminary screening of the disease, hence reducing the number of samples sent to be processed in the laboratory. The broad vision in developing new isothermal assays, is the insertion of an on-site detection tool in a plant disease diagnostic "toolbox" of nucleic acid extraction methods and detection technologies that can be combined and used as required, depending on the epidemiologic issue to be addressed. Certainly, as more tools become available more informed plant disease management decisions could be taken.