Rapid detection of viral, bacterial, fungal, and oomycete pathogens on tomatoes with microneedles, LAMP on a microfluidic chip, and smartphone device.

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Abstract

Rapid detection of plant diseases before they escalate can improve disease control. Our team has developed rapid nucleic acid extraction methods with microneedles (MN) and combined these with LAMP assays for pathogen detection in the field. In this work, we developed LAMP assays for early blight (*Alternaria linariae, A. alternata,* and *A. solani*) and bacterial spot of tomato (*Xanthomonas perforans*) and validated these LAMP assays and two previously developed LAMP assays for tomato spotted wilt virus and late blight. Tomato plants were inoculated and disease severity was measured. Extractions were performed using MN and LAMP assays were run in tubes (with hydroxynaphthol blue) on a heat block, or on a newly designed microfluidic chip on a heat block or a slide heater. Fluorescence on the microfluidic chip slides was visualized using EvaGreen and photographed on a smartphone. Plants inoculated with *X. perforans* or tomato spotted wilt virus tested positive prior to visible disease symptoms, while *P. infestans* and *A. linariae* were detected at the time of visual disease symptoms. LAMP assays were more sensitive than PCR and the limit of detection was 1 pg of DNA for both *A. linariae* and *X. perforans*. The LAMP assay designed for early blight detected all three species of *Alternaria* that infect tomato and is thus an

Alternaria spp. assay. This study demonstrates the utility of rapid MN extraction followed by LAMP on a microfluidic chip for rapid diagnosis of four important tomato pathogens.

Introduction

Crop pests and diseases cause between 20-30% yield losses on staple crops (Savary et al. 2019). As the Earth's population is projected to reach 10 billion by the year 2050, the need to maximize food production and minimize the impact of plant pathogens and pests on crop yields will become paramount (Ristaino et al. 2021). Pathogen detection in pre-symptomatic or initial stages of infection is key for effective disease forecasting and management to improve crop yields (Canton 2021; Fenu and Malloci 2021). This is particularly important for pathogens such as *Phytophthora infestans* (Mont.) de Bary, the causal agent of late blight of potato and tomato, which can devastate a field in as little as a week if left untreated (Fry 2008).

In recent years different technologies have been developed to detect plant disease in these critical early presymptomatic stages including isothermal molecular assays, leaf diffuse reflectance spectroscopy, or hyperspectral imaging (Brittain 2018; Clark et al. 2022; Gold et al. 2020; Zhou et al. 2023). Isothermal reactions are performed at a single temperature instead of the multiple cycles of heating and cooling needed for traditional thermal cycling amplification techniques, and thus can be incorporated more readily into rapid diagnostics. Some common isothermal methods include helicase-dependent amplification (HDA), recombinase polymerase amplification (RPA) and loop-mediated amplification (LAMP), all of which rely on amplification of the target DNA (Zhao et al. 2015). Another isothermal approach is CRISPR/Cas, which relies on cleavage of the target DNA (Gosavi et al. 2020, Shymanovich et al. 2024). Rapid detection assays have been developed for many plant pathogens, including *P. infestans* (Ristaino et al. 2020), tomato spotted wilt virus (TSWV) (Paul et al. 2021; Zhang et al.

2021), *Dickeya dianthicola* (Ocenar et al. 2019), *Phytophthora cinnamomi* (Dai et al. 2019) and *Phytophthora capsici* (Yu et al. 2019), among others.

We recently developed a smartphone-based system for running LAMP reactions, where changes in fluorescence are used as an indicator of pathogen presence/absence (Paul et al. 2021). A smartphone-based LAMP assay was paired with a new rapid extraction technique using microneedle (MN) patches to puncture and quickly extract DNA and RNA from a tomato leaf, decreasing the time to diagnosis (Paul et al. 2019). This newly designed isothermal amplification system can be used to detect multiple pathogens simultaneously, is designed to give results based on a colorimetric or fluorescence change, and has the potential to become a versatile tool for in-field plant pathogen detection. The system is universally applicable for detection of any kind of microbe once pathogen-specific LAMP primers are identified. To further explore the utility of this system, we selected tomatoes as a crop to target for the development of new field-ready assays and focused on four important plant diseases of tomato that are responsible for significant crop loss: early blight caused by three fungal species (*Alternaria* species including *A. linariae* (Neerg.) E.G. Simmons*, A. alternata,* Kessler, and *A. solani* (Ell. and Mart.), late blight caused by the oomycete *Phytophthora infestans*, bacterial spot caused by *Xanthomonas perforans* Jones et al., and tomato spotted wilt virus, caused by TSWV (**Supplemental Fig. 1**).

Tomatoes, of both processing and fresh market types, are the second most consumed vegetable in the US behind potatoes and are valued at over 1.85 billion dollars. North Carolina ranks 6th in the nation in tomato production and grows more than 4,000 acres annually (Webb et al. 2022). Production of greenhouse-grown tomatoes is also on the rise. Plant pathogens on tomatoes are a persistent threat under field and greenhouse conditions.

Our previous LAMP detection work focused on *P. infestans* and TSWV (Paul et al. 2021; Ristaino et al. 2020). Additional detection assays for these pathogens have been previously developed using a variety of platforms, including PCR (e.g. Trout et al. 1997), real-time PCR (e.g. Lees et al. 2019; Roberts et al 2000; Debreczeni et al. 2011), LAMP (e.g. Hansen et al. 2016; Khan et al. 2017; Lees et al. 2019), RPA (e.g. Lee et al. 2021), and CRISPR/Cas13a (e.g. Zhang et al. 2021). None is a rapid assay capable of being deployed in a field setting.

Early blight and bacterial spot are also common leaf diseases on tomatoes. Both diseases can be caused by several closely related species, including *A. alternata, A. linariae,* and *A. solani* for early blight (Adhikari et al. 2021), and *X. vesicatoria*, *X. euvesicatoria*, and *X. gardneri* for bacterial spot (Araújo et al. 2012). Previous detection assays have focused on identification of one or multiple species within these complexes. For the detection of *A. solani*, for example, PCR, LAMP, and real-time PCR assays have been developed (e.g., Adhikari et al. 2021; Khan et al 2018; Kumar et al. 2013; Lees et al. 2019; Leiminger et al. 2015). In addition, assays have been developed to distinguish between the four bacterial spot-causing pathogens using PCR (e.g., Araújo et al. 2012), real-time PCR (Strayer et al. 2016), or RPA assays (Strayer-Scherer et al. 2019).

To control late blight, early blight, and bacterial spot, growers use more than 15 pesticide applications per season on field grown fresh market tomatoes (Meadows, 2024). While synthetic pesticides offer an effective means of crop protection, there are many detrimental downstream health effects from excessive pesticide use and some plant pathogens have developed resistance to synthetic pesticides (Saville et al. 2015). Insect transmitted viruses and their vectors are significant problems for tomato producers in the United States, causing unacceptably large losses (Riley et al. 2018). In addition, resistance-breaking strains of TSWV have been recently

identified in NC (Lahre et al. 2023; Shymanovich et al. 2024). Better and more rapid diagnostic assays could help reduce pesticide application by improving timing of applications when pathogens are present.

The objectives of this study were to: 1) Develop LAMP assays for the detection of *Alternaria* spp. and *X. perforans* and validate their specificity and sensitivity; 2) Test the LAMP for detection of either *A. linariae, X. perforans, P. infestans* or TSWV on inoculated tomatoes assays as disease progresses over time using MNs; and 3) Develop and test a microfluidic chip for running LAMP assays on a smartphone device.

METHODS

Sampling and DNA extraction

Forty fungal, bacterial, oomycete, and viral isolates were used in this study, spanning 24 species that are known common pathogens of tomato (**Supplemental Table 1**). For the purposes of inoculations, testing, and validating the LAMP assays both *in vitro* and *in vivo*, we selected four plant pathogens including *P. infestans* isolate NC14-1, *A. linariae* isolate JD1B, *X. perforans* isolate 19-027, and TSWV strain CA-WT.

