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TITLE:

Detection of *Phytophthora capsici* in Irrigation Water using Loop-Mediated Isothermal Amplification

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KEYWORDS:

Phytophthora capsici, Irrigation water, Zoospore detection, LAMP assay, Filter paper, Rapid detection, DNA extraction, In-field diagnosis

SUMMARY:

We developed a method to detect *Phytophthora capsici* zoospores in water sources using a filter paper DNA extraction method coupled with a loop-mediated isothermal amplification (LAMP) assay that can be analyzed in the field or in the lab.

ABSTRACT:

Phytophthora capsici is a devastating oomycete pathogen that affects many important solanaceous and cucurbit crops causing significant economic losses in vegetable production annually. *Phytophthora capsici* is soil-borne and a persistent problem in vegetable fields due to its long-lived survival structures (oospores and chlamydospores) that resist weathering and degradation. The main method of dispersal is through the production of zoospores, which are single-celled, flagellated spores that can swim through thin films of water present on surfaces or in water-filled soil pores and can accumulate in puddles and ponds. Therefore, irrigation ponds can be a source of the pathogen and initial points of disease outbreaks. Detection of *P. capsici* in

irrigation water is difficult using traditional culture-based methods because other microorganisms present in the environment, such as *Pythium* spp., usually overgrow *P. capsici* making it undetectable. To determine the presence of *P. capsici* spores in water sources (irrigation water, runoff, etc.), we developed a hand pump-based filter paper (8-10 µm) method that captures the pathogen's spores (zoospores) and is later used to amplify the pathogen's DNA through a novel loop-mediated isothermal amplification (LAMP) assay designed for the specific amplification of *P. capsici.* This method can amplify and detect DNA from a concentration as low as 1.2 x 10^2 zoospores/mL, which is 40 times more sensitive than conventional PCR. No cross-amplification was obtained when testing closely related species. LAMP was also performed using a colorimetric LAMP master mix dye, displaying results that could be read with the naked eye for on-site rapid detection. This protocol could be adapted to other pathogens that reside, accumulate, or are dispersed via contaminated irrigation systems.

INTRODUCTION:

Recycling water in farms and nurseries is becoming increasingly popular due to the increase in water costs and environmental concerns behind water usage. Many irrigation methods have been developed for growers to reduce the spread and occurrence of plant disease. Regardless of the source of the water (irrigation or precipitation), runoff is generated, and many vegetable and nursery growers have a pond to collect and recycle runoff[1]. This creates a reservoir for possible pathogen accumulation favoring the spread of pathogens when the recycled water is used in irrigate crops[2-4]. Oomycete plant pathogens particularly benefit from this practice as zoospores will accumulate in water and the primary dispersive spore is self-motile but requires surface water[5-7]. *Phytophthora capsici* is an oomycete pathogen that affects a significant number of solanaceous and cucurbit crops in different ways[8]. Often, the symptoms are damping-off of seedlings, root and crown rot; however, in crops such as cucumber, squash, melon, pumpkin, watermelon, eggplant and pepper, entire harvests may be lost due to fruit rot[9]. Although there are known methods of detecting this plant pathogen, most require an infection to have already taken place which is too late for any preventative fungicides to have a significant effect[10].

The traditional method to test irrigation water for the detection and diagnosis of targeted microorganisms is an antiquated approach when speed and sensitivity are crucial to success and profitable crop production[11,12]. Plant tissue susceptible to the targeted pathogen (e.g., eggplant for *P. capsici*) is attached to a modified trap that is suspended in an irrigation pond for extended period before being removed and inspected for infection. Samples from the plant tissue are then plated on semi-selective media (PARPH) and incubated for culture growth, then morphological identification is performed using a compound microscope[13]. There are other similar detection methods for other plant pathogens using selective media and plating small amounts of contaminated water before sub-culturing[14,15]. These methods require anywhere from 2 to 6 weeks, several rounds of sub-culturing to isolate the organism, and experience on *Phytophthora* diagnostics to be able to recognize the key morphological characters of each species. These traditional methods do not work well for detection of irrigation water contaminated by *P. capsici* due to factors such as interference by other microorganisms that also present in the water sources. Some fast-growing microorganisms like *Pythium* spp. and water-borne bacteria can overgrow on the plate making *P. capsici* undetectable[16,17].

The purpose of this study was to develop a sensitive and specific molecular method that can be used in both field and laboratory settings to detect *P. capsici* zoospores in irrigation water. The protocol includes the development of a novel loop-mediated isothermal amplification (LAMP) primer set able to specifically amplify *P. capsici*, based on a 1121-base pair (bp) fragment of *P. capsici*[18,19]. A previously developed LAMP primer from Dong et al. (2015) was used in comparison to the assay that was developed for this study[20].

The LAMP assay is a relatively new form of molecular detection that has been demonstrated to be more rapid, sensitive, and specific than conventional polymerase chain reaction (PCR)[21]. In general, conventional PCR assays cannot detect under 500 copies (1.25 pg/µL); in contrast, previous studies have shown that the sensitivity of LAMP can be 10 to 1,000 times higher than conventional PCR and can easily detect even 1 fg/µL of genomic DNA[22,23]. Additionally, the assay can be carried out rapidly (often in 30 min) and on-site (in the field) by using a portable heating block for amplification and a colorimetric dye that changes color for a positive sample (removing the need for electrophoresis). In this study, we compared the sensitivity of PCR and LAMP assays using a filter extraction method. The proposed detection method allows researchers and extension agents to easily detect the presence of *P. capsici* spores from different water sources in less than two hours. The assay is proven to be more sensitive than conventional PCR and was validated in situ by detecting the presence of the pathogen in the irrigation water used by a grower. This detection method will allow growers to estimate the presence and population density of the pathogen in various water sources that are being used for irrigation, preventing devastating outbreaks and economic losses.

PROTOCOL:

1. On-site detection of *Phytophthora capsici* from irrigation water using portable loop-mediated isothermal amplification

1.1. Setting up the pump and filter

1.1.1. Attach a filtering flask to a tube that is connected to a hand pump so that when the pump is activated, air will be pulled in through the mouth of the filtering flask.

1.1.2. Fit the Buchner funnel into the rubber stopper to the mouth of the filtering flask and fit the appropriately sized piece of filter paper into the Buchner funnel so that air is pulled through the filter paper. The filter paper should have a retention size of 15 µm.

NOTE: The filter paper must fit to the edges of the Buchner funnel so that minimal water will flow around the filter paper.

1.2. Water sampling and filtering

1.2.1. Take water samples from the targeted source. Water may have small amounts of debris but not significant sediment or soil.

1.2.2. Pour up to 1,000 mL of test water over the filter paper placed inside the Buchner funnel slowly enough to prevent overflow, while the hand pump (or vacuum) is being used to create a suction to pull the water through.

NOTE: There is no minimum amount of water that can be tested using this method, and although it at least 50 mL is suggested, 1000 mL is the maximum for this method.

1.2.3. Using forceps, remove the filter paper from the Buchner funnel and cut it into small pieces with sterile scissors. Add as many pieces (8-12) as can be submerged in the amount of extraction buffer (400 µL for magnetic bead-based extraction) required by the protocol into a 1.5 mL tube. Save remaining pieces of filter paper for processing after the first set has been extracted.

1.2.4. Vortex or otherwise agitate the pieces of filter paper and lysis buffer for 10 s every minute for 5 min. Then, using forceps, remove the filter paper losing as little of the lysis buffer as possible. Repeat this step with the remaining pieces of filter paper until all pieces have been vortexed/agitated and soaked in the lysis buffer.

1.3. Magnetic bead-based extraction of DNA from filter paper

1.3.1. To the 1.5 mL tube (which now contains approximately 200-300 µL of lysis buffer), add 20 µL of proteinase K and 10 µL of 10 ng/µL RNase.

1.3.2. Incubate at room temperature for 15 min, vortex or shake the tube every 3 min.

1.3.3. Add 500 µL of magnetic beads with the binding buffer to the sample and mix well by shaking. Then incubate for 5 min at room temperature.

1.3.4. Place the tube in the magnetic separator rack for 2 min until all beads have been pulled to the magnet. Remove and discard the supernatant.

1.3.5. Remove the tube from the magnetic separator. Add 500 µL of Wash Buffer 1 and re-suspend beads by shaking the tube vigorously. Wait for 30 s and then place the tube back in the magnetic separator. Wait for 2 min until all beads have been pulled towards the magnet before removing and discarding the supernatant.

NOTE: When waiting for the magnetic beads to magnetize to the separator, we recommend inverting the tube, which can dislodge magnetic beads stuck to the cap and the sides of the tube and result in a greater number of beads being attached.

1.3.6. Repeat step 1.3.5 with 500 µL of Wash Buffer 2.

1.3.7. Repeat step 1.3.5 with 500 µL of 80% ethanol.

1.3.8. Airdry the magnetic bead pellet for 15 min at room temperature (18-27 °C) with the lid open. If temperatures do not permit, incubate in a gloved hand with the cap open for 15 min.

1.3.9. Remove the tube from the magnetic separator. Add 50 µL of elution buffer and re-suspend the beads by pipetting up and down for 1 min.

1.3.10. Place tube back in a magnetic separator. Wait 2 min before transferring the supernatant without disturbing the beads to a separate tube for DNA storage.

