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Development of real-time isothermal amplification assays for on-site detection of *Phytophthora infestans* in potato leaves

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Abstract

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. xx:xxx-xxx.

Real-time Loop-Mediated Isothermal Amplification (LAMP) and Recombinase Polymerase Amplification (RPA) assays were developed targeting the ITS2 region of the ribosomal DNA of *Phytophthora infestans*, the potato late blight causal agent. A rapid crude plant extract (CPE) preparation method from infected potato leaves was developed for on-site testing. The assay's specificity was tested using several species of *Phytophthora* and other potato fungal and oomycete pathogens. Both LAMP and RPA assays showed specificity to *P. infestans* but also to the closely related species *P. andina*, *P. mirabilis*, *P. phaseoli* and *P. ipomoeae*, although not reported as potato pathogen species. No cross-reaction occurred with *P. capsici* or with the potato pathogens tested, including *P. nicotianae* and *P. erythroseptica*. The sensitivity was determined using *P. infestans* pure genomic DNA added into healthy CPE samples. Both LAMP and RPA assays detected DNA at 50 fg/ul and were insensitive to CPE inhibition. The isothermal assays were tested with artificially inoculated and naturally infected potato plants using a Smart-DART platform. The LAMP assay effectively detected *P. infestans* in symptomless potato leaves as soon as 24 h post-inoculation. A rapid and accurate on-site detection of *P. infestans* in plant material using the LAMP assay will contribute to improved late blight diagnosis, early detection of infections and facilitate prompt management decisions.

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

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1 ## Introduction

2 Late blight, caused by the oomycete *Phytophthora infestans* (Mont.) de Bary, has
3 historically been an important disease in solanaceous plants and remains a major constraint to
4 the production of potato and tomato worldwide (Fry et al., 2015; Kamoun et al., 2015).
5 Potato late blight infections are considered a perpetual threat to global food security causing
6 considerable economical losses estimated at billions of US Dollars yearly (Cooke et al., 2012;
7 Haverkort et al., 2009). Recently, the appearance of highly aggressive genotypes of *P.*
8 *infestans*, which are resistant to the most widely used phenylamide fungicides, and the spread
9 of A1 and A2 mating type isolates, resulting in sexual recombination, have caused severe
10 outbreaks (Chowdappa et al., 2015; Cooke et al., 2012; Danies et al., 2014; Fry and
11 Goodwin, 1997; Fry et al., 2013; Gisi et al., 2011; Peters et al., 2014). Potato late blight is a
12 polycyclic disease with multiple short infection-sporulation cycles, under favorable
13 conditions. Consequently, disease progress can certainly be influenced by the amount of
14 initial inoculum and weather conditions favoring the completion of sporulation-infection
15 cycles. Late blight has the potential to progress rapidly which challenges disease
16 management, thus potato growers who are risk averse rely mainly on scheduled preventive
17 fungicide applications. However, in practice, time to disease onset varies significantly from
18 one year to another (Fall et al., 2015b). Regardless of the late blight management strategy;
19 conventional, integrated or organic, being able to detect early infections is crucial for disease
20 management. Determining the occurrence or risk of initial infection is achieved using
21 weather-based forecasting systems (Arora et al., 2014; Cooke et al., 2011; Small et al.,
22 2015) or airborne monitoring devices (Fall et al., 2015b). Being a destructive disease, late
23 blight tolerance level is very low (Stein and Kirk, 2002), hence early detection methods could
24 be helpful to accurately and rapidly confirm the presence of *P. infestans* and to trigger
25 management actions.