DNA was extracted using a hexadecyltrimethylammonium bromide (CTAB) method (May and Ristaino 2004). For CTAB extraction, fresh mycelia was placed in sterile 1.5 mL microcentrifuge tubes to which 150 µL of extraction buffer (0.35 M sorbitol, 0.1 M Tris, 0.005 M EDTA, pH 7.5, and 0.02 M sodium bisulfite) was added and each sample was macerated using a Konte pestle. Nuclei lysis buffer (150 µL; 0.2 M Tris, 0.05 M EDTA, pH 7.5, 2.0 M NaCl, 2% CTAB, and 60 µL of 5% sarkosyl (N-Lauroylsarcosine) was added and tubes were vortexed and incubated at 65 ºC for 15 to 30 min in a water bath. After incubation, one volume (~300 µL) of 24:1 chloroform:isoamyl alcohol was added to each tube and tubes were

centrifuged for 15 min at 13,000 rpm at room temperature on an Eppendorf 5425 mini centrifuge using a FA-24x2 rotor (Eppendorf, Hamburg, Germany) or a Baxter Biofuge13 using a Heraeus Sepatech 3743 rotor (Heraeus Group, Hanau, Germany). The aqueous phase was removed to a new tube and the chloroform extraction was repeated. DNA was precipitated overnight at –20 °C in 0.1 volume of 3M sodium acetate (pH 8.0) and 2 volumes of cold 100% ethanol. The supernatant was then discarded and pellets were washed twice with 70% ethanol and dried at room temperature. DNA was suspended in Tris-EDTA (TE; 10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

For the tomato inoculation studies, DNA was extracted from infected leaves using MN. The MN patches were made from 10% polyvinyl alcohol (PVA) as described previously (Paul et al. 2019). To create the patches, molds were placed in a 6-well deep tissue culture plate containing inverted 50 mL Falcon tube caps to create a concave surface. The molds were covered with 800 µL 10% PVA and spun in an Eppendorf 5810R centrifuge using a high-speed A-2-DWP-AT plate rotor (Eppendorf, Hamburg, Germany) at 4200rpm for 25 minutes at room temperature. Patches were then left to dry overnight. To extract DNA, a patch was pressed into the leaf on a suspect lesion for approximately ten seconds and then removed. DNA was washed off the patch using 60 µL of molecular-grade water (for RNA extraction of TSWV) or TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) (for DNA extraction) (**Fig. 1A**).

LAMP reactions for *X. perforans* and *Alternaria* spp.

LAMP primers previously developed for *P. infestans* and TSWV (Paul et al. 2021; Ristaino et al. 2020) were used for this study (**Supplemental Table 2)**. New primers were designed to target *A. linariae* and *X. perforans* using PrimerExplorer version 5 (Eiken Chemical Co., Tokyo, Japan☺. We selected the *β-tubulin* (*β-tub*) gene for the development of *Alternaria*

spp. LAMP primers (Genbank Accession no. Y17078.1) and utilized PCR primers reported to be species-specific for *A. linariae* for comparison (Adhikari et al. 2021). Considering the results discussed below, the assay developed using these primers will be referred to as the *Alternaria* spp. assay for the rest of the text. *A. linariae* was used as the representative species in the host inoculation and LAMP assays described below.

We generated PCR amplicons using primers that amplify nucleotide sequences for a hypothetical protein (Araújo et al. 2012) and used the subsequent sequence data to design LAMP primers for *X. perforans* (**Supplemental Table 2)**. Primer candidates were bioinformatically evaluated against sequences of closely related species for species specificity and to evaluate the position of informative SNPs. The primer sets selected for further testing showed a larger number of SNPs close to the 3' ends and for lower (more negative) values of the Gibbs free energy change (ΔG). In addition, the 3' end of F2/B2 and the 5' end of F1c/B1c were checked to ensure the ΔG was more negative than -4 kcal/mol. Final primer alignments are shown in **Supplemental Fig. 2**.

Sensitivity tests for the *Alternaria* spp. and *X. perforans* assays were performed with CTAB DNA extractions from pure mycelia or bacterial cultures, respectively, using 10-fold serial dilutions starting with 10 ng/µL for *A. linariae* (isolate JD1B) and 1 ng/µL for *X. perforans* (isolate 19-027) as measured on a Qubit-4 fluorometer (Invitrogen, Carlsbad, CA). Specificity tests for the *Alternaria* spp. and *X. perforans* assays were performed with 1 ng/µL of CTAB-extracted DNA from multiple fungal and bacterial species commonly found on tomatoes (**Supplemental Table 1**). This panel included bacterial species commonly found on tomato: *X. euvesicatoria* (XE), *X. vesicatoria* (XV), *X. gardneri* (XG), *Pseudomonas syringae* (PS), *Pectobacterium carotovorum (PC), Clavibacter michiganensis* (CM), *Rasltonia solanacearum*

(RS), and five strains of *X. perforans* (XP) (**Supplemental Table 1**). TSWV was not included in the specificity tests.

Standardized LAMP protocols for four pathogens

LAMP protocols for all four pathogens were standardized based on our previously developed LAMP reactions for *P. infestans* and TSWV (Paul et al. 2021; Ristaino et al. 2020). Reaction mixes included EvaGreen fluorescent dye for fluorometric visualization (Biotium, Fremont, CA) and hydroxynaphthol blue for colorimetric visualization (HNB; Honeywell Fluka, Charlotte, NC). Positive reactions using HNB are indicated by a color shift from violet or dark blue to sky blue (**Fig. 2D)**.

All primers were diluted to 100 µM using TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). For the *X. perforans* and TSWV assays, primer mixes were made using 20 µL each of F3 and B3, 40 µL of loop primers or TE buffer (if only one loop primer is used), and 160 µL each of FIP and BIP. The primer mix for the *Alternaria* spp. assay was made using 5 µL each of F3 and B3, 10 µL each of LF and LB, 20 µL each of FIP and BIP, and 30 µL of TE buffer. The primer mix for the *P. infestans* assay was made using 10 µL each of F3 and B3, 40 µL each of LB and LF, and 100 µL each of FIP and BIP. All primer mixes were prepared ahead and frozen in aliquots needed for eight reactions. The master mix for each 25 µL reaction contained 2.5 µL of 10X isothermal amplification buffer (New England Biolabs, Ipswich, MA), 1.25 µL of 100 mM magnesium sulfate (New England Biolabs, Ipswich, MA), 3.5 µL of dNTPs (10 mM each) (Apex Bioresearch Products, Genesee Scientific, El Cajon, CA), 2 µL of 5M Betaine (Sigma-Aldrich, St. Louis, MO), 2.5 µL (1.5 µL for *P. infestans*) of primer mix, 1.2 µL of 2.5 mM HNB, 1.25 µL of 20X EvaGreen, 1 µL of 8 U/µL Bst 2 WarmStart DNA polymerase (New England Biolabs, Ipswich, MA), 0.5 µL of reverse transcriptase 15 U/µL (New England Biolabs, Ipswich, MA)

(for TSWV detection only), and 1µL of DNA (**Supplemental Table 3)**. LAMP reactions were performed on a Bio-Rad CFX96 real-time machine or on Bio-Rad T100 conventional thermal cycler with a hold at 65 °C for 30 minutes followed by a deactivation step at 80 °C for 5 min.

Detection of mixed infections with *A. linariae* and *X. perforans* DNA by LAMP

To test if mixtures of the two pathogens could be detected with our LAMP methods we ran a blind test. CTAB-extracted DNA samples (1 ng/µL via Qubit 4) for *A. linariae* and *X. perforans* and a 1:1 mixture of both pathogen DNA samples was prepared and coded numerically by one investigator. The second investigator performed blind tests on the numeric samples using colorimetric LAMP reactions for *A. linariae* and *X. perforans* and the generalized protocol. Tests were conducted with three samples and repeated an additional two times. For each 25 µL LAMP reaction, 2 µL of DNA sample or mixture was added.