NOTE: Here the experiment can be paused before moving on. Extracted DNA should be stored on ice or in a -20 °C freezer.

1.4. Application of newly developed LAMP assay

1.4.1. Prepare LAMP primer mix using 0.2 µM of each F3 and B3 primer, 0.8 µM of each Loop-F and Loop-B primer, and 1.6 µM of each FIP and BIP primer (**Table 2**).

1.4.2. Add the following LAMP solution to a single PCR tube, or to each individual tube in the 8 tube strip: 2.5 µL of primer mix (step 1.4.1), 12.5 µL of LAVA LAMP master mix, 1 µL of extracted DNA, and 9 µL of ddH_2O. The total volume is 25 µL.

NOTE: If using the portable amplification instrument (e.g., Genie III) with colorimetric dye (e.g., Warmstart), refer to section 2.4.2- 2.4.3.

1.4.3. Designate two tubes as positive and negative controls. For the positive control, use either a control provided by the LAVA LAMP kit, or a known positive DNA sample. For the negative control, use ddH_2O. For both, substitute the 1 µL of extracted DNA for 1 µL of the positive control or ddH_2O.

1.4.4. Set the samples into a heat block (or portable amplification instrument) set to 64 °C for 45 min.

NOTE: Other extraction methods can be used here instead of magnetic bead based extraction. CTAB and a commercial DNA extraction kit were both successfully tested using the standard protocols and substituting the filter paper pieces for the plant sample[24,25]. Results were compared in **Table 2**. If concentration of DNA can be quantified, use between 1-10 ng genomic DNA.

1.5. Visualization of results

1.5.1. If performed in a laboratory setting, view the amplification products by loading 5 µL of each sample into a 1% agarose gel, running them in a gel electrophoresis machine, and imaging them in a UV imaging machine.

1.5.2. If a colorimetric dye (e.g., Warmstart) was used, view the color change to determine results as positive or negative.

1.5.3. If a portable amplification instrument (e.g., Genie III) was used, view the amplification graph on the screen to determine the results.

1.6. Application of previously developed PCR assay

NOTE: If using a conventional PCR assay, steps 1-3 remain the same, and the following steps should be applied in place of steps 1.4 and 1.5.

1.6.1. Add 1 µL of each DNA extraction to individual tubes containing the following components: 12.5 µL of Green PCR master mix, 9.5 µL of ddH_2O, and 1 µL of forward and reverse primers (**Table 2**).

1.6.2. Spin down each sample using a microcentrifuge and place tubes into a thermal cycler.

1.6.3. Use the following thermal cycler settings in accordance to the previous publications: 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min.

1.6.4. Run the product in a 1% agarose gel. Observe the presence of bands under UV light where positive reactions will have a band size of ~508 bp.

1.7. Traditional method of detection

NOTE: There are multiple methods of selective plating for pathogen detection, and the following is a general protocol for *P. capsici*.

1.7.1. First, obtain a healthy eggplant fruit (a susceptible host for *P. capsici*) and surface sterilize by washing the fruit surface with 70% isopropyl alcohol.

1.7.2. Place the eggplant fruit into milk crates with a flotation device (polyethylene foam or other) and deploy into irrigation ponds. Secure each bait trap to a single point and leave the traps in the water reservoir (recycled irrigation water) for at least 7 days or until fruit rot symptoms are observed. Collect the fruit and transport it to the laboratory.

1.7.3. Rinse and dry the fruit in a sterile hood before removing small pieces of infected tissues and place them on a plate of PARPH medium amended with 25 mg/L pentachloronitrobenzene,

0.0005% pimaricin, 250 mg/L ampicillin, 10 mg/L rifampicin and 50 mg/L hymexazol. Incubate plates at 25 °C for 5 days.

1.7.4. View plates under a compound microscope for traditional morphological identification at 4 days after isolation.

2. Determining the detection limit of zoospore concentration

2.1. Making zoospore suspension

2.1.1. Incubate *P. capsici* on V8 agar (100 mL of V8 juice, 900 mL of ddH_2O, 1 g of $CaCO_3$) plates for 1 week at 26 °C. Multiple plates can be used to obtain a larger amount of zoospore suspension.

2.1.2. Incubate the plates under continuous light at room temperature for 3 days to stimulate sporulation.

2.1.3. Flood the plates by adding 15 mL of ddH_2O to each plate and place them in a 4 °C fridge for 25 min. Then return to room temperature for 30 min.

2.1.4. Agitate plates to dislodge zoospores and pipette the solution into a single 50 mL tube from all the plates.

2.1.5. To obtain an accurate estimate of zoospore concentration, add 10 µL of the spore suspension onto a hemocytometer and observe under a microscope to count zoospores and estimate the average concentration.

2.2. Serial dilution

2.2.1. Add 1 mL of the spore suspension and 9 mL of ddH_2O to a separate tube. Repeat this step for as many 10-fold dilutions as desired.

2.2.2. Spore solutions are then submitted for DNA extraction based on the previous protocol using the filtration method.

NOTE: If a larger volume of suspension is desired, double the volume: 2 mL of spore suspension and 18 mL of ddH_2O.

2.3. Detection of zoospore concentration limit

2.3.1. Evaluate spore detection limit by running each serial dilution individually through the assay until clearly positive results are no longer observed. Once the final dilution is obtained, dilute by a factor of 2 (4.8 to 2.4 in this example) and run the assay again to get a more accurate detection limit.

2.4. Development and optimization of the LAMP method

NOTE: LAMP primers were designed based on a 1121-base pair (bp) fragment of *P. capsici* (Li et al.[19]) as shown in **Supplementary Figure 2**.

2.4.1. If colorimetric dye (e.g., Warmstart) is used, use the following the solution: 2.5 µL of primer mix, 12.5 µL of colorimetric dye, 0.5 µL of Green fluorescent dye, 1 µL of extracted DNA, and 8.5 µL of ddH_2O. The total volume is 25 µL.

2.4.2. When using the portable amplification instrument with LAVALAMP mastermix, have an initial step of 95 °C for 3 min as recommended by the manufacturer, but this is not required. A final annealing step is not required to observe the color change or amplification graph. Do not run a warmstart step if the commercial colorimetric dye is to be used.

2.4.3. View LAMP assay results in one of the following methods: run samples on a 1% agarose gel or view using a UV imaging machine with the naked eye or view at the Genie III real-time amplification screen.

2.4.4. Optimize the temperature of the LAMP assay by using the portable amplification instrument and analyzed using the real-time amplification graph for speed and level of sensitivity. Run samples with unique temperatures to determine the fastest amplification with the highest level of sensitivity.

2.4.5. Determine the detection limit of the LAMP developed assay by making a serial dilution of extracted DNA (as with spore suspension in step 2.2.1) and maintaining reaction conditions as previously described for the LAMP reaction for each dilution.

2.5. Detection limit determination and comparison with conventional PCR method

2.5.1. Use DNA extracted in steps in section 1.3 to compare the detection level of conventional PCR with that of the LAMP assay.

2.5.2. Add 1 µL of DNA to a PCR tube that contained 1 µL of both forward and reverse PCR primers (**Table 2**), 12.5 µL of Green PCR Mastermix, and 9.5 µL of ddH_2O for a total of 25 µL.

2.5.3. Amplify samples in a thermal cycler using the following conditions: 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min.

2.5.4. Run samples in an electrophoresis machine on a 1% agarose gel and view on a UV imaging machine. The excepted band size was 508 bp.

REPRESENTATIVE RESULTS

Optimization of LAMP method

In this study, we detected the presence of *Phytophthora capsici* in irrigation water using a portable loop-mediated isothermal amplification (LAMP) assay. First, the proposed LAMP assay was optimized by testing different LAMP primer concentrations [F3, B3 (0.1–0.5 µM each); LF, LB (0.5–1.0 µM each) and FIP, BIP (0.8–2.4 µM each)], durations (30–70 min), and temperatures (55–70 °C). The final LAMP primer mix used in this study was: 0.2 µM of each F3 and B3 primer, 0.8 µM of each Loop-F and Loop-B primer, 1.6 µM of each FIP and BIP primer. Optimization of reaction temperature was performed in the portable amplification instrument (e.g., Genie III) by determining what temperature performed the fastest reaction with no additional negative amplification. The optimal temperature was confirmed to be 64 °C (data not shown). The optimal time for running the assay at 64 °C was 45 min, as the lowest concentrations that were positive for detection (1.2×10^2 spores/mL) still amplified by 40 min, while higher concentrations amplified at 20 min (**Figure 2 D**). The amplified LAMP products were further observed on 1% agarose gel stained with a nucleic acid stain to confirm amplification. All reactions were repeated at least three times.

Isolates of *P. capsici* were taken from Tennessee, Florida, and Georgia and were submitted to the same protocol described in the methods. All samples of *P. capsici* isolates were amplified successfully in all runs of the assay (**Figure 3**).