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

36 Isothermal nucleic acid amplification technologies have a significant advantage over PCR-
37 based methods, as they can be implemented in a single step process at a constant temperature
38 (Li and Macdonald, 2015). Removing the need for thermal cycling allows for on-site
39 diagnostics to be carried out using small and portable instruments (Chang et al., 2012). The
40 most commonly used isothermal technique is Loop-Mediated Isothermal Amplification
41 (LAMP), known to be rapid, accurate and requiring the use of a strand displacing polymerase
42 to amplify DNA, typically *Bst* polymerase (Notomi et al., 2000). The amplification relies on a
43 set of four to six primers, specially designed to recognize six to eight distinct regions of a
44 target gene, resulting in high efficiency and specificity (Tomita et al., 2008). Moreover,
45 LAMP has been shown to be tolerant to inhibitory substances present in biological samples,
46 hence, simple and rapid sample preparation methods, without DNA purification steps, are
47 sufficient for LAMP assays (Kaneko et al., 2007; Niessen, 2014). Recently, several
48 *Phytophthora* species-specific assays have been developed using different methods to detect
49 LAMP products (Chen et al., 2013; Dai et al., 2012; Dong et al., 2015; Tomlinson et al.,
50 2010). Hansen et al., (2016) developed colorimetric LAMP assays for the detection of *P.*

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51 *infestans* using hydroxynaphthol blue in a closed-tube reaction. Since LAMP reaction
52 generates up to 10^9 self-replicating amplicons within a 1 hour reaction (Notomi et al., 2000),
53 it is often strongly advised not to open completed LAMP reaction tubes to detect LAMP
54 products due to the risk of contamination. Therefore, monitoring the isothermal LAMP
55 reaction in real-time has been described using double-strand DNA binding dye (Keremane et
56 al., 2015), or FRET-based assimilating probes technology as previously described by Kubota
57 et al., (2011) for a sequence-specific detection (Kubota and Jenkins, 2015; Tanner and
58 Evans, 2014). Moreover, the use of portable devices for real-time monitoring of the LAMP
59 reaction allows performing on-site diagnostics and in-field testing.

60 Another relatively new and promising isothermal technique that can be implemented for
61 on-site diagnostics is Recombinase Polymerase Amplification (RPA). RPA uses recombinase
62 and co-enzymes, which form complexes with the primers, to facilitate the annealing of
63 primers into a double stranded template and initiate the amplification (Piepenburg et al.,
64 2006). Several types of RPA kits (TwistDx Ltd., Cambridge, UK) are available to develop
65 specific assays through the design of a primer pair (30-35 bp), and a fluorescent probe with
66 unique structure (46-52 bp), namely TwistAmp™ exo probe, for real-time detection (TwistDx-
67 Limited, 2016). Recently, Miles et al., (2015) used RPA approaches to develop a genus-
68 specific assay for detection of *Phytophthora* spp., and other assays for *P. ramorum* and *P.*
69 *kernoviae* species detection.

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

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

80 Materials and methods

81 **Fungal and oomycete isolates.** DNA of the different fungal and oomycete isolates and *P.*
82 *infestans* cultures were provided from different sources listed in Table 1. Cultures of *P.*
83 *infestans* isolates were maintained on Rye B Agar medium (Caten and Jinks, 1968) and kept
84 at 20°C and 12h photoperiod. Approximately, 2 mg of fresh mycelium was collected from the
85 surface a 10-day-old culture, using a sterile scalpel, and DNA was extracted using a DNeasy
86 plant mini kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. DNA
87 of the different fungal isolates and of *P. infestans* was quantified using a NanoDrop 2000
88 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and kept at -20 ºC until
89 use in the sensitivity and specificity tests.

90 **Design of LAMP primers and assimilating probe.** Sequences of the ITS region of
91 *Phytophthora* and *Pythium* species (Coffey et al., 2009) were compared to identify potential
92 regions specific to *P. infestans*. The LAMP target sequence was selected in the ITS2 region
93 and includes the *P. infestans* specific region previously described by Fall et al., (2015b).
94 Three LAMP set candidates were designed using the online software Primer ExplorerV4
95 (Eiken Chemicals, Tokyo, Japan), and their analytical sensitivity was compared. The LAMP
96 primers of the best performing LAMP set are listed in Table 2. Additionally, the designed
97 Loop Backward (LB) primer was modified by adding assimilating probe sequences, as
98 developed by Kubota et al., (2011), to detect only LAMP amplicons of *P. infestans* in real
99 time. The LB primer sequence was attached to a supplementary sequence bearing a

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100 fluorescent label (6-carboxyfluorescein) at the 5' end. The latter sequence is complementary
101 to a Quencher strand (Qstrand) carrying a Black Hole Quencher-1 (BHQ) at the 3' end (Table
102 2). The LAMP primers and assimilating probe were synthesized by Integrated DNA
103 Technologies (Coralville, IA, USA).