In vivo detection experiments using tomatoes.

One week prior to experiments, three week old tomato seedlings (cultivar Mountain Fresh Plus) were placed in a growth chamber at 23 °C with a 16 h light/8 h dark schedule to acclimate for experiments involving *P. infestans*, *X. perforans*, and *A. linariae*. For experiments involving TSWV, two-week-old seedlings were placed in the chamber shortly before inoculation. Plants were watered every day with a nutrient solution formulated by the NC State University Phytotron (Saravitz and Chiera 2019). At each experiment's start, eight similarly developed plants were selected and randomly assigned as follows: four to the inoculated treatment and four to the non-inoculated control group. We conducted these experiments twice with *X. perforans*, *P. infestans*, *A. linariae,* and TSWV infections.

Pathogen inoculum preparation.

Inoculum of *X. perforans* was prepared from an actively growing culture (isolate XP19-027) collected from Rowan County, NC. The bacterial suspension was diluted to optical density 0.07 OD (Spectronic 21, Bausch & Lomb, Laval, Canada), which corresponds to 10^8 CFUs per mL. For *A. linariae* experiments, a conidia suspension was collected by dry brushing a 2 week old culture (isolate JD1B) plated on V8 agar (0.2 g $CaCO_3$, 100 mL V8 juice, 20 g Difco Bacto Agar (BD Diagnostics, East Rutherford, NJ), 1 L dH_2O) using a cell spreader and leaving the plate exposed for 24-72 hours. The plate was then brushed with 2 mL of sterile water and the water collected. Conidia density was calculated under a microscope with a hemacytometer, and conidia were diluted to 2000 conidia/mL. For *P. infestans* experiments, an isolate of *P. infestans* (isolate NC14-1, US-23 genotype) was maintained on detached tomato leaves (cv. Mountain Fresh Plus). A leaf with active *P. infestans* sporulation was vortexed in 10 mL of distilled water to harvest sporangia, then the sporangia were quantified using a hemocytometer and adjusted to 10,000 sporangia/mL. The inoculum of TSWV was prepared from several young leaves from tomato plants infected with a wild-type strain collected in California (Shymanovich et al. 2024). TSWV infected leaves were ground in an ice-cold mortar with 5-10 mL sodium sulphite (63 mg per 50 mL tap water) buffer.

Pathogen inoculations.

In the experiments with *X. perforans*, *P. infestans*, and *A. linariae*, each pathogen-inoculated plant was sprayed with 2 mL of corresponding inoculum suspension. Each control plant was sprayed with 2 mL of distilled water. All plants were covered with plastic bags to prevent cross contamination and maintain humidity. Disease ratings were performed on days 0, 2, 3, 4, 5, 6 and 7. Percent leaf area diseased was used to measure disease severity based on a modified Horsfall-Barratt scale (**Supplemental Table 4**). Plants were inoculated with TSWV

using a mechanical rubbing procedure. The ground TSWV infected tissue was applied to plants with cotton applicators by rubbing them onto tomato leaves sprinkled with carborundum to wound the leaf (Shymanovitch et al. 2024). Ten minutes later, plants were sprayed with distilled water to remove the remaining carborundum. Control plants were mock inoculated with the buffer only. Plants in pairs from one treatment group were placed in BugDorm-4E3074 Insect Rearing Cages (MegaView Science Co., Taichung, Taiwan).

MN nucleic acid extraction.

DNA was extracted from the inoculated and control plants over time using MN extractions on days 0, 2, 4, and 7 after inoculation. A MN patch was pressed hard on the leaf placed on a solid surface (**Fig. 1A**) and rinsed with 60 µL of TE buffer. Double-distilled water was used for rinsing TSWV from the MNs (Paul et al. 2021). Care was taken to avoid moving sample DNA on gloves. Because TSWV disease develops slowly, ratings and MN extractions were performed 0, 2, 4, 7, 9, 11, and 14 days after inoculation. Plant height was measured to the youngest leaf petiole at each measurement time. Disease severity ratings considered plant height, leaf size, and color. We used a TSWV disease rating scale based on plant height to an upper leaf base. Disease severity ratings were: 0 = none, 1 = slight stunting (10-15% compared to control), 2 = significant stunting (20-25% compared to control) and smaller young leaves, 3 = strong stunting (25-50% compared to control) and yellowish leaves, 4 = severe stunting (50-75% compared to control) and some necrosis, 5 = dwarf, stunting (> 75% compared to control), dying.

For all pathogens, the area under the disease progress curve (AUDPC) for disease severity was calculated using R. v. 4.2.0 (R Core Team 2022) and the *agricolae* library v. 1.3-5 (de Mendiburu 2021).

LAMP tests with MN extractions.

For LAMP reactions, 2 µL of MN extract was added to a 23 µL master mix. We used 2 µL DNA of CTAB extractions made from the plated cultures of each pathogen for positive controls. Positive control RNA for TSWV was extracted from infected leaves with a Total RNA (Plant) Kit (IBI Scientific, Dubuque, Iowa) via manufacturer instructions and 2 µL was used per reaction. Two µL of molecular grade water was used for no template controls (NTC). LAMP results were visualized either by gel electrophoresis with 5 µL of LAMP product on a 2% agarose gel with 1M TAE buffer at 130V, by green fluorescence and Cq values on a Bio-Rad real-time machine, or by colorimetric color change from violet/dark blue to sky blue in tubes.

Detection accuracy by LAMP and PCR.

To test the detection accuracy of our new assays, inoculation tests were performed with 10 detached leaves for each pathogen infection group (*A. linariae* or *X. perforans*) and 10 detached leaves that served as the non-inoculated control. Fresh tomato leaves were placed in inverted 15% water agar plates such that the water agar was suspended over the leaves to provide humidity, with one leaf per plate. The leaves were then sprayed with 0.5 mL of inoculation suspension or DI water. Inoculation suspensions were prepared the same as for the whole plant inoculation experiments. Plates were sealed with parafilm and kept in ambient light in the lab at room temperature for 7 days (*X. perforans*) or 14 days (*A. linariae*). Each leaf was monitored for infection throughout the experiment. MN extractions were performed on the final incubation day (day 7 or day 14) and real-time and colorimetric LAMP and conventional PCR were performed. Previously we noticed that MN extractions from *A. linariae*-infected leaves degraded quickly in storage, so we ran assays the same days as the MN extractions.

PCR tests with MN extractions from *X. perforans* and *A. linariae* whole plant inoculation experiments.

To evaluate performance of the *X. perforans* and *Alternaria* spp. assays, we compared detection results from the two new LAMP assays to traditional PCR testing. Conventional PCR tests were performed by using previously described PCR primers for *A. linariae* and *X. perforans* (**Supplemental Table 2**). For 25µL reactions we used 2.5 µL 10X PCR buffer (Apex Bioresearch Products, Genesee Scientific, El Cajon, CA), 1.25µL dNTPs (2 mM each) (Apex Bioresearch Products, Genesee Scientific, El Cajon, CA), 1 µL of each forward and reverse 10 µM primers, 0.9 µL $MgCl_2$ (50 mM) (Apex Bioresearch Products, Genesee Scientific, El Cajon, CA), 0.125 µL BSA (20 mg/mL) (Thermo Scientific, Waltham, MA), 0.1 µl Taq (5U/µL) (Apex Bioresearch Products, Genesee Scientific, El Cajon, CA) and 16.125 µL molecular grade water. For each reaction we added 2 µL of microneedle extracted DNA. Gel electrophoresis was run with 5 µL of PCR product on 1% agarose gel with 1M TAE buffer at 130V. For *A. linariae* a 483 bp band was detected while for *X. perforans,* a 197 bp band was detected.