Detection and sensitivity testing of *Phytophthora capsici* in irrigation water using portable LAMP assay

We standardized this filter paper-based LAMP method under laboratory conditions using a serial dilution of *P. capsici* spore suspensions (**Figure 1**). Serial dilutions were made from a *P. capsici* spore suspension starting at 4.8×10^4 zoospores/mL and run with the LAMP assay in triplicate. Spore concentrations are shown rather than DNA concentration due to the method involved for DNA extraction. CTAB DNA extraction of the highest spore concentration was 4.5 ng/µL measured by Nanodrop, and the magnetic bead DNA extraction protocol yielded 3.8 ng/µL[26]. The newly designed LAMP primer set could detect a concentration as low as 1.2×10^2 spores/mL (**Figure 2B, 2C,** & **2D**) with all methods of extraction. The sensitivity shown in the graph of amplification on the portable amplification instrument was reflective of that shown in the UV image with no additional sensitivity. The same serial dilution was run in a LAMP reaction using the colorimetric dye to determine the level of sensitivity to the naked eye for field detection. The lowest observable concentration was 4.8×10^3 spores/mL (**Figure 2C**).

To evaluate the ability of this assay using real-world samples, water samples were collected from seven ponds used for commercial vegetable production in Tift County, Georgia (**Table 1, Figure 5,** and **Supplementary Figure 1**. Out of the 7 ponds, 3 showed positive LAMP results (P1, P4, and P6) (**Figure 6A, 6B, & 6C**). These results suggest that the portable filter paper-based LAMP method could be very useful for detection of the pathogen even with a low zoospore concentration. This demonstrates the applicability of LAMP as a more sensitive detection assay than PCR for screening irrigation water contamination by *P. capsici.*

Comparative analysis of different methods: Traditional baiting, conventional PCR, and the portable LAMP-based assay

In order to compare the detection sensitivity of LAMP with conventional PCR, the DNA extracted from the serial dilution of spore suspensions were run in a PCR reaction. Results showed that conventional PCR was 40x less sensitive than LAMP which could detect zoospore concentration as low as 4.8 x 10^3 spores/mL (**Figure 2A**). Additionally, the DNA samples obtained from filtered irrigation pond water were tested using conventional PCR, and only one of the three positive samples (P4) was successfully amplified as expected band size plus significant contaminates resulting in some smearing and unspecific bands (**Figure 6D**). **Table 3** displays the differences between detection methods using such variables as time, cost, sensitivity, and preparation required. LAMP was the least expensive method among these three methods and it was also the fastest, ranging from 30-60 min for amplification (DNA extraction excluded). Conventional PCR ranged from 120-180 min for amplification (DNA extraction excluded).

Finally, to determine the specificity of the primers, samples of closely related oomycete pathogens (*Phytophthora sansomeana*, *Phytophthora sojae*, *Phytophthora cinnamomi*, *Phytophthora palmivora*, *Pythium ultimum* var*. ultimum*, *Phytopythium vexans*, *Phytopythium helicoides*, and *Pythium aphanidermatum*) were obtained, DNA was extracted using the same protocol for consistency and evaluated by the new LAMP assay with a positive and negative control (**Figure 4**) to determine specificity of the primers. All non-target samples were negative using the optimized 64 °C for 45 min. This was observed on the real-time amplification graph and imaged on a 1% agarose gel under UV light.

Figure captions:

Figure 1: Diagram showing the different steps involved in *Phytophthora capsici* from a serial dilution of a concentrated spore suspension under laboratory conditions.

Figure 2: Laboratory optimization of the limit for detection of *Phytophthora capsici*. (A) Conventional PCR assay was carried out using specific *P. capsici* primers on serial dilution factors and visualized on 1% agarose gel. 1, Ladder; 2-7, 4.8 x 10^4, 4.8 x 10^3, 4.8 x 10^2, 2.4 x 10^2, 1.2 x 10^2 spores/mL, respectively and 7, negative water control. (**B**) LAMP assay serial dilution factors visualized on 1% agarose gel. 1-6, a decreasing spore concentration: 4.8 x 10^4, 4.8 x 10^3, 4.8 x 10^2, 2.4 x 10^2, 1.2 x 10^2 spores/mL, and 7, negative water control. (**C**) LAMP results visualized using the colorimetric dye. 1-6, a decreasing spore concentration: 4.8 x 10^4, 4.8 x 10^3, 4.8 x 10^2, 2.4 x 10^2, 1.2 x 10^2 spores/mL, and 7, negative water control. (**D**) LAMP results visualized on the amplification graph. Red = 4.8 x 10^4, Dark blue = 4.8 x 10^3, Orange = 4.8 x 10^2, Light blue = 2.4 x 10^2, Green = 1.2 x 10^2 spores/mL, Pink = Negative control (other Phytophthora species), Yellow = ddH2O. (**E**) A standard curve showing quantification of the values shown in the real-time results. Ln (Spore count) is shown on the X-axis, and minutes to amplification on the Y-axis. (**F**) LAMP assay with published primer (Dong et al. 2015) on serial dilution factors visualized on 1% agarose gel. 1-6, a decreasing spore concentration: 4.8 x 10^4, 4.8 x 10^3, 4.8 x 10^2, 2.4 x 10^2, 1.2 x 10^2 spores/mL, and 7, negative water control.

Figure 3: Amplification of *P. capsici* DNA from various locations. (**A**) LAMP results visualized on 1% agarose gel. 1, PC_TN1; 2, PC_TN2; 3, PC_FL1; 4,PC_FL2; 5, PC_GA1; 6, PC_GA2; N, Negative control. Samples 1 and 2 were isolated from TN; samples 3 and 4 isolated from FL; samples 5 and 6 were isolated from GA. (**B**) Results visualized using the colorimetric dye. 1, PC_TN1; 2, PCTN2; 3, PC_FL1; 4,PC_FL2; 5, PC_GA1; 6, PC_GA2. (**C**) Results visualized on the amplification graph. Red, PC_TN1; Orange, PCTN2; Yellow, PC_FL1; Green, PC_FL2; Dark blue, PC_GA1; Light blue, PC_GA2; Pink, negative control.

Figure 4: Specificity determination of LAMP assay using DNA from non-target species *P. capsici.* (**A**) LAMP assay reaction with related non-target species on agarose gel and visualized on 1% agarose gel. L, Ladder; 1, *Phytophthora sansomeana*; 2, *Phytophthora sojae*; 3, *Phytophthora cinnamomi*; 4, *Phytophthora palmivora*; 5, *Pythium ultimum* var. *ultimum*; 6, *Phytopythium vexans*; 7, Negative control; 8, *Phytophthora capsica.* (**B**) LAMP results visualized using the colorimetric dye. 1, *Phytophthora sansomeana*; 2, *Phytophthora sojae*; 3, *Phytophthora cinnamomi*; 4, *Phytophthora palmivora*; 5, *Pythium ultimum* var. *ultimum*; 6, *Phytopythium vexans*; 7, Negative control; 8, *Phytophthora capsici* (**C**) LAMP results visualized on the amplification graph. Red = *Phytophthora capcisi*, all other non-target species samples were not amplified.

Figure 5: Pictures showing the sampling and processing of recycled water for the detection of *Phytophthora capsici* in the field.

Figure 6: Results from on-site detection of *Phytophthora capsici* in irrigation water sources. (**A**) Agarose gel showing results from the LAMP amplification of tested water from seven farm in South Georgia. Sample names from left to right: P1, P2, P3, P4, P5, P6, P7, Negative control, N. (**B**) LAMP results visualized using warmstart colorimetric dye of field samples: P1, P2, P3, P4, P5, P6, P7, Negative control, N. (**C**) Results from LAMP amplification of field samples using graph. Red: P1, Green: P2, Purple: P3, Yellow: P4, Blue: P5, Orange: P6, Pink: P7, Negative control, N. (**D**) Agarose gel showing conventional PCR results of the amplification using specific primers PC-1/PC-2 (note than only one site tested positive in comparison to three in LAMP).

Table 1. Detection of irrigation water from Southern GA

Table 2: Primers used in this study

Table 3. Comparison of methods for detection of P. capsici

Supplementary Figure 1: Location of Tift County, GA and samples of irrigation water taken from various locations from within the state. Positive samples were shown as red dots.

Supplementary Figure 2: Design of LAMP primer set. Arrows show direction of how primers are read.

DISCUSSION

The testing of irrigation water for phytopathogens is a crucial step for growers using irrigation ponds and recycle water[27]. Irrigation ponds provide a reservoir and breeding ground for a number of phytopathogens as excess irrigation water is directed from the field to the pond carrying with it any pathogens that may have been present[16,27]. The traditional method for detection of a plant pathogens in a large water source is to set a bait for the pathogen by using susceptible host tissue (e.g., fruit, leaves) suspended in the pond and wait for an infection to take place, then remove the fruit/leaves and confirm the diagnosis with microscopy or molecular methods[13,14]. These methods are limiting due to the amount of time required to run the detection test (2 weeks or longer), and the labor and equipment required. Additionally, extensive experience and knowledge in visual diagnosis, pathogen morphology, and taxonomy are required for accurate results. Molecular techniques such as PCR, qPCR, and DNA hybridization require significantly less time (3-4 h) than the traditional methods of detection; however, they require expensive equipment and a laboratory setting. Additionally, these techniques do not allow for the processing of large volumes of water. Serological assays too, fall short in their detection ability due to non-target positive reactions, and no species-specific assays for Phytophthora species have been developed. Loop-mediated isothermal amplification (LAMP) has recently been used as an on-site diagnosis technique for rapid and sensitive detection of multiple pathogens as the assay only requires a single temperature rather than a thermal cycler[28,29]. LAMP can be run in the field using a colorimetric dye for visual confirmation or using a real-time amplification machine for results in less than one hour[30].