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

109 **LAMP reaction.** The LAMP assays for *P. infestans* detection were performed in 25 μL
110 reaction mixtures using the Isothermal Master Mix without intercalating dye (Catalog No.
111 ISO001-nd, Optigene, Inc., Horsham, UK), containing the GspSSD DNA polymerase. The
112 optimized reaction mixtures contained 1.6 μM FIP and BIP, 0.2 μM of the F3 and B3
113 primers, 0.8 μM of the loop primer LF, 0.08 μM of the assimilating probe FLB strand, 0.12
114 μM of the assimilating probe Q-strand (Table 2), and 3μl template DNA. LAMP
115 amplifications were carried out at 65°C for a period of 30 minutes, and real-time fluorescence
116 values of the assimilating probe were measured every 30 seconds either using a real-time
117 PCR instrument (Mx3005P QPCR System, Agilent Technologies, Santa Clara, CA, USA) for
118 standard curve analysis, or a Smart-DART instrument (Diagenetix Inc., Honolulu, HI, USA)
119 for CPE preparation and real-time isothermal detection.

120 **RPA reaction.** The RPA assay was performed in a 50 μl reaction volume using a
121 TwistAmp exo kit (TwistDx Ltd., Cambridge, UK), 0.42 μM of each of the primers F2 and
122 R2, and 0.12 μM of the exo-probe P2, 14 μM magnesium acetate and 29.5 μl TwistAmp
123 rehydration buffer. A mastermix was prepared and distributed into 200 μl reaction tubes, each
124 containing lyophilized enzymes of the TwistAmp exo kit to which 3 μl of template DNA was

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125 added. Finally, magnesium acetate (2.5 µl) was pipetted into the tube lids before closing. To
126 initiate the reactions, closed tubes were mixed by inversion then centrifuged briefly and
127 immediately placed into the real-time PCR instrument for standard curve analysis, or the
128 Smart-DART instrument for CPE preparation and real-time isothermal detection. RPA
129 amplifications were carried out at 39°C for a period of 30 minutes and real-time fluorescence
130 values of the exo-probe were measured every 30 seconds.

131 **Specificity of the isothermal assays.** The specificity of the LAMP and RPA assays was
132 tested on several isolates of 24 species of *Phytophthora*, as well as other potato fungal and
133 oomycete pathogens (Table 1), and one no template control consisting of nuclease-free water
134 (Integrated DNA Technologies, Inc. Coralville, IA, USA). Before use, DNA concentration of
135 the different isolates was diluted to 20 pg/µl.

136 **Sensitivity of the isothermal assays.** The limits of detection of LAMP and RPA assays
137 were determined using serial dilutions of the above-mentioned pure genomic DNA obtained
138 from *P. infestans* cultures and DNA serial dilutions incorporated into non-diseased CPE
139 samples, for monitoring of plant extract inhibition within the assays. DNA decimal dilutions
140 ranging from 0.5 ng/µl to 5 fg/µl were prepared in both TE buffer pH.8 (Integrated DNA
141 Technologies, Inc. Coralville, IA, USA) and in healthy CPE samples, similarly. Each sample
142 from pure DNA dilutions and from DNA dilutions added to healthy CPE samples was tested
143 in three technical replicates. Standard curves were constructed by plotting DNA serial
144 dilutions with the correspondent Ct values with a fluorescence threshold set manually.
145 Additionally, the LAMP and RPA amplifications were scored based on the Reaction time
146 (Rt), in order to record any delay in the reaction due to plant extract inhibition. The use of a
147 real time PCR instrument to carry out isothermal reactions requires the conversion of the
148 qPCR instrument output Cycle threshold (Ct) to Rt (min.) through the application of a time
149 multiplier. Since for both real-time LAMP and real-time RPA reactions fluorescence was