Development of a microfluidic chip for smartphone-based detection.

In previous work, we described a smartphone-based detection system utilizing a square 4-celled reaction chip run on a heating slide and analyzed using a smartphone camera (Paul et. 2021). In this work, we redesigned the PDMS slide system to work with a new reaction chip that decreased the potential for contamination and lowered the reaction volume. For microfluidic chip fabrication, a 3D model of the microfluidic mold was first designed with the Autodesk Inventor software. The mold was then printed by Proto Labs, Inc (Morrisville, NC). (**Supplemental Fig. 3A**). Six microfluidic chips can be made simultaneously with the 3D-printed mold. Each chip consists of four microfluidic channels (**Supplemental Fig. 3B)**, and the length, width, and height of each channel were 16 mm, 0.8 mm, and 2 mm, respectively. To fabricate the PDMS microfluidic chip, Sylgard elastomer, curing agent, and charcoal powder were mixed in a weight

ratio of 100:10:1 and vacuumed for 15 minutes to remove air bubbles from the mixture. The mixture was then poured into the mold and cured overnight between 70 and 80°C. After curing, the PDMS layer was separated from the mold and cut into six chip pieces with a razor blade. Finally, PDMS pieces were treated with plasma using a Harrick Plasma Cleaner (model PDC-32G, Harrick Scientific Products, Pleasantville, NY) to permanently attach them to glass microscope slides trimmed to approximately 2.5 cm wide by 3.8 cm long (**Supplemental Fig. 3C)**.

LAMP reactions and imaging of microfluidic chips.

Each of the four pathogen LAMP reactions were evaluated on PDMS chips heated on an AmplifyRP heat block (Agdia Inc, Elkhardt, IN) (**Fig. 1B**). We used MN extractions from the whole plant inoculation experiments described previously and confirmed with a real-time LAMP as positive for each pathogen alongside negative samples collected from the uninoculated control plants. Each slide contains four linear reaction wells that were assigned as follows: 1 = positive pathogen control with CTAB DNA extraction 1ng/µL, 2 = MN extraction from an infected tomato leaf, 3 = MN extraction from healthy control tomato leaf, 4 = NTC (**Fig. 1C**). A standard LAMP master mix was prepared as described previously. Two µL of DNA or water was added to each reaction tube as described above and 22 µL of the final mix was transferred into each reaction well through small openings on the backside of the PDMS (**Supplemental Fig. 3B**). After loading reagents, the backside of the PDMS chip was sealed with a piece of PCR film. The loaded microfluidic chip was heated one of two ways: (1) on an AmplifyRP heat block surface at 65 °C for 30 min (**Fig. 1B**) or (2) on a heat slide cartridge (*P. infestans* only) (Paul et al., 2021). The chip was photographed under blue light on the smartphone-based device using fixed settings (manual photo mode, WB auto, 1/2 s exposure, ISO 50) (**Fig. 1C**). A black stand was

made from a Petri dish lid covered with black fabric for imaging in the smartphone to prevent background reflection. The smartphone device has a blue LED light with a wavelength of 470 nm for fluorescence excitation and an optical lens for filtering fluorescent wavelengths (543 ± 27 nm).

Images were analyzed with ImageJ software to detect differences in fluorescence compared to an estimated threshold. We used the "split image" function, and only "green" images were analyzed. We recorded "mean intensity" in relative fluorescence units (RFU) from each reaction well. To estimate a threshold value, we ran twenty no template control (NTC) reactions in five chips and measured the RFU of each reaction after 30 minutes. The threshold value was estimated as mean RFU_{NTC} + 3SD. Positives were identified if the test sample RFU was greater than the calculated threshold value. Negatives had a RFU lower than the calculated threshold value. Visual assessments were compared with calculations.

RESULTS

Alternaria spp. LAMP assay

The detection limit for the *Alternaria* spp. LAMP was 1 pg after 30 min (**Fig. 2A, B**). The melting temperature of the LAMP amplicons was 88.5 °C (**Fig. 2C**). Positive reactions are characterized by a distinct color change to light blue (**Fig. 2D**) and or ladder-like bands as visualized on a gel (**Fig. 2E**). The *Alternaria* spp. LAMP assay was approximately 100 times more sensitive than PCR as only 10 ng, 1 ng, and 100 pg DNA samples produced a positive reaction using PCR (**Fig. 2E-F**). Specificity tests indicated that while the *Alternaria* spp. LAMP assay detects *A. linariae*, it also detects closely related *A. alternata* and *A. solani*, which also cause early blight on tomato. However, the *Alternaria* spp. LAMP assay did not amplify any

other fungal or bacterial pathogens of tomato tested, including the four species of *Xanthomonas* that cause bacterial spot (**Supplemental Table 1**).

***X. perforans* LAMP assay**

Likewise, the same detection limit of 1 pg of DNA was observed for *X. perforans* after a 30-minute LAMP reaction at 65 °C (**Fig. 3A,B)** and the melting temperature of this amplicon was 88 °C (**Fig. 3C**). The *X. perforans* LAMP assay was approximately 100 times more sensitive than PCR, which only amplified the 1 ng and 100 pg samples (197 bp bands) (**Fig. 3F**). Our LAMP assay showed specificity with all strains of *X. perforans* evaluated and did not react with any of the other bacterial or fungal pathogens of tomato that were included in testing (**Supplemental Table 1, Supplemental Figs. 4, 5**).

Detection of target pathogen in mixed infections

LAMP tests correctly detected *A. linariae* and *X. perforans* presence in separate and mixed infected leaf samples in blind tests (**Supplemental Table 5**). The color of positive reactions changed to sky blue, while negative reactions remained dark blue or violet in color.

Detection accuracy of LAMP tests for *Alternaria* spp. and *X. perforans* detection

The newly designed LAMP assays for *Alternaria* spp. and *X. perforans* showed high detection accuracy (**Supplemental Fig. 6**). The *Alternaria* spp. assay was only evaluated against *A. linariae* for detection accuracy and not the other early blight causing *Alternaria* species. LAMP tests detected the pathogen in nine out of ten infected samples (90% detection rate), while only one sample was positive by PCR (10% detection rate) (**Supplemental Fig. 6A-C**). *Xanthomonas perforans* was detected in all ten samples by LAMP assay and the detection rate was 100%, while detection rates by PCR were lower (70%) (**Supplemental Fig. 6D-F**).

Detection of target pathogens in whole plant inoculation assays

Initial symptoms of early blight on tomato were first observed 3 days after inoculation (dai) and by day 7 lesions covered up to 25-50% of total leaf area (**Fig. 4A**). The AUDPC was 60.4 on day 7. Individual lesions progressed from less than 1 mm in diameter on day 3 to 1-2 mm on day 4, and some reached up to 3-4 mm by day 7 (**Supplemental Fig. 1**). Only one sample taken 2 dai from *A. linariae* inoculated plants was positive by LAMP prior to visible symptoms (presymptomatic detection rate 13%) (**Fig. 4E**). The same plant also tested positive 4 dai, when the lesions were larger (> 2 mm). *Alternaria linariae* was detected in six out of eight inoculated plants (75%) by LAMP 7 dai (**Fig. 4E**). Positive LAMP results were obtained from lesions >2 mm in diameter. No positive LAMP reactions were observed in either the control plants or the NTCs LAMPs.

Disease symptoms caused by *X. perforans* on tomato plants developed rapidly over time in our experiments (**Fig. 4B, Supplemental Fig. 1**). First symptoms were observed 3 dai and included spots that expanded up to one third of the total leaf area and the AUDPC was 45.5 by 7 dai. Interestingly, LAMP tests from MN extracted DNA from inoculated plants were positive 2 dai on all plants. Thus, LAMP assay detected presymptomatic *X. perforans* with 100% accuracy (**Fig. 4F**). By 4 dai, LAMP detection remained 100% and then decreased to 62% by 7 dai, due to a decrease in bacterial populations in older lesions. In contrast, PCR tests detected the pathogen at rates of 87.5% 75% and 50% by 2 dai, 4 dai and 7 dai, respectively (**Fig. 4F**).