The goal of this experiment was to develop a rapid and sensitive method to detect the presence of *Phytophthora capsici* in water sources either on-site or in a laboratory. To increase the speed of detection and to combat the limitations of the previously mentioned methods for detecting *P. capsici* in irrigation water, we designed a method using filter paper to capture the spores and extract their DNA from a larger volume of water. After spores were captured using the filter paper technique and DNA was extracted, the presence of the pathogen was confirmed based on a newly designed LAMP primer set specific to *P. capsici*. Detection sensitivity and specificity was compared using LAMP and PCR. In all 3 replications and with all of the zoospore concentrations, LAMP was a quicker and more sensitive detection method (**Table 3**). This method is not limited by having a small sample volume as traditional methods, as this method can be test up to 1 L of water at a single time, increasing the chances of pathogen detection. It was noted in testing that pouring irrigation water slowly through the Buchner funnel at a speed of no more than 40 mL per second increased the spore capture ability of the filter paper.

To validate the detection protocol, water samples from the field where *P. capsici* was suspected to be present were also taken (**Supplementary Figure 1**) to test the designed method with a practical scenario. Out of the 7 farms tested, 3 were positive for the presence of *P. capsici* using the LAMP assay (**Figure 6A-6C**) while only one farm was positive when using the conventional PCR assay (**Figure 6D**), showing LAMP as a more sensitive assay for this method of testing irrigation water. Although, this filter based LAMP assay could detect DNA from a concentration as low as 1.2×10^2 zoospores/mL which was significantly less than the original sensitivity of this assay (0.01 ng genomic DNA equivalent to ~5 spores) with unfiltered zoospore suspension. The previous LAMP assay by Dong et. al. (2015)[20] was run on the same serial dilution and the level of

sensitivity was the same (**Figure 2F**). The level of detection for a PCR assay with unfiltered spore solution have also shown a higher level of detection (equivalent ~10 spores) which was very similar to the previous PCR based findings[10]. The spore detection limit of the traditional baiting method of detection was not checked as a single spore could cause infection depending on its individual ability. The decreased sensitivity found in both LAMP and PCR assays is likely due to some spores flowing through or around the filter paper, or once attached to the filter paper, unable to be extracted with 100% efficiency. Nevertheless, this new LAMP and filter system can process and analyze a much higher volume of water than previous methods and confers a higher level of specificity and speed for in-field detection.

Of the extraction methods used, magnetic bead-based extraction was the most rapid and did not require the use of external machines such as a bead beater or centrifuge making it useful for in-field extractions and compliments the portable feature of the LAMP assay. The CTAB based method yielded the highest concentration of DNA but took the longest amount of time, while the commercial plant DNA extraction kit (e.g., DNeasy) was second in both time required and DNA concentration acquired.

With both CTAB and the commercial plant DNA extraction kit, during the homogenization of filter paper it was noted that homogenization was more successful and yielded a higher concentration if the CTAB solution (or extraction buffer) was added to the tube with the pieces of filter paper before bead beating or hand homogenization commenced. Bead beating was done 3 times for one minute each, but vortexing and agitating the tube in between each round was necessary for complete homogenization in all extraction methods. Importantly, the filter paper is subject to getting stuck on the sides or bottom of the tube, so it is crucial to make sure the filter paper is off the walls so that it gets homogenized.

The total time required for amplification is 90-120 min and can be easily done in the field for on-site diagnosis. This filter method is also designed to filter significant amounts of water to increase the possible chances for the detection of the pathogen. This method is also applicable to many pathogens that can accumulate in a water source, particularly genera such as: *Pythium*, *Phytophthora*, *Fusarium*, and bacteria; the only change required will be the development of an equally specific LAMP primer set for the targeted pathogen[31].

A significant output of this work is the development of a highly sensitive and rapid filter paper-based LAMP assay for the detection of *P. capsici* in irrigation water sources. We expect that this study will lead to an increase in awareness of contamination of recycled irrigation water, eventually improving management of Phytophthora associated diseases, and consequently reduce production costs and increase crop yield. Such information is highly needed to improve vegetable production sustainability and enhance profitability of vegetable productions.

ACKNOWLEDGMENTS

This work received the financial support of Georgia Commodity Commission for Vegetables project ID# FP00016659. The authors thank Dr. Pingsheng Ji, University of Georgia and Dr. Anne

Dorrance, Ohio State University for providing pure cultures of *Phytophthora spp*. We also thank Li Wang and Deloris Veney for their technical assistance throughout the study.

DISCLOSURES

The authors have nothing to disclose or any conflicts of interest.