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150 measured every 30 sec., we considered Rt values equal to two times Ct values, *i.e*. Rt = 2Ct
151 (min.). A cut-off of Rt value, to determine whether samples were positive or negative, was
152 defined based on the results of the detection limit and tests with infected plant materials.

153 **Crude plant extract preparation method.** Crude plant extracts (CPE), which can be
154 obtained in the field with minimal laboratory equipment, were used as alternatives to DNA
155 extractions. Briefly, a single potato leaf disc (about 13 mg plant material) was cut using a
156 handheld paper punch (0.6 mm), placed in 200 µl PCR strip-tubes containing 150 µl of
157 extraction buffer: 20 mM Tris-HCl; pH 8.0, 2 mM EDTA and 1% TritonX100 (Keremane et
158 al., 2015), and heated in the Smart-DART device for 10 min at 90°C. The samples were
159 centrifuged for a few seconds in a micro-centrifuge and the lysate was diluted 1/10 in TE
160 buffer pH 8.0. The obtained CPE samples were used promptly, within an hour after
161 preparation, for the LAMP and RPA reactions. For later use, the freshly cut leaf disc was
162 placed in a 2 ml screw cap tube containing 150 µl of isopropanol 100 % and stored at – 20°C.
163 The latter leaf disc was left to dry on a filter paper for 5 minutes before proceeding to the
164 CPE preparation. The paper punch was cleaned using ethanol 70% between samplings to
165 avoid sample contamination. Healthy CPE samples were prepared from non-infected potato
166 leaf discs and used in LAMP and RPA assays as negative control.

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

173 **Plants inoculation and sampling.** Leaflets from 3-week-old greenhouse-grown potato
174 plants cv. Russet Burbank (second leaves from the top of the plant) were inoculated by

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175 placing a 20 µl droplet of *P. infestans* inoculum on the upper surface of each leaflet. Five
176 plants were inoculated and placed in closed plastic cages in the bottom of a growth chamber
177 (PGC20 growth chamber; Conviron, Winnipeg, MB, Canada) with incubation conditions of
178 18°C and 95% relative humidity. A first cycle of 24h of darkness was applied to promote
179 infection followed by a photoperiod of 10h for 6 days. Sampling was performed before the
180 inoculation (T0), at 1 day post-inoculation (dpi), 2 dpi, 3 dpi and 6 dpi. At each time point, a
181 single leaf disc was excised from the inoculated area using a handheld paper punch (0.6 mm)
182 and washed with distilled water, in order to remove any inoculum residue from the leaf disc
183 before the extraction following the CPE preparation method. The handheld paper punch was
184 cleaned with ethanol 70% between each sample. Two inoculated leaflets per plant were not
185 sampled and kept for symptom observation. Similarly, non-inoculated leaf discs were
186 collected from three potato plants, on which droplets of distilled water were placed, and
187 maintained in a separate growth chamber under the same incubation conditions. The
188 inoculation experiment was conducted twice. LAMP and RPA assays were conducted using
189 the Smart-DART instrument and their sensitivity (early detection) was determined. Healthy
190 CPE samples were included as negative control and positive control samples were prepared
191 by adding *P. infestans* pure DNA (50 pg/ µl) into healthy CPE.