Disease symptoms in *P. infestans* inoculated tomato developed very rapidly (**Supplemental Fig. 1**). Symptoms were first observed 3 dai and lesions reached up to 20-35% of total leaf area within the next four days (**Fig. 4C**). The AUDPC was 55.1 by 7 dai. The first positive LAMP tests from MN extracted DNA were obtained from samples collected 4 dai when lesions reached about 0.5 cm^2 or more on the leaf (**Supplemental Fig. 1**). By 7 dai, when lesions

were greater than 1 cm^2 on leaves, 75% of samples tested positive by LAMP (**Fig. 4G**). All reactions from NTCs and non-inoculated leaves were negative.

Disease symptoms caused by TSWV were evaluated by the degree of stunting of the inoculated plants when compared to the control plants. Disease progressed slower in TSWV inoculated plants than for the three other pathogens tested (**Fig. 4D**). Visible stunting was observed 7 dai (**Supplemental Fig. 1**) and five out of eight inoculated plants had 10-15% stunting compared to the control plants by 9 dai (**Fig. 4D**). Plant growth was reduced by 15-25% by 14 dai, in seven of eight inoculated plants compared to the control plants and the AUDPC was 71.1. TSWV was detected by rtLAMP in three inoculated plants 2 dai (**Fig. 4H**). However, systemic infection had occurred by 7 dai and TSWV was detected by LAMP in three plants, only one of which was among the positive detections from 2 dai. Over time, five, six, and seven plants were positive by rtLAMP at 9, 11, and 14 dai, respectively and the pathogen was detected in all seven plants with symptoms by 14 dai (**Fig. 4H**). The rtLAMP assay detected TSWV as early as two days prior to visible symptoms. All reactions from NTCs and non-inoculated plants were negative, indicating no false positive LAMP results were obtained.

Detecting tomato pathogens with a smartphone-based device

All four pathogens were detected with LAMP assays run either in tubes or on a heat block. We also detected *P. infestans* by running the LAMP assay in the microfluidic chips on a heating slide powered by either fixed mains in a building or a portable battery suitable for field use.

We visualized all four LAMP reactions on the smartphone device. We compared the RFU from positive and negative controls and developed a threshold value for calling a reaction positive from the image on the Android smartphone (**Supplemental Fig. 7**). Positive reactions

displayed a solid green color (**Supplemental Fig. 7A)** and appeared white on the non-fluorescent original image(**Supplemental Fig. 7B**). Negative reactions were transparent green or transparent white by visual examination, respectively. The estimated mean RFU_{NTC} was 56.9±5.56, so the estimated threshold value equaled 76.59. Visual assessments corresponded with these calculations. All positive control samples and MN extractions from inoculated plants were determined to be positive. All reactions with uninoculated control plants and NTCs were determined to be negative (**Supplemental Table 6**).

Discussion In this work, we have expanded the utility of smartphone technology coupled with LAMP diagnostics for use in early detection of four tomato diseases: early blight, bacterial spot, tomato spotted wilt, and late blight. We showed that bacterial, fungal, oomycete, and viral infections of tomato can each be detected within 30 minutes using MN-extracted nucleic acids followed by LAMP reactions. We used portable heating equipment, including either a slide heater with a smartphone-based device reader or a portable heat block. The inoculation experiments with the four tomato pathogens showed that our methods detected bacterial spot and TSWV at presymptomatic stages and early and late blight at early symptomatic stages of disease. Moreover, we developed an improved microfluidic chip and generalized our LAMP protocols for smartphone-based fluorescence detection.

To the best of our knowledge, we report the first LAMP assay for the detection of *X. perforans* and the first general LAMP assay that detects the three common *Alternaria* species that occur infect tomato. The LAMP assay we developed was initially targeted for *A. linariae*, but over the course of the study we found the LAMP assay also amplified two other important *Alternaria* species which are also known to cause early blight on tomato: *A. alternata* and *A. solani* (Adhikari et al. 2021). While some LAMP assays have been previously developed to

detect only *A. solani* (e.g., Edin, 2012; Kumar et al. 2013; Lees et al. 2019) or only *A. alternata* (e.g. Yang et al. 2019, Liu et al. 2022), to the best of our knowledge no LAMP assay has been developed that detects all three. However, it should be noted that while the assay was able to detect all three species from pure mycelium extractions, it has not been evaluated on plants infected with either *A. solani* or *A alternata*, which cause either leaf lesions or leaf and stem lesions on tomato, respectively. *A. alternata* can also cause mild infection or co-occur with other pathogens, typically as a secondary infection. While *A. alternata* can be managed through the selection of resistant cultivars, the use of fungicides is still needed for control of the other two species. Knowing whether an oomycete, bacterial or fungal pathogen has caused the disease is useful to help growers make effective management decisions.

Previously, LAMP assays for the detection of *X. euvesicatoria* (Larrea-Sarmiento et al. 2018), *X. gardneri* (Stehlíková et al. 2020) and more recently duplex LAMP for simultaneous detection of *X. euvesicatoria* and *X. vesicatoria* (Beran et al. 2022) were reported. In those reports authors tested their methods with BioRanger, a portable device that allows real-time detection of two fluorophores. Like other LAMP assays for *Xanthomonas* spp. detection, our new assay has high specificity and sensitivity. Compared to a conventional PCR, our LAMP assay was 100 times more sensitive and allowed detection to levels as low as 1 pg of target DNA. Moreover, our assay can detect *X. perforans* with 100% accuracy from pre-symptomatic leaves. However, because *X. perforans* is associated with warmer climates, this assay may have less utility in cooler areas where other species, such as *X. gardneri*, are more prevalent on tomato.

The new *Alternaria* spp. LAMP assay designed in our study was sensitive and detected up to 1 pg of *A. linariae* DNA. Our assay can detect early blight from small lesions of *A. linariae*

(two millimeters in diameter) with 90% accuracy. For comparison, we used PCR primers that were designed to amplify large-spored *Alternaria* species, which included *A. linariae* and excluded *A. solani* (Adhikari et al. 2021). Interestingly, we also noticed that both the *Alternaria* spp. LAMP and *A. linariae* PCR tests worked best with very fresh DNA extractions.

The nucleic acid extractions with MN allowed us to process eight samples from inoculation experiments within five minutes while providing sufficient nucleic acid quality for LAMP assays. These MN extractions provided positive results at initial stages of TSWV and *X. perforans* infection (2 dai) and for *A. linariae* and *P. infestans* at 4 dai. Therefore, MN patches are a very rapid and convenient tool for in-field nucleic extractions.

As a part of this study, we standardized the master mix recipe we used to allow for interchangeability between pathogen assays, which simplifies in-field use, requiring only a change in the primers used and/or the addition of reverse transcriptase for TSWV detection. In addition, standardization of the assay simplified efforts to translate the assays to a more field ready format

To improve our smartphone-based detection system, we designed a new microfluidic chip and tested different heating methods. The new microfluidic chip design includes small apertures for reaction mix delivery and reduces issues from leakage and sample overflow contamination (**Supplemental Fig. 3B**). We tested the LAMP assays in these microfluidic chips using either a slide heater as previously described (Paul et al. 2019) or a commercially available heating block. For field applications, both these devices can be run from a charged portable battery or plugged into a cigarette lighter or 12 V electric plug on the back of a truck. However, further improvements to the slide heater on the smart phone device are still needed to make it field-ready and scalable.