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Figure 1

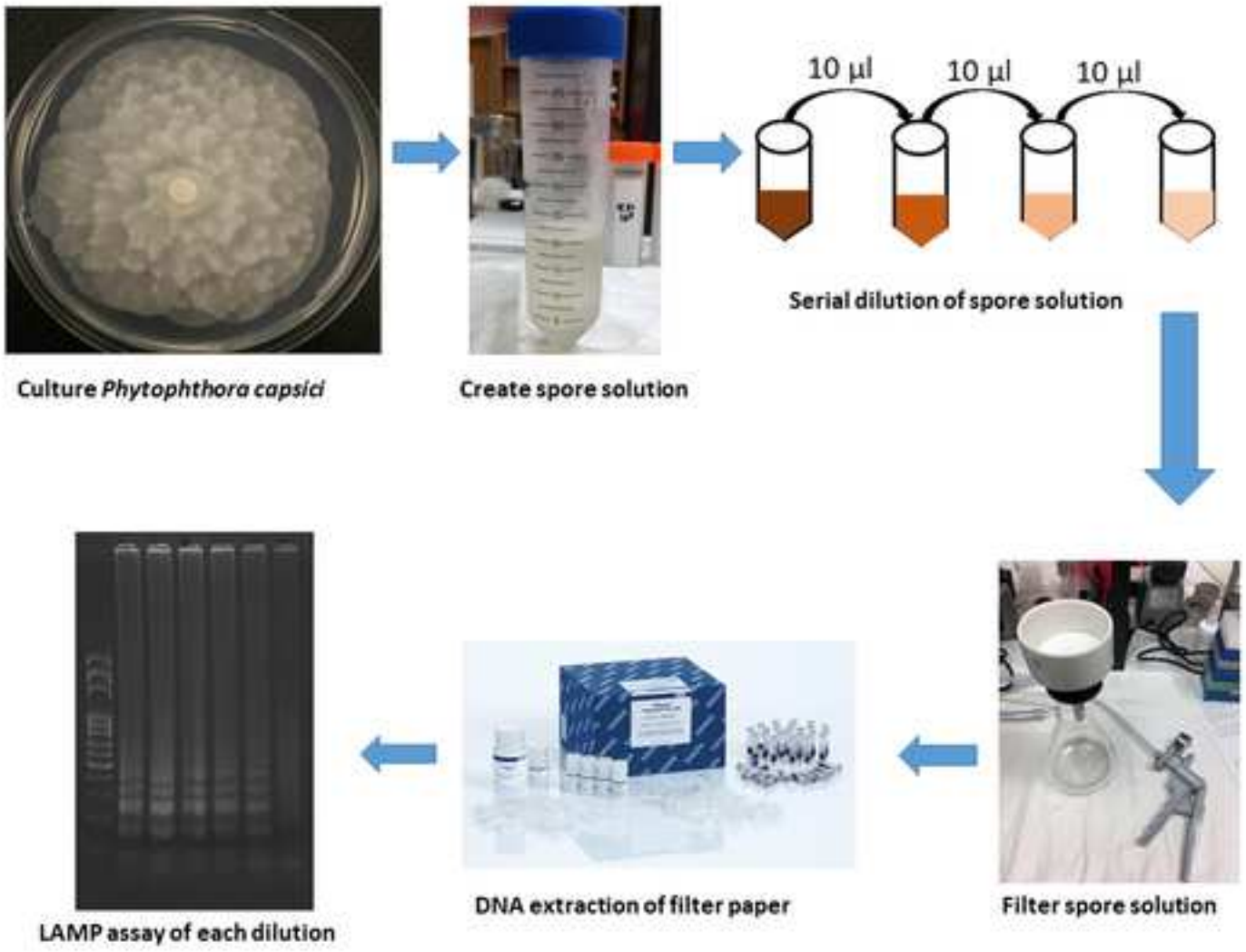

Figure 2

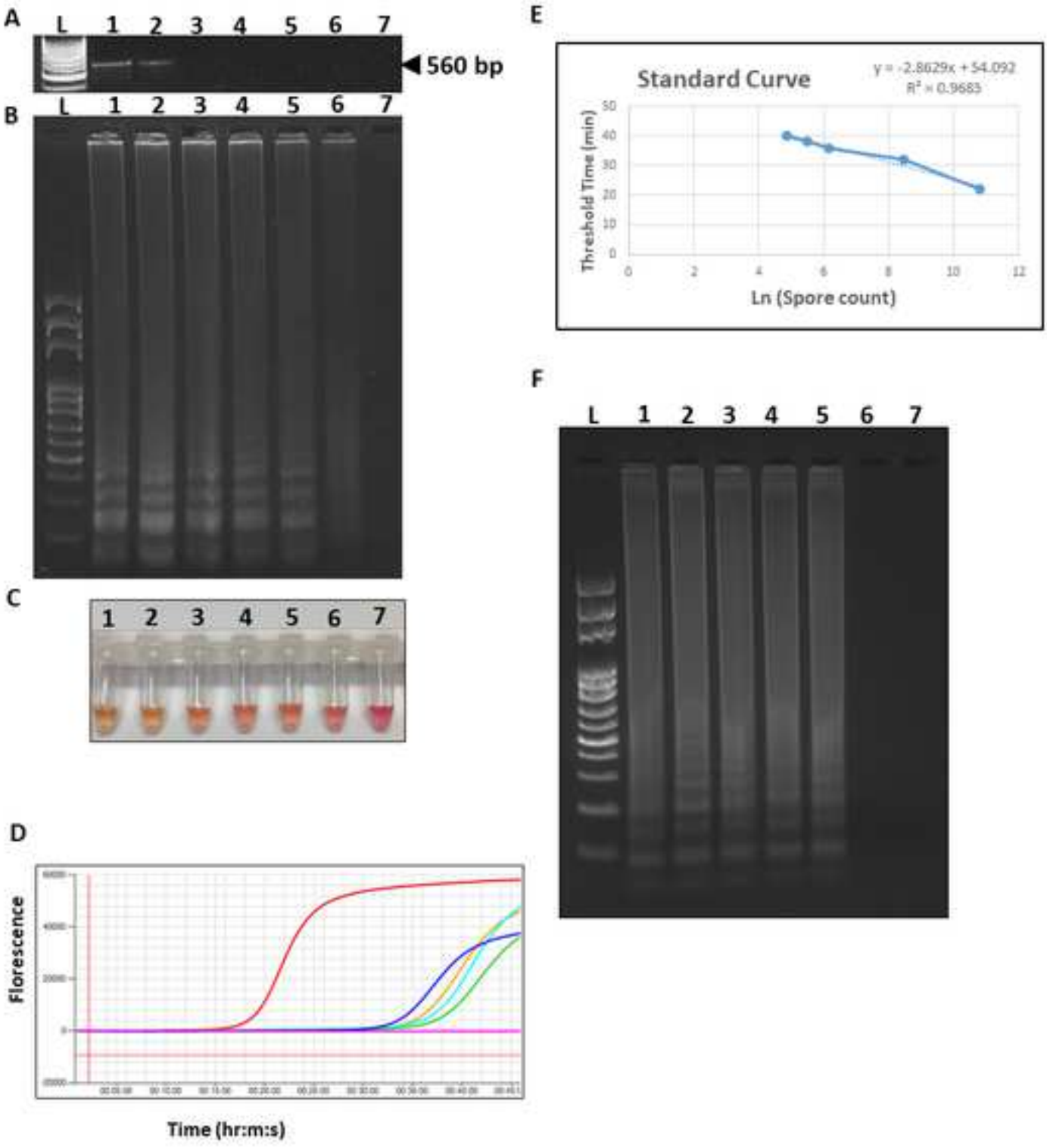

Figure 3

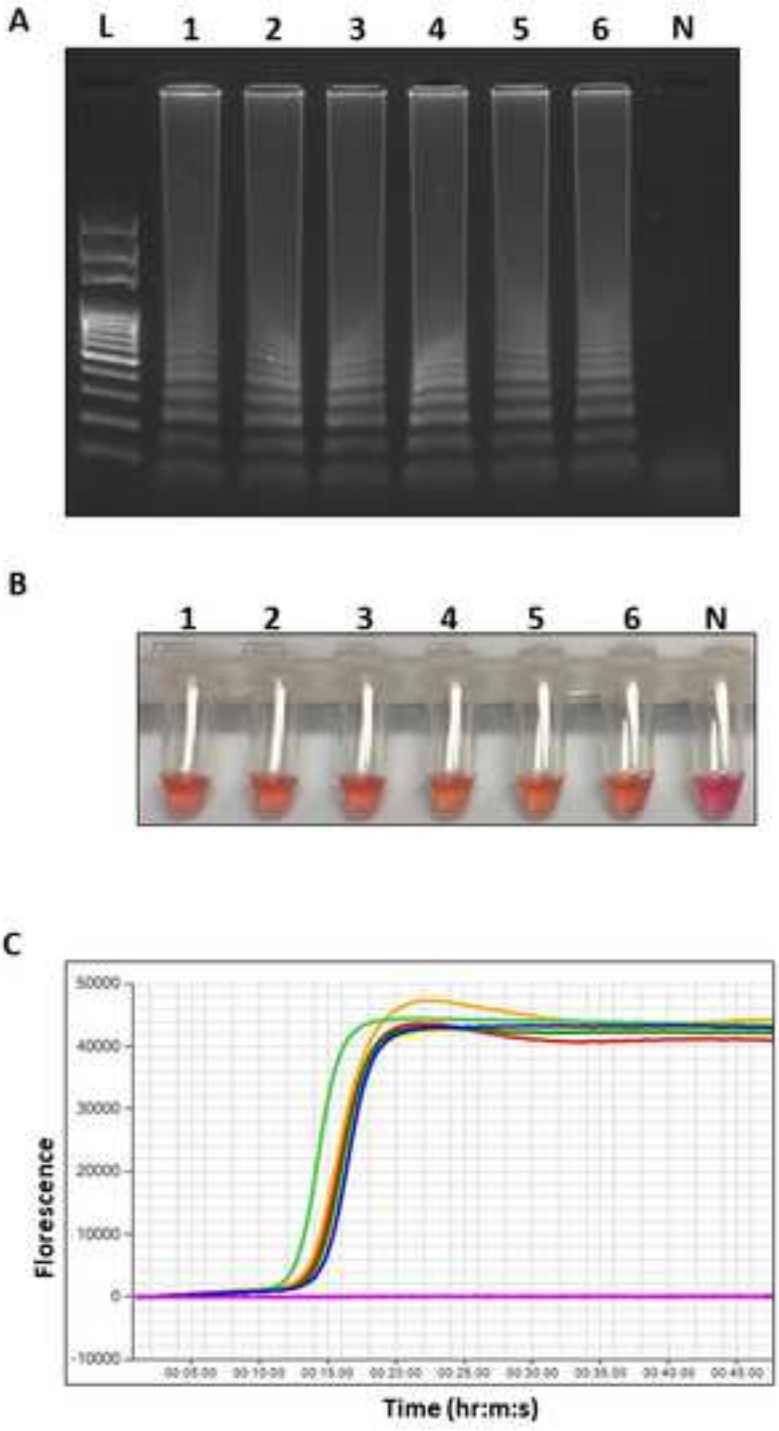

Figure 4

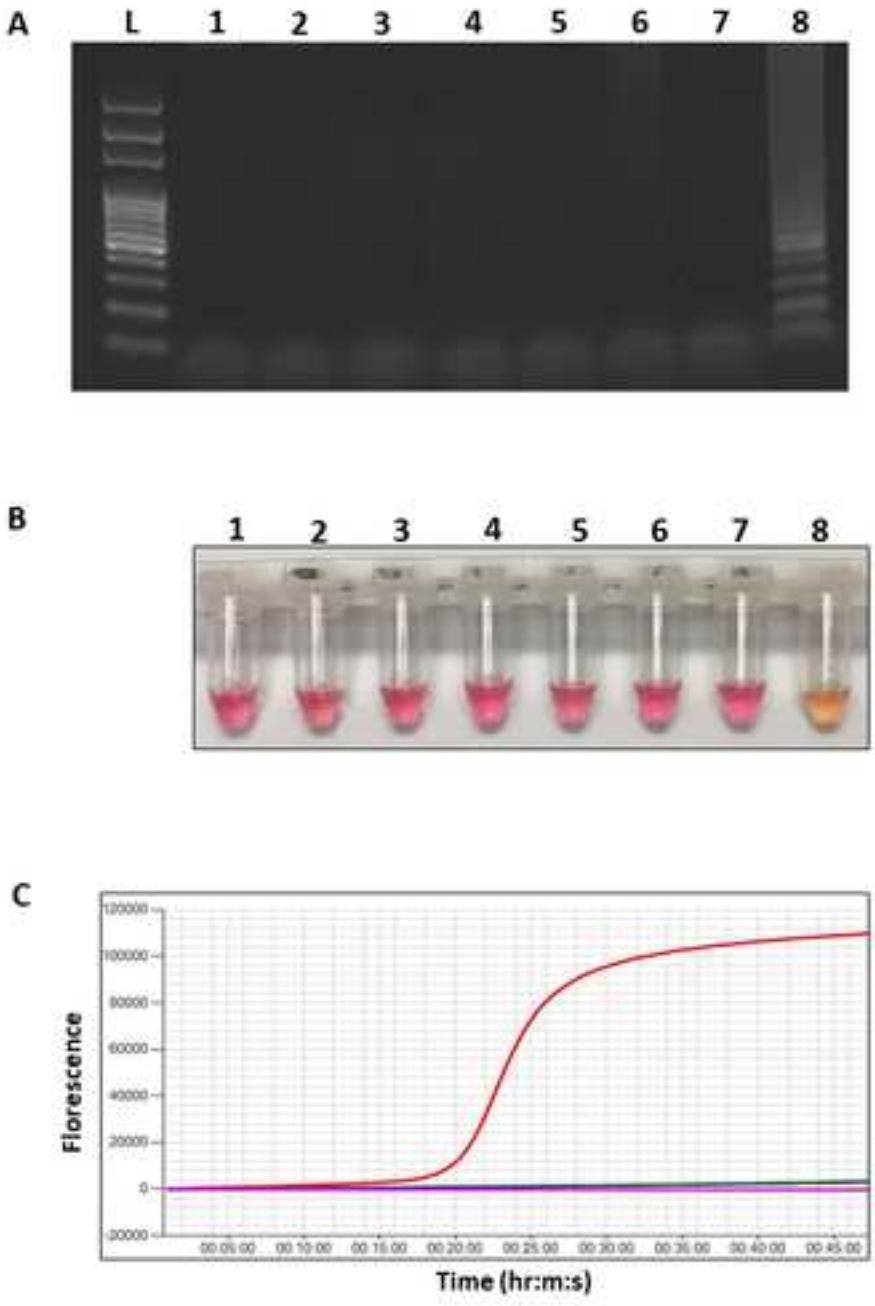

Figure 5

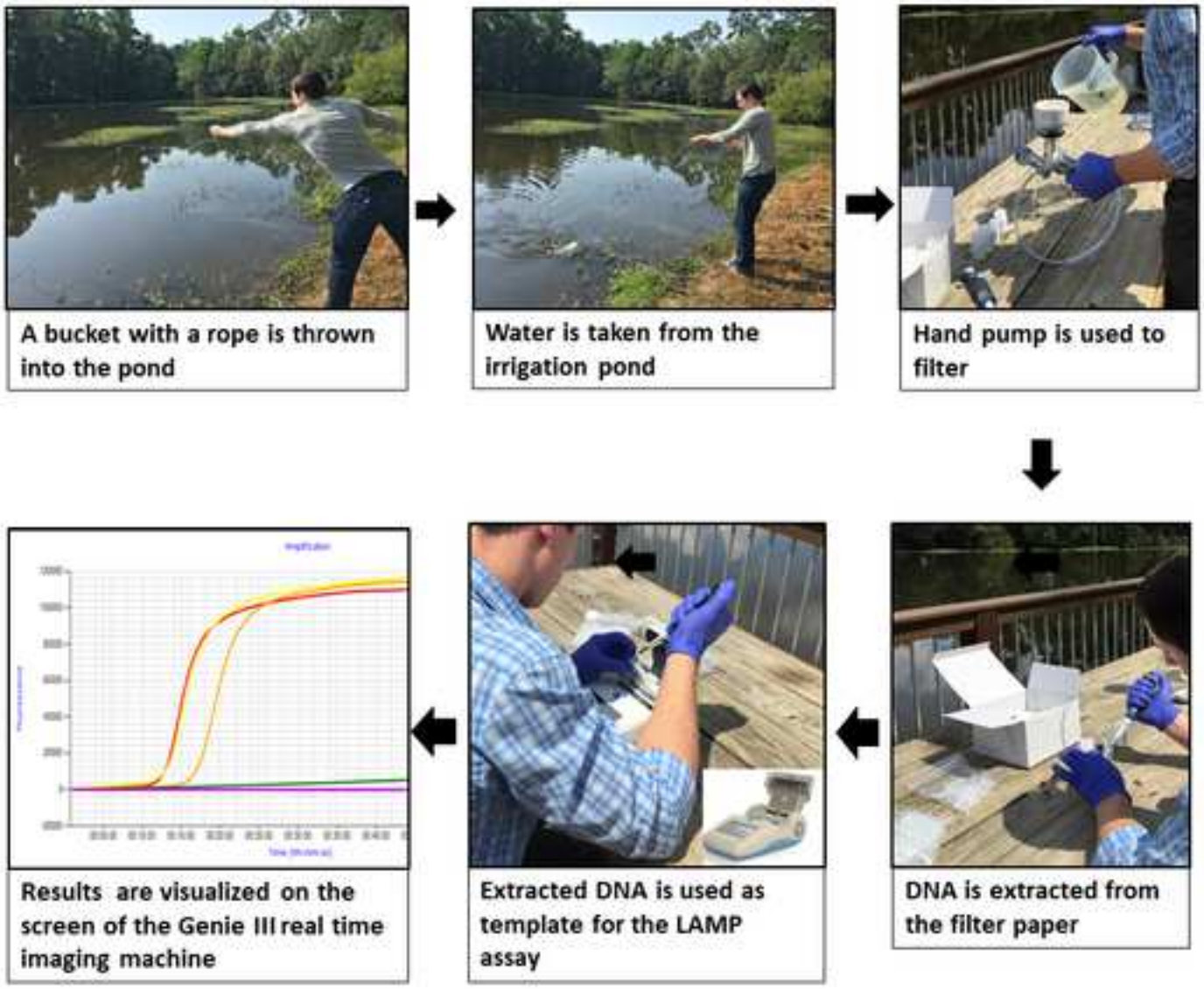

Figure 6

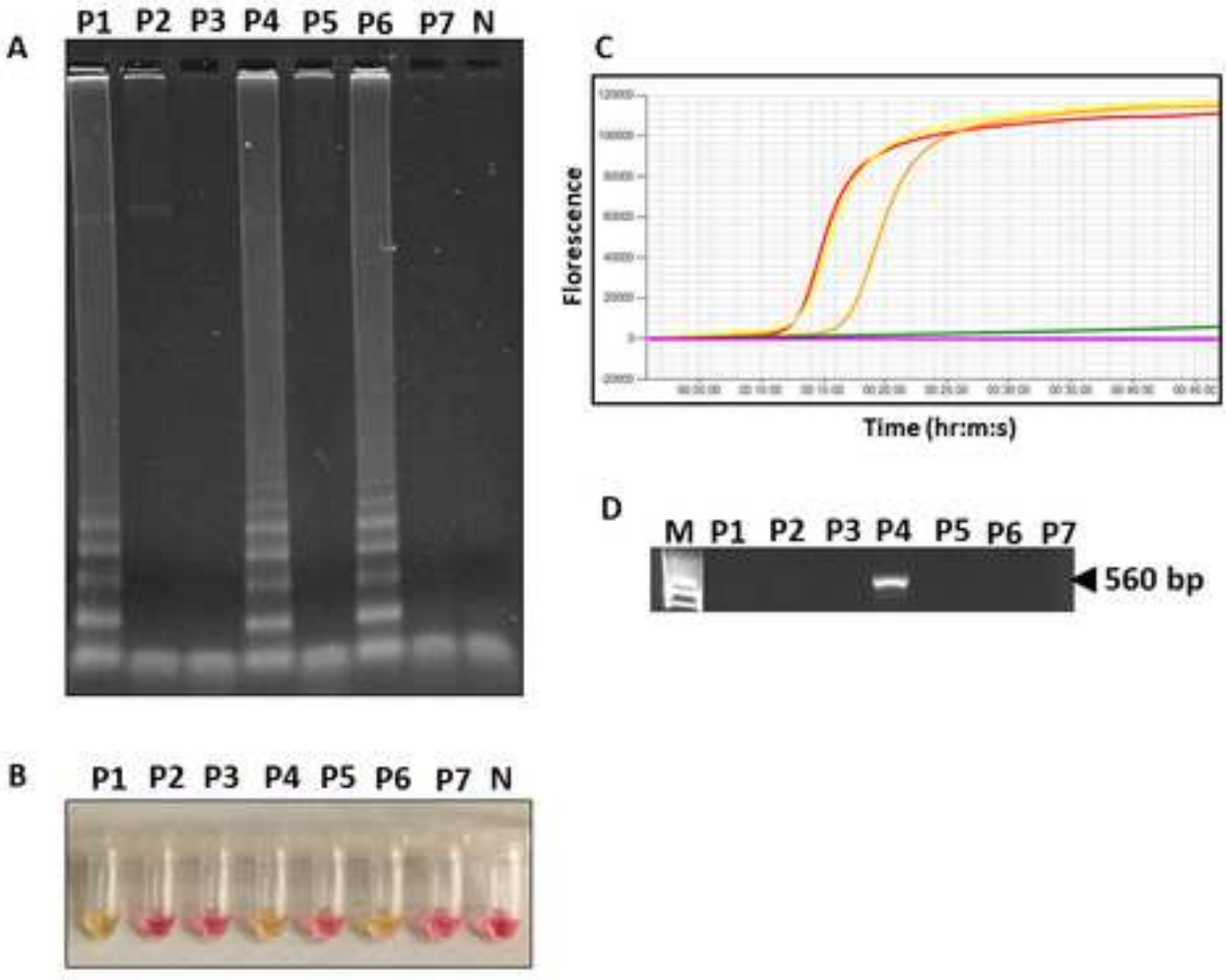

Table 1. Detection of irrigation water from Southern GA

Pond name	County, State	Target crops for irrigation	PCR Detection	LAMP Detection	History of Disease (Y/N)
P1	Tift, GA	Vegetables	-	+	N
P2	Tift, GA	Vegetables	-	-	N
P3	Tift, GA	Vegetables	-	-	N
P4	Tift, GA	Vegetables	+	+	N
P5	Tift, GA	Vegetables	-	-	N
P6	Tift, GA	Vegetables	-	+	N
P7	Tift, GA	Vegetables	-	-	N

Table 2: Primers used in this study

Primer type	Primer name	Sequence 5'-3'	Source
LAMP	PCA3-F3	TGTGTGTGTGTTCGATCACA	This study
	PCA3-B3	TTTTTGCGTGCGTCCAGA	This study
	PCA3-FIP	GACACCAAGCACTCGTACTOGTTTTTACAATTGTG	This study
	PCA3-BIP	AGAACGAGTATTCGGCGGCGTTTTGAAAAAGGAC	This study
	PCA3-LF	TGTCGAATGGATTTGCGATCTT	This study
	PCA3-LB	ATACGCAGGTCATTTGACTGAC	This study
PCR	PC-1	GTCTTGTACCCTATCATGGCG	Zhang et al., 2006
	PC-2	CGCCACAGCAGGAAAAGCATT	Zhang et al., 2006

Table 3. Comparison of methods for detection of *P. capsici*.