192 **Field samples.** Potato late blight infections were monitored from July to September 2016.
193 A total of 24 potato leaf samples were collected from fields with and without visible
194 symptoms of late blight infections in New-Brunswick and Quebec provinces (Canada),
195 respectively. The samples collected from fields where late blight was reported were classified
196 as true positives. Whereas samples collected from late blight-free potato fields but showing
197 symptoms similar to those caused by *P. infestans* (Fig. 1) were classified as true negatives.
198 To confirm the presence or absence of late blight, collected leaves were placed in humid
199 chambers (plastic bags containing damp paper tissue) to monitor eventual sporulation of *P.*

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200 *infestans*. Leaf discs were excised from the edge of the lesions and were processed following
201 the above-mentioned CPE preparation method. LAMP and RPA assays were conducted using
202 the Smart-DART instrument and the true positive, true negative, false positive, and false
203 negative proportions were defined and used to determine the reliability of the assays. For the
204 LAMP and RPA reactions, healthy CPE samples were included as reaction negative control
205 and positive control samples were prepared by adding *P. infestans* pur DNA (50 pg/ µl) into
206 healthy CPE.

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

217 Results

218 **Specificity of the LAMP and RPA assays.** When tested for *P. infestans* specificity, LAMP
219 and RPA assays presented a cross-reaction with the taxonomically closely related species *P.*
220 *andina*, *P. mirabilis*, *P. phaseoli* and *P. ipomoeae*. However, no cross-reaction occurred with
221 the other potato oomycete and fungal pathogens tested including *P. erythroseptica* and *P.*
222 *nicotiana* (Table 1).

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223 **Sensitivity of LAMP and RPA assays.** The sensitivity of the optimized isothermal assays
224 was determined using *P. infestans* pure DNA serial dilutions ranging from 0.5 ng/µl to 5
225 fg/µl. The limit of detection of both LAMP and RPA assays was 50 fg/µl. Similarly, plant
226 extract had limited effect on amplification of pure DNA that was added into healthy CPE
227 samples. *P. infestans* detection occurred at all the concentrations up to 50 fg/µl, in both
228 LAMP (Fig.2A and 2B) and RPA assays (Fig.3A and 3B). However, a slight delay of Rt
229 values of 1 to 2 minutes for LAMP and 30 sec to 1 min for RPA assays was noted in samples
230 containing plant extract. Considering the obtained limit of detection, the reaction time of the
231 LAMP and RPA assays was 20 minutes and 10 minutes, respectively. The obtained standard
232 curves, plotting DNA serial dilutions with the correspondent Ct values, presented a linear
233 correlation with a regression coefficient (R^2) of 0.980 for LAMP and 0.963 for RPA, when
234 using *P. infestans* pure DNA serial dilutions (Fig. 2C and Fig. 3C). Moreover, when pure
235 DNA was added to healthy CPE, R^2 values were 0.972 and 0.943 for LAMP and RPA,
236 respectively (Fig.2D and Fig.3D).

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

243 **Field samples.** The reliability of the developed assays was determined on a total of 24
244 potato leaf samples collected from fields with and without visible late blight symptoms
245 (Table 4). A single leaf disc was tested per sample. The LAMP assay reported 11 out of 12
246 true positives and 11 out of 12 true negatives, and thus an overall accuracy of 91.7%. Despite

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247 a high value of true negatives (12/12), the RPA assay presented a low proportion of true
248 positive samples (4/12).

249 Discussion

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

254 When tested for their specificity to *P. infestans*, the real-time isothermal assays presented a
255 limited cross-reaction with its closely related species. The real-time LAMP and RPA assays,
256 designed to target *P. infestans* ITS2 region, also amplified DNA from *P. mirabilis*, *P.*
257 *phaseoli*, *P. ipomoeae* and *P. andina.* This is due to the high similarity (99.9%) of the
258 ribosomal DNA internal transcribed spacer regions among *Phytophthora* species (Blair et al.,
259 2012; Raffaele et al., 2010). Similar results are often reported in molecular assays developed
260 for the detection and quantification of *P. infestans* (Fall et al., 2015b; Judelson and Tooley,
261 2000; Lees et al., 2012; Tooley et al., 1997) and are considered of minor consequence as
262 these species do not infect potato or tomato (Goss et al., 2011; Lees et al., 2012). Notably,
263 the LAMP and RPA assays did not cross-react with *P. nicotianae* and *P. erythroseptica,* nor
264 with the other known potato infecting fungal species.