We also developed an easier assessment method of analyzing images from the microfluidic chip retrieved from the smartphone. We improved and simplified the method for computationally determining positive/negative results on the smartphone. In previous iterations, before and after images were used to calculate the positive/negative threshold, which required careful imaging that was not always possible in a field setting (Paul et al. 2019). By using the non-template negative control and the negative threshold, we simplified the process and provided a more direct method for developing machine-learning algorithms for automatic image sensing in future projects.

In general, the LAMP-based diagnostics coupled with MN extractions are less time consuming than traditional extraction methods. While LAMP reactions have been noted previously for their tendency to produce false positives through amplicon contamination and primer dimerization, they confer several advantages over PCR, including speed and ability to be adapted to field conditions (Larrea-Sarmiento et al. 2019, Paul et al. 2021). To prevent false positive results, we suggest running three replicates to test unknown samples and at least two negative control reactions.

Our work has expanded the targets and opportunities for rapid in-field diagnostics of tomato pathogens. Two newly developed LAMP assays for *Alternaria* species and *X. perforans* detection have excellent specificity and sensitivity. We showed that quick and easy microneedle extractions work well for LAMP assays and demonstrated the potential for two pathogens, *X. perforans* and TSWV, to be detected at the presymptomatic stage. These LAMP reactions can be run in tubes on a heating block or heat slide charged from a portable battery and assessed visually on a smart phone. Next steps include scaling the imaging device and importing data

from the field LAMP assays into a database for mapping disease occurrences and further testing some of the LAMP assays in NC tomato fields.

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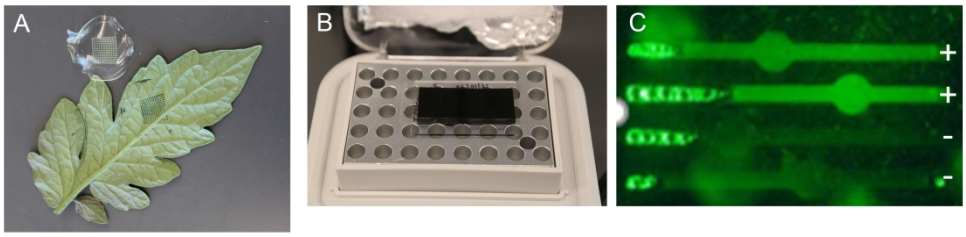

Fig. 1. Process for running an in-field LAMP. A, DNA extraction from a tomato leaf with a microneedle patch, B, running the PDMS microfluidic chip on a heating block and C, microfluidic chip with positive fluorescence reaction observed in chip after LAMP reaction. The lanes from top to bottom are: CTAB-extracted *P. infestans* DNA; DNA from a *P. infestans*-infected tomato leaf; DNA from a healthy leaf; no template control (NTC). Both the CTAB-extracted *P. infestans* DNA and *P. infestans*-infected leaf DNA are positive, while the healthy leaf DNA and the NTC are negative.

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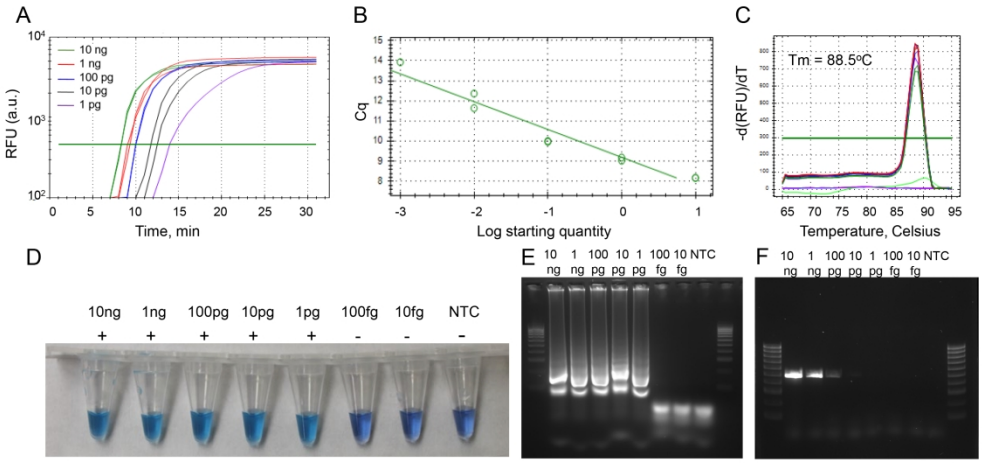

Fig. 2. *Alternaria* spp. LAMP reactions using *A. linariae* (A-E) run for 30 minutes. A, Real-time (rt) amplification at different DNA concentrations, B, standard curve for DNA dilution series, C, melting peak temperature for LAMP amplicon, D, colorimetric reactions for DNA dilution series (blue: positive; purple: negative), E, gel electrophoresis of LAMP products from a DNA dilution series and F, and gel electrophoresis for *A. linariae* PCR products from a DNA dilution series.

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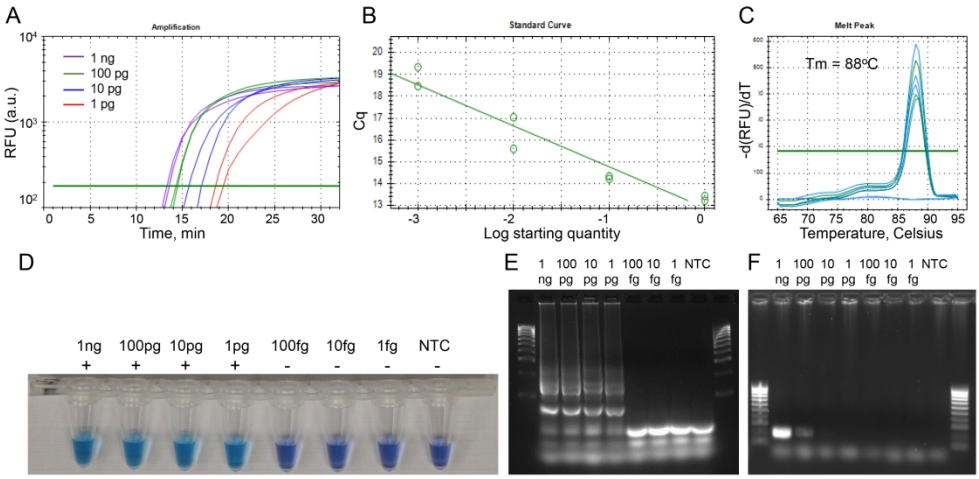

Fig. 3. *Xanthomonas perforans* LAMP reactions (A-E) run for 30 minutes. A, Real-time amplification at different DNA concentrations; B, standard curve for DNA dilution series C, melting peak temperature for LAMP amplicon; D, colorimetric reactions for DNA dilution series; E, gel electrophoresis of LAMP products from a DNA dilution series; and F, gel electrophoresis of *X. perforans* PCR products from a DNA dilution series

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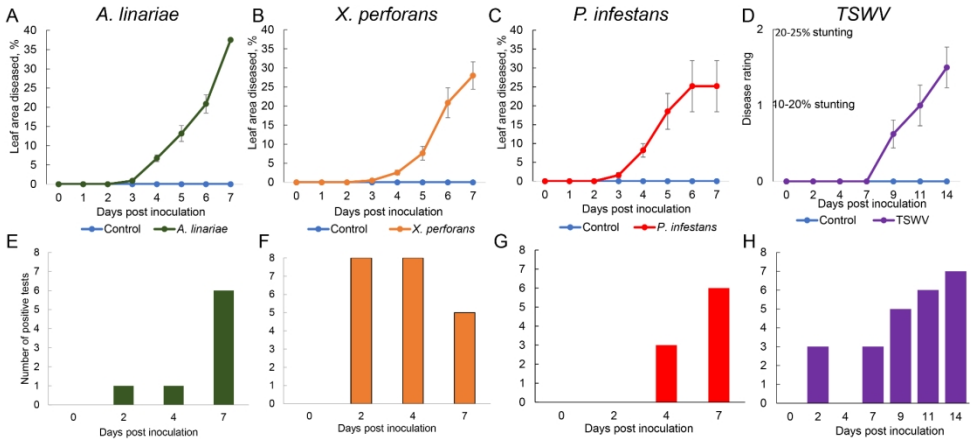


Fig. 4. Disease progress curves from tomato plant inoculation assays for A, *Alternaria linariae*, B, *Xanthomonas perforans*, C, *Phytophthora infestans* and, D. TSWV. Figs. A-D show mean disease severity ±SE of midpoints from each rating. LAMP test results from microneedle extracted nucleic acids sampled from tomato leaves shown over time for E, A. linariae, F, X perforans, G, P. infestans, and H, TSWV.