Parameters	Traditional	Conventional PCR	LAMP
Sensitivity	NA	4.8 X 10^2 spores/ml	1.2 X 10^2 spores/ml
Time	2 weeks or longer	2-3 hours (not including DNA	30 mins - 1 hour (not including DNA
Preparation	• Media creation • Plating and	• Spore collection using Filter paper • DNA extraction	• Spore collection using Filter paper • DNA extraction
Materials	• Autoclave • Media and plates • Incubation room • Flow hood • Eggplant	• Thermal cycler • Agar gel • Gel Doc	• Heat block or • Genie III
Cost	$5.00 per trap	$0.60 per reaction	$0.75 per reaction

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials5.7AF.xlsx

Name of Material/Equipment	Company	Catalog Number
Agarose gel powder	Thomas Scientific	C997J85
Buchner funnel	Southern Labware	JBF003
Bullet Blender	Next Advance	BBX24
Centrifuge 5430	Eppendorf	22620509
Chloroform	Fischer Scientific	C298-500
CTAB solution	Biosciences	786-565
Dneasy Extraction Kit	Qiagen	69104
Filter Flask	United	FHFL1000
Filter Paper	United Scientific Supplies	FPR009
Gel Green 10000X	Thomas Scientific	B003B68 (1/EA)
Genie III	OptiGene	
Hand pump	Thomas Scientific	1163B06
Iso-amyl Alcohol	Fischer Scientific	BP1150-500
LAVA LAMP master mix	Lucigen	30086-1
Magnetic bead DNA extraction	Genesig	genesigEASY-EK
Magnetic Separator	Genesig	genesigEASY-MR
polyvinylpyrrolidone	Sigma Aldrich	PVP40-500G
Primers	Sigma Aldrich	
Prism Mini Centrifuge	Labnet	C1801
T100 Thermal Cycler	Bio-Rad	1861096
UV Gel Doc	Analytik Jena	849-00502-2
Warmstart Colorimetric Dye	Lucigen	E1800m
Wide Mini ReadySub-Cell GT Cell	Bio-Rad	1704489EDU
70% isopropanol	Fischer Scientific	A451-1

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Date: 04/15/2020

Dr. Alisha DSouza
Senior Review Editor, JoVE

Re: JoVE61478 "Novel method for detection of Phytophthora capsici in irrigation water using loop-mediated isothermal amplification".

Dear Editor,

Thank you and three reviewers for the insightful comments and suggestions on our manuscript "JoVE61478". We have carefully discussed all the points among the authors and are now ready to respond to these comments point-by-point. Below I try to recite each of your comments in the order appeared in your email, and insert our responses right beneath of in blue. Changes to the manuscript are viewable within the revised manuscript by red color.

Comments from Editor:

Comment 1: Superfluous statements

Our response: Corrected in the title.

Comment 2: Protocol detail:

Our response: Details were added to the manuscript in Red.

Comment 3: Protocol numbering:

Our response: Numbers for the method section were changed, and details of number changes were reflected in the methods.

Comment 4: Protocol Highlight:

Our response: The protocol was highlighted and is between 2-2.5 pages in length and notes have been excluded while sub-headings have been included.

Comment 5: Discussion:

Our response: Additional statements of limitations, important steps, and references to the method specifically were included in the discussion. See lines 411 and 420.

Comment 6: Figures:

1): Provide each figure as individual PNG file:

2): Remove the figure and table legends from files and move to representative results:

3): Fig 2d expand axis tick size:

Our response:

1: Figures are now submitted individually.

2: Figure captions were removed from figures and added to the manuscript.

3: Figure 2d was enlarged

Comment 7: Tables:

Our response: Tables should also have been submitted individually.

Comment 8: References:

Supplementary Files should be attached with your submission. Please list the items on lines 463-471 in the reference list instead and use superscripted citation numbering in text. Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units…

Our response: These were moved to the references and abbreviations were made for non SI units.

…….

Reviewer #1 (Comments for the Author):

Major:

Comment 1: How's this LAMP assay compared the previous LAMP assay designed for P. capsici (Dong et al. 2015)? Suggest to add this 2015 paper to the introduction section.

Our response: Thank you, we ran the other LAMP assay with our dilution factor samples and had the same level of sensitivity which is shown in a new supplemental figure. This is included in the supplemental figure 3. Clarification of sensitivity was added on lines 93 and 431.

Comment 2: How many biological replications were run to determine the detection limit?

Our response: All tests were run in triplicate, we believe this to be shown in results line: 292.

Comment 3: Line 275: What other methods did you use to compare with LAMP for detecting P. capsici in the pond water samples?

Our response: This assay was tested using LAMP and was compared to conventional PCR. Ponds were not tested with baiting method, however comparisons were made to previously done research.

Comment 4: The CTAB and Qiagen DNA extractiosn methods were only mentioned in Results and Discussion, but not in the protocol?

Our response: They have been added in note form on line 178 to the protocol, but not in detail as the tests were made to limit confusion by choosing only the most field-optimal detection method for this assay. References for these methos protocol have also been added to reflect this.

Minor:

Comment 1: There are some typos in the manuscript (e.g. line 39)

Our response: More editing has been done for grammar and is reflected in red text.

Comment 2: Line 266: Please edit the sentence. The graph of amplification itself cannot be identical to the gel doc image, but they can provide the same detection sensitivity.

Our response: Thank you, this section was edited and rearranged. See line 305 for the changes made.

…….

Reviewer #2 (Comments for the Author):

Comment 1: Species reported as tested are Red = Phytophthora sansomeana, Orange = Phytophthora sojae, Purple: Phytophthora cinnamomi, Green = Phytophthora palmivora, Pink =Pythium irregulare, Dark blue = Phytophthora capsici. I think these are few in comparison to species that are considered genetically near to the target and could represent a false positive for the assay… In addition in the Figure 2F there is an amplification curve (dark green?) that is starting to be amplified. Since higher amplification minutes in LAMP correspond to lower DNA concentrations, its amplification starting at 45 minutes could be due to low concentration of that DNA. If in higher concentration it is recognized before (e.g. 15 min) it could be a problem for the assay…

Our response: DNA of these samples were standardized with one another before running them on the Genie III at a significant amount (100-150ng) and at no point in any of the 3 runs in triplicate did this sample amplify in the gel runs, nor in the Genie real time results did it amplify before 45 minutes. New tests and figures have been done and created to answer this as well (figures 2, 3, and 4).

Comment 2: Since P. capsici is recognized as a species having an high genetic variability in its population and your assay is designed on a gene that is present in single copy in P. capsici genome, I would suggest to test several isolates of the target species with many different origins in order to exclude false negative results…

Our response: We added another figure (3A-C) that includes more isolates of P. capsici from a larger geographic distribution.

Minor:

Comment 1: I think that your protocol and results could be clearer if you put first methods and data regarding the optimization of LAMP (both in lab and on field samples) and then the application on-site of your assay…

Our response: Rearrangement of the mentioned sections were done, as shown by lines 279-316 and various locations in the methods section.

Comment 2: Line 39: a D of detection is missed

Our response: Line 39 was corrected (line 43).

Comment 3: Line 87: Even if the novelty is not a requirement for the publication I think you have to cite in the introduction that other LAMP-based method were developed for P. capsici "Dong, Z., Liu, P., Li, B., Chen, G., Weng, Q., & Chen, Q. (2015). Loop-mediated isothermal amplification assay for sensitive and rapid detection of Phytophthora capsici. Canadian Journal of Plant Pathology, 37(4), 485-494."

Our response: This was changed in the introduction and a citation was added. Additionally a Supplementary figure was added showing this assay, and in lines 93 and 431.

Comment 4: Line 119: Does it works with every kind of water sample? There could be problems if some waters are contaminated by soil or mug?

Our response: Samples should not have much soil but some debris is okay as long as the water flowing through the filter paper is not hindered. This was added to line 124.

Comment 5: Line 117: 250-1000ml depending on what? How can I choose how much I need?

Our response: Depending on the source of the water some people may have access to lots of water or simply a small amount of run off. Our method can take up to 1L of water but does not have a minimum amount. This was added as a note in the methods section; line 129.

Comment 6: Line 121: What kind of lysis buffer? Does it works with every kind?

Our response: This lysis buffer was specific to the extraction kit in the protocol, this was added in the methods section on line 129 and a note was made for this section for other extraction methods on line 178.

Comment 7: Line 154: you can cite Table 2 as you made for PCR

Our response: This was added to the appropriate section now on line 167

Comment 8: Line 164: why do you need an initial warmstep at 95°C of 3 minutes with the Genie III? You can start your run at 65°C….