265 The targeted ITS region is known to be in high copy number, thus improving the sensitivity
266 of detection (Bilodeau et al., 2014). Moreover, LAMP and RPA isothermal technologies are
267 efficiently able to generate billions of DNA copies within 40 to 60 minutes (Li and
268 Macdonald, 2015; Notomi et al., 2000). The two developed isothermal assays showed their
269 ability to detect very low quantities of *P. infestans* DNA, *i.e.* 50 fg/µl, and proved to be
270 insignificantly affected by plant extract inhibition when DNA was spiked into healthy CPE

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271 samples. The sensitivity of LAMP and RPA assays compares favorably with previously
272 described *P. infestans* qPCR and LAMP assays, such as: 2 pg (Llorente et al., 2010), 1 pg
273 (Böhm et al., 1999), 100 fg (Lees et al., 2012), 2 pg for LAMP ITSII and 200 pg for LAMP
274 Rgn86_2 assays (Hansen et al., 2016). Within the obtained limit of detection, the reaction
275 time (Rt) of the LAMP and RPA assays was 20 minutes and 10 minutes, respectively.
276 However, after 25 minutes LAMP reaction, we observed a DNA amplification background
277 from the lowest *P. infestans* DNA dilution (5 fg/µl) and the healthy CPE samples. Based on
278 these results, a cut-off value of 25 minutes for the LAMP assays was used and any
279 amplification that occurred beyond this limit (*i.e.* Rt> 25 min.) was considered negative.

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

290 In the inoculation experiments we performed, the LAMP assay detected *P. infestans*
291 infections from asymptomatic leaves as soon as 1 day after the inoculation, whereas the RPA
292 assay detected the infections 3 days after the inoculation, which corresponds to the
293 appearance of the first small lesions on the leaves. Consequently, based on these results the
294 RPA assay should not be used for early detection or to confirm late blight infections. These
295 results highlight the sensitivity and the potential of *P. infestans* early detection using the

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296 developed LAMP assay. Moreover, the tests carried out on field samples provided
297 information on the reliability of the assays, highlighting the better suitability of the LAMP for
298 on-site early detection of *P. infestans* infections. In commercial potato fields, late blight is
299 difficult to manage mostly because of the potentially high rate of disease development under
300 favorable conditions, consequent losses and a suggested action threshold as low as 1% of
301 diseased leaf area (Stein and Kirk, 2002). Various disease decision support systems (DDSS)
302 have been developed to time fungicide applications including initiation and interval between
303 applications (UC-IPM, 2016). To a large extent, these systems improved disease
304 management. However, because they are weather-based, risk estimation could be improved
305 by adding field observations. Recently, Fall et al., (2015a) studied the spatial distribution of
306 *P. infestans* airborne sporangia and showed that risk estimation from DDSS could be greatly
307 improved by adding information on airborne inoculum and disease onset. These authors
308 reported that the first field observations of late blight symptoms occurred about a week after
309 the first significant peak of airborne sporangia concentration. However, *P. infestans*
310 overwinters in stored tubers (seed potatoes) and in infected potatoes discarded in cull piles,
311 and in infected volunteer potato plants. Consequently, it is expected that initial disease will
312 appear as foci and that detection of the first infections might be difficult, requiring that a
313 large number of plants are observed and that proper symptom identification be performed in a
314 timely fashion. The real-time isothermal tool developed in this study could be used for
315 training scouts for early detection of late blight symptoms, and for *in situ* confirmation of any
316 symptoms, especially in the early stages of the disease, providing pathogen-based
317 information for proper fungicide application. This detection requires further validation and
318 implementation, notably for results interpretation in terms of disease management decisions,
319 and for monitoring of infected seed tubers during storage. Besides, a large scale sampling
320 methodology should be defined to have better information on the spatial distribution of the

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321 first infections in the field, for example: a bulked samples approach as described by Villari et
322 al., (2016) could be useful in a large scale plant-screening plan with a limited number of
323 samples to be tested.