228x102mm (220 x 220 DPI)

Supplemental Table 1. Plant pathogen species, isolate numbers, and collector of isolates were used in this study to evaluate specificity of LAMP for detection of *Alternaria* species and *Xanthomonas perforans*.

Species	Isolate	Collector	LAMP Detection of *A. linariae*	LAMP Detection of *X. perforans*
Alternaria linariae	JD1B[a]	I. Meadows	+	-
Alternaria linariae	25	T. Adhikari	+	NT[b]
Alternaria linariae	3	T. Adhikari	+	NT
Alternaria linariae	60	T. Adhikari	+	NT
Alternaria alternata	19-068	I. Meadows	+	NT
Alternaria alternata	T-25	T. Adhikari	+	NT
Alternaria solani	P44	T. Adhikari	+	NT
Alternaria solani	P270	T. Adhikari	+	NT
Fusarium sambucinum	LRSJ27-LY	R. Loria	-	-
Fusarium solani	Caco-2	R. Loria	-	-
Fusarium sp.	F5	J. Ristaino	-	-
Helminthosporium solani	12-SS2	R. Loria	-	-
Helminthosporium solani	19-SS-2TI	R. Loria	-	-
Pythium aphanidermatum	1	J. Kerns	-	-
Pythium aphanidermatum	2	J. Kerns	-	-
Rhizoctonia solani	R-109	J. Ristaino	-	-
Rhizoctonia solani	AG3NC1	M. Cubeta	-	-
Sclerotium rolfsii	SR-DD-5	J. Ristaino	-	-
Sclerotium rolfsii	SR-DD-10	J. Ristaino	-	-
Sclerotinia sclerotiorum	SS-DD-1	J. Ristaino	-	-
Sclerotinia sclerotiorum	TI	P. Weingartner	-	-
Verticillium albo-atrum	462	R. Rowe	-	-
Verticillium dahliae	21	R. Rowe	-	-
Phytophthora cinnamomi	P10	M. Gallegly	-	-
Phytophthora infestans	GA 21-1	I. Meadows	-	-
Phytophthora infestans	GA 21-2	I. Meadows	-	-
Phytophthora infestans	NC14-1[a]	R. Gardner	-	-

Xanthomonas euvesicatoria	15-015	I. Meadows	-	-
Xanthomonas vesicatoria	56	D. Ritchie	-	-
Xanthomonas gardneri	15-019	I. Meadows	-	-
Pseudomonas syringae	DC3000	H. McMillan	-	-
Pectobacterium carotovorum	BP9128	C. Bull	-	-
Clavibacter michiganensis	CMS2	D. Mills	-	-
Ralstonia solanacearum	JC-57	F. Louws	-	-
Xanthomonas perforans	15-018	I. Meadows	-	+
Xanthomonas perforans	19-027[a]	I. Meadows	-	+
Xanthomonas perforans	19-031	I. Meadows	-	+
Xanthomonas perforans	19-041A	I. Meadows	-	+
Xanthomonas perforans	NC-71	F. Louws	-	+
Tomato spotted wilt virus	CA-WT[a]	C. Cespedes	-	-

[a] isolate used for the inoculation experiments
[b] NT= not tested

Supplemental Table 2. Primer sequences used for TSWV, *Phytophthora infestans*, *Xanthomonas perforans* and *Alternaria* spp. LAMP in this study.

Assay	Pathogen	Primer name	Primer sequence (5' – 3')	Reference
LAMP	TSWV *NsM*	F3_TSWV	TCAAGCCTATGGATTACCTCT	Paul et al. 2021
		B3_TSWV	TCTCACTGTAATGTTCCATAGC	
		FIP_TSWV	GGTCGATCCCGAGATCCTTGTAGCT TCAGTTGATAGCTTTGAG	
		BIP_TSWV	ACACCAGGGAAGCCTTAGGAACCTT CTTCACCTGATCTTCATT	
		LoopF_TSWV	AGCCAAGACAACACTGATCAT	
		LoopB_TSWV	AAGTTTGCACTGTGCTGAAA	
LAMP	*P. infestans* ITS	F3_Pinf	CTCCAAAAGTGGTGGCATTG	Ristaino et al. 2020
		B3_Pinf	GCAACAGCAAAGCCGATTC	
		FIP_Pinf_HPLC[a]	TCTCCATTAACGCCGCAGCAGTGGA CGCTGCTATTGTAGC	
		BIP_Pinf_HPLC[a]	CGTGGTATGGTTGGCTTCGGCATGG TTCACCAGTCCATCAC	
		LoopF_Pinf	ACAAACCGGTCGCCAACTC	
		LoopB_Pinf	ATGCGCTTATTGGGTGATTTTCCTG	
LAMP	*X. perforans* [b] XPE_07070	XP15F3	ACACGACTTCCGAACGTAGT	This study
		XP15B3	CCCGCTTTTCTGACAGGTC	
		XP15FIP	GCCCGTCGTGTTGATGGAGCTTATC TCCCACACCGCGATA	
		XP15BIP	CGTCCGCAGTGGAATGCCATTTCGA GGTGATCGGTGATGC	
		XP15Loop	CCGGGTTGTAGTTACACGGC	
LAMP	*Alternaria* spp *β-tub*	F3_Alter_BT	AACTCGGACGAGACCTTCT	This study
		B3_Alter_BT	GAAGTGGAGACGGGGGAA	
		FIP_Alter_BT	CCGTACGAGGGGTTGTTCAGCGCAT TGACAACGAGGCTCTC	
		BIP_Alter_BT	CCACCGTCATGTCGGGTGTCGACGG CCAACTTCCTCAG	
		LoopF_Alter_BT	GTCCTCATGCAGATGTCGTA	
		LoopB_Alter_BT	ACCACCTGCCTGCGTT	

PCR	*X. perforans* [b] XPE_07070	BS-XpF	GTCGTGTTGATGGAGCGTTC	Koenraadt et al. 2009
		BS-XpR	GTGCGAGTCAATTATCAGAATGTGG	Araujo et al. 2012
PCR	*A. linariae* calmodulin-encoding gene	OAtF4	TGCGGCTTGCTGGCTAAGGT	Adhikari et al. 2021
		OAtR2	CAGTCGATGCGGCCGTCA	

[a] Primers must be HPLC purified instead of the standard desalting procedure to ensure species specificity and sensitivity.
[b] For *X perforans*, genome XPE_07070 was used at positions 1612629-1613216.

Supplemental Table 3. Protocol for LAMP reaction master mix

Reagent	**Stock concentration**	**Volume per reaction, µL**
Isothermal Amplification Buffer	10 X	2.5
dNTPs (each)	10 mM	3.5
MgSO4	100 mM	1.25-1.5
Betaine	5 M	2
HNB	2.5 mM	1.2
EvaGreen	20 X	1.25
Bst DNA polymerase	8 U/µL	1
WarmStart reverse transcriptase (use for TSWV RT-LAMP only)	15 U/µL	0.5
Primer mix	100 mM	2.5 (for TSWV) 1.5 (for *P. infestans, Alternaria* spp.*, X. perforans*)
Water (molecular grade)	-	up to 25 µL

Supplemental Table 4. Horsfall-Barratt Scale [a] used for disease severity ratings. Categories broaden and narrow to account for human bias to over- or under-estimate disease.