Our response: This is addressed on line 251 as the manufacturer recommends the extra warmstart for LAVALAMP mastermix, but for others it does not.

Comment 9: Line 87: how much of the product have you loaded on the gel to obtain a good result?

Our response: 5uL. This was added to step 5 of the protocol on line 184.

Comment 10: Line 145: at room temperature (18-27°C)…. What if in the field you have lower temperatures?

Our response: Thank you, this was changed on line 158.

Comment 11: Line 229: Development of LAMP assay for P. capsici
- I think it would be clearer to put in this chapter the LAMP mixture (concentration and doses of primers, etc) that you have optimize and referring to this in the other part of the protocol (e.g. when talking about LAMP application)
- You say that 1ul of DNA template is enough for the LAMP detection…..what is the best concentration of DNA to be used?

Our response: This has been added to line 182, although 1uL is standardized so that varying DNA extraction concentrations could be used.

Comment 12: - Line 236: 4.3 what do you mean with identical LAMP reactions with unique temperatures? Explain better or rewrite….

Our response: This sentence has been re-written for clarity; line 260.

Comment 13: - Line 239: 4.4 a) Detection limit of DNA concentration, I think is better "Detection limit of the LAMP developed assay" b) Are the serial dilutions of target DNA in water? Explain. What is the lowest and the highest DNA concentration comprise in the serial dilution test? c) DNA extracted from what? Pure cultures? d) "using the same protocol parameters for the LAMP reaction for each dilution" could be changed in "maintaining reaction conditions as previous described".

Our response: A) corrected in line 262 B) yes, this is written in the protocol in line 233 2.2.1 and shown in results C) This has been added to the protocol in 2.2.2. line 235 D) Corrected in line 240.

Comment 14: Line 258: "First, we standardized this filter paper-based LAMP method under laboratory conditions using a serial dilution of P. capsici spore suspensions". For the effectiveness of your LAMP method, I think that first you have to demonstrate that the new LAMP assay can work properly in term of sensitivity and specificity, so first of all I would put the development and optimization results.

Our response: The results section was modified during revision and the sensitivity of the LAMP assay does come first now. Line 279.

Comment 15: Line 275: "This demonstrates the applicability of LAMP as a more sensitive assay", more sensitive of what?

Our response: Modified in line 316.

Comment 16: Line 284: "with significant contaminates present" Explain better…

Our response: Modified in line 325

Comment 17: Line 291: I would change "using" with "testing"

Our response: Modified in line 277

Comment 18: Line 295: Change Optimization of temperature in optimization of amplification temperature or of reaction temperature

Our response: Modified in line 285.

Comment 19: Line 297: The optimal temperature was confirmed to be 64 °C. So, why have you used 65°C in the application protocol (Line 163, 4.4)?

Our response: Modified in the protocol on line 175.

Comment 20: Line 298: as the lowest concentrations that were positive for detection still amplified by 40 minutes, while higher concentrations amplified at 20 minutes. 1) Explain what are the highest and the lower concentrations 2) are you talking about DNA of the target species? 3) Are you talking of DNA extracted from pure cultures?

Our response: Modifications were added to the results section (line 279 & 288-290) and references to the figures were added.

Comment 21: Line 304: (Phytophthora and Pythium species), I think it would be useful to list all the species you have tested. At what DNA concentration have you tested non-target DNA? It could be useful to test the specificity using the same DNA concentration for target and non-target strains, allowing to have a valuable comparison of your detection results…

Our response: A list was added to the results section as well as noting the DNA concentration, and included in the results on line 332 and in figure 4.

Comment 22: Line 326: Loop-mediated….in describing LAMP methods I think you have to cite also the one developed for P. capsici "Dong, Z., Liu, P., Li, B., Chen, G., Weng, Q., & Chen, Q. (2015). Loop-mediated isothermal amplification assay for sensitive and rapid detection of Phytophthora capsici. Canadian Journal of Plant Pathology, 37(4), 485-494.". Also, in the discussion part a comparison between the results they have obtained and your results (specificity, sensitivity…) could be useful to further demonstrate the efficiency of your method…

Our response: This was additionally addressed in the sections above.

……..

Reviewer #3 (Comments for the Author):

Comment 1: Abstract part: 1, first word "P hytophthora", get rid of the extra space.

Our response: This was corrected.

Comment 2: 2, "etection of P. capsica", capitalize the first letter.

Our response: This was corrected.

Comment 3: Page 5-117, "250-1000 ml of test water", the range is a bit wide, as accurate as possible, and all the samples for each tests need to be consistent.

Our response: This was modified in the text (line 129).

Comment 4: Page 6-163, "65 °C for 60 minutes", based on your results, I think here should be changed to "64°C for 45 minutes".

Our response: Correct, thank you, this was modified (line 288).

Comment 5: Page 7-191, 7-208 and 11-333, "Phytophthora capsici" change to "P. capsici"

Our response: These changes were made except for the final one as it was the first mention of the pathogen in the section.

Comment 6: Page 7-208, "V8 agar", you should describe the components in detail.

Our response: Corrected on line 221.

Comment 7: Page 10-290, I think this part (Optimization of LAMP method) should be moved forward the first part of RESULTS.

Our response: Thank you, this is reflected in the results.

Comment 8: Page 10-304, you can add some main strains that isolated from irrigation water for the primer specific test.

Our response: Thank you, a list was added on line 332.

Comment 9: Page 10-307, you determined the response time of 45 min, so, In you figure 2 and 4, you should delete the data after 45 min.

Our response: Corrected axes on specific images.

Comment 10: For Figure 2 and 4, you should express the A,B,C…respectively in the text. For example, Page 9-265, you should change "Figure 2" to "Figure 2, B,C,&D". Please modify all similar cases.

Our response: Corrected throughout the manuscript, thank you.

Comment 11: Figure 2C, why is the reaction liquid transparent in the 7, negative water control, not light Red?

Our response: Thank you, this was additionally changed in the figures.

Comment 12: Figure 2D, I think this figure is not good. You need do this work again. I think there should be some sort of quantitative curve. The detection time interval between adjacent concentration gradients should increase. If you do not understand, you can look up some articles about LAMP quantitative graphs.

Our response: We have added a quantification curve to the results which reflect the data that is shown in the figure in question. We have left the previous figure as it is helpful when interpreting the data.

Comment 13: Page 13-399, 13-419, 13-433, 13-436, 14-446, 14-448 and 14-454, you should write down all the authors' names. The format should be consistent.

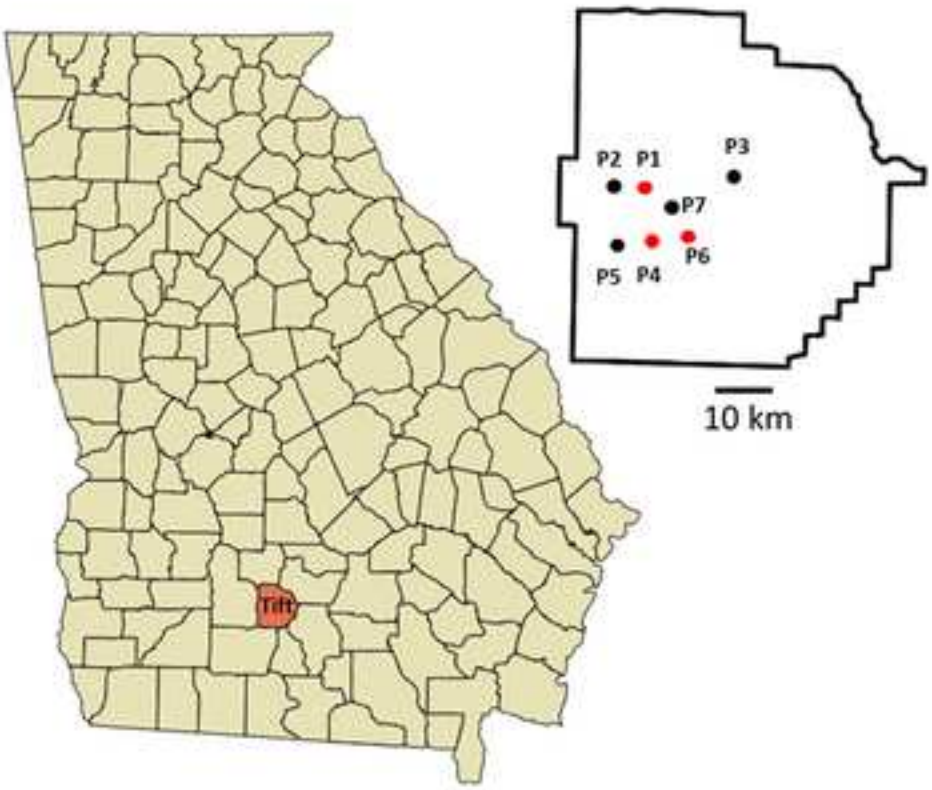
Our response: Corrected as suggested.

We hope that these changes adequately address all of the points raised by the editor and the reviewers. We are looking forward to hearing from you about your decision.

Sincerely,

Emran Ali
Director, Plant Molecular Diagnostic Lab
Department of Plant Pathology
University of Georgia

Supplementary Figure 1



Supplementary Figure 2