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

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

Figure 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 0.5 ng/ul to 5 fg/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 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 0.5 ng/ul to 5 fg/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.

Figure 4: Lesions progression of *Phytophthora infestans* on inoculated potato plants and LAMP and RPA detection over incubation time. dpi: days post-inoculation

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Table 1. Isolates of *Phytophthora* species as well as 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	+	+

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Table 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	+	+

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Table 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.

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Table 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*<u>CGTGATGGACTGGTGAACCATG</u>	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,

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Table 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.

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Table 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			RPA		
	Positive	**Negative**	Total	**Positive**	**Negative**	Total
With LB	11	1	12	4	8	12
Without LB	1	11	12	0	12	12
Total	12	12	24	4	20	24

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Figure 1.

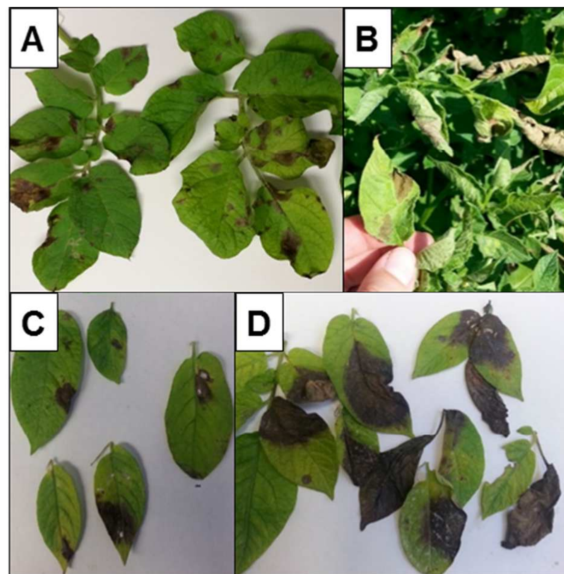

Figure 1: Potato leaf samples collected for the reliability test of the LAMP and RPA assays. Samples were collected from **A, B)** potato fields without visible symptoms of late blight but showing symptoms similar to *P. infestans* lesions, and from **C, D)** potato fields with late blight infections.

1

Figure 2.

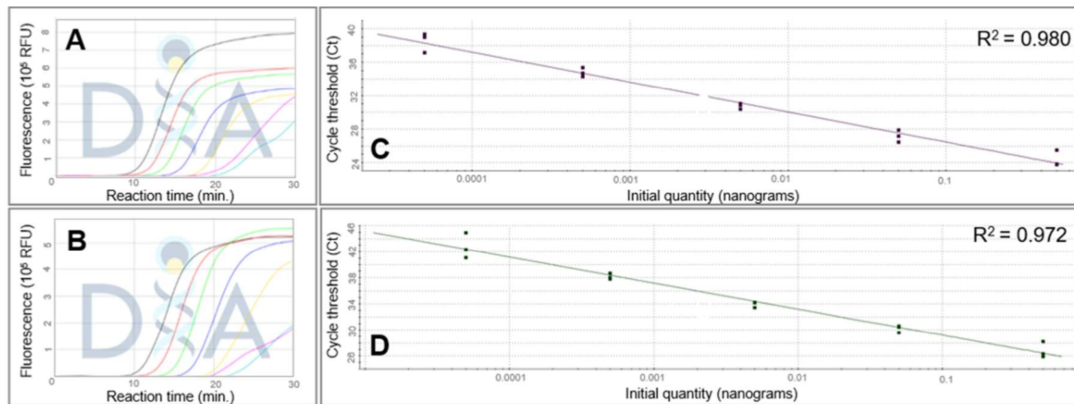

Figure 2: Sensitivity of LAMP assay. Smart-DART amplification curve generated in the LAMP assay using 10 fold serial dilutions of *P. infestans* **A)** pure DNA ranging from 0.5 ng/ul to 5 fg/ul and **B)** DNA serial dilutions incorporated into healthy crude plant extract. Standard curve obtained in 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.