Rating	**%LAD**	**Midpoint[b]**
0	0	0
1	<1%	1%
2	1-3%	2%
3	3-6%	4.5%
4	6-12%	9%
5	12-25%	18.5%
6	25-50%	37.5%
7	50-75%	62.5%
8	75-87%	81%
9	87-94%	90.5%
10	94-97%	95.5%
11	97-100%	98.5%
12	100%	100%

[a] Horsfall, J. G and Barratt, R. W. 1945. An Improved Grading System for Measuring Plant Disease. Phytopathology **35**: 655(Abstract).

[b] Data are converted to midpoint percentage ratings for statistical analysis & graphs.

Supplemental Table 5. Detection of *Alternaria linariae* or *Xanthomonas perforans* by LAMP either singly or in mixed samples with both pathogens

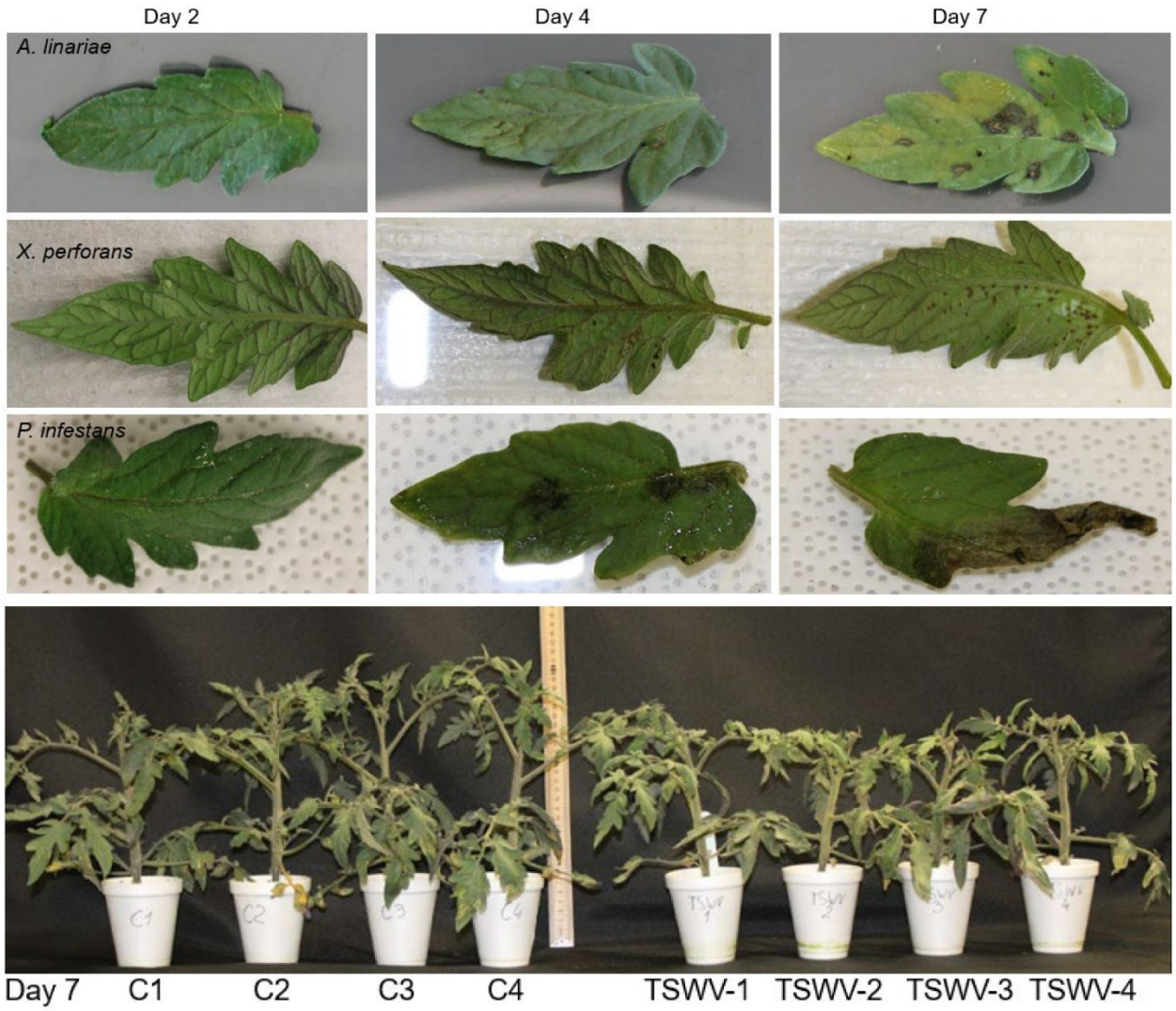
Sample	***Alternaria* linariae**	***X. perforans***
A. linariae only	+	-
X. perforans only	-	+
A. linariae & *X. perforans mixture*	+	+

Three subsamples evaluated were either positive or negative respectively for each pathogen alone or in mixture.

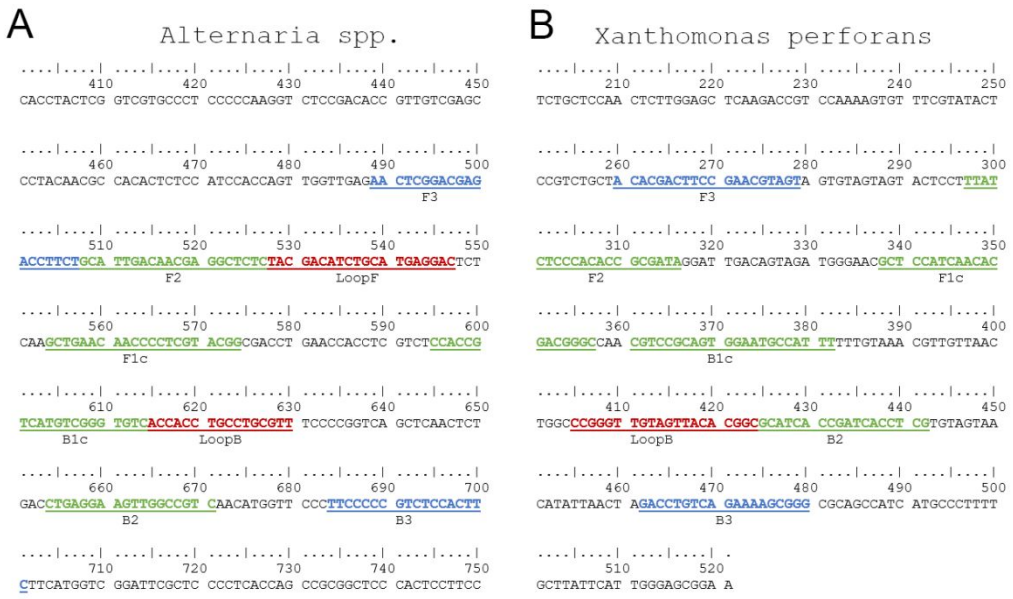
Supplemental Table 6. Relative fluorescence units (RFU) measurements from smartphone reader for four tomato plant pathogen LAMP reactions.

LAMP assay	Samples	RFU[a]	Detection
TSWV	PC	77.72	positive
	MN	77.65	positive
	Healthy	52.39	negative
	NTC	57.43	negative
Alternaria spp.	PC	136.75	positive
	MN	82.59	positive
	Healthy	66.50	negative
	NTC	70.89	negative
X. perforans	PC	151.63	positive
	MN	115.00	positive
	Healthy	74.98	negative
	NTC	70.66	negative
P. infestans	PC	112.46	positive
	MN	115.88	positive
	Healthy	48.52	negative
	NTC	52.28	negative