2

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Figure 3.

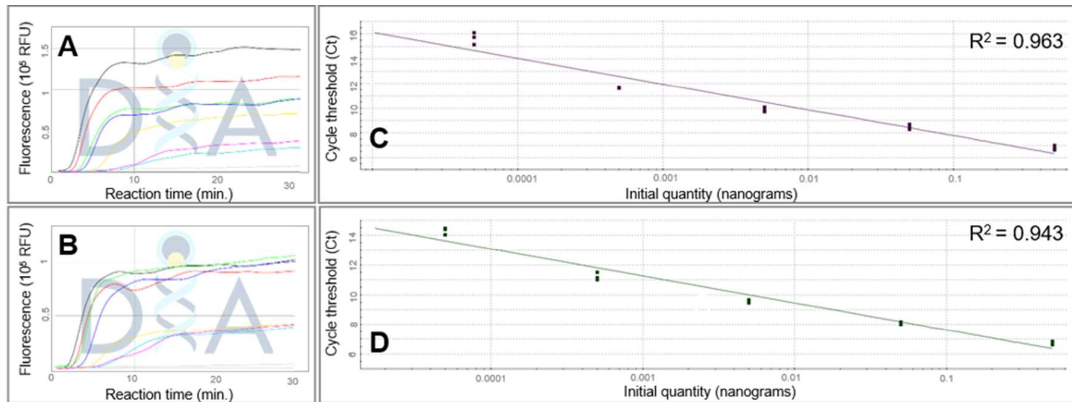

Figure 3: Sensitivity of RPA assay. Smart- DART amplification curve generated in the RPA assay using 10 fold serial dilutions of *P. infestans* **A)** pure DNA ranging from 0.5 ng/ul to 5 fg/ul and **B)** DNA serial dilutions incorporated into healthy crude plant extract. Standard curve obtained in a 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.

3

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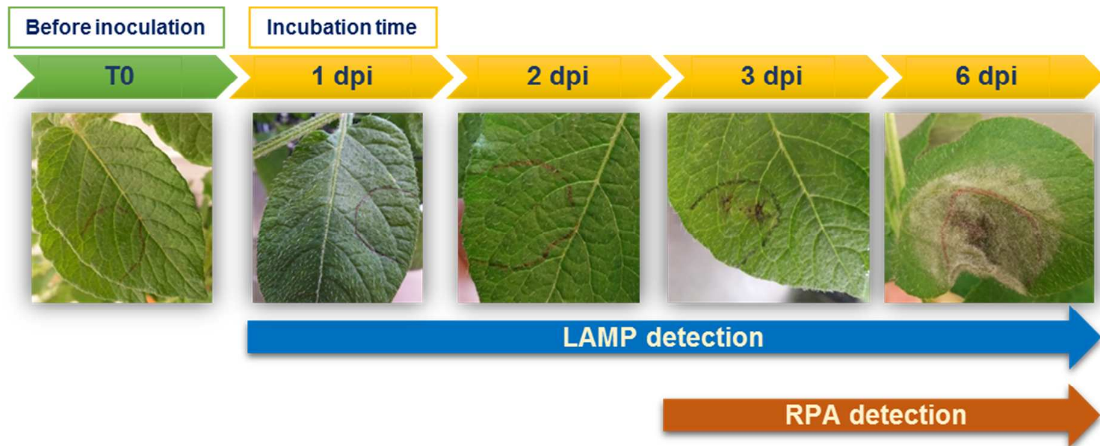

Figure 4: Lesions progression of *Phytophthora infestans* on inoculated potato plants and LAMP and RPA detection over incubation time. dpi: days post-inoculation

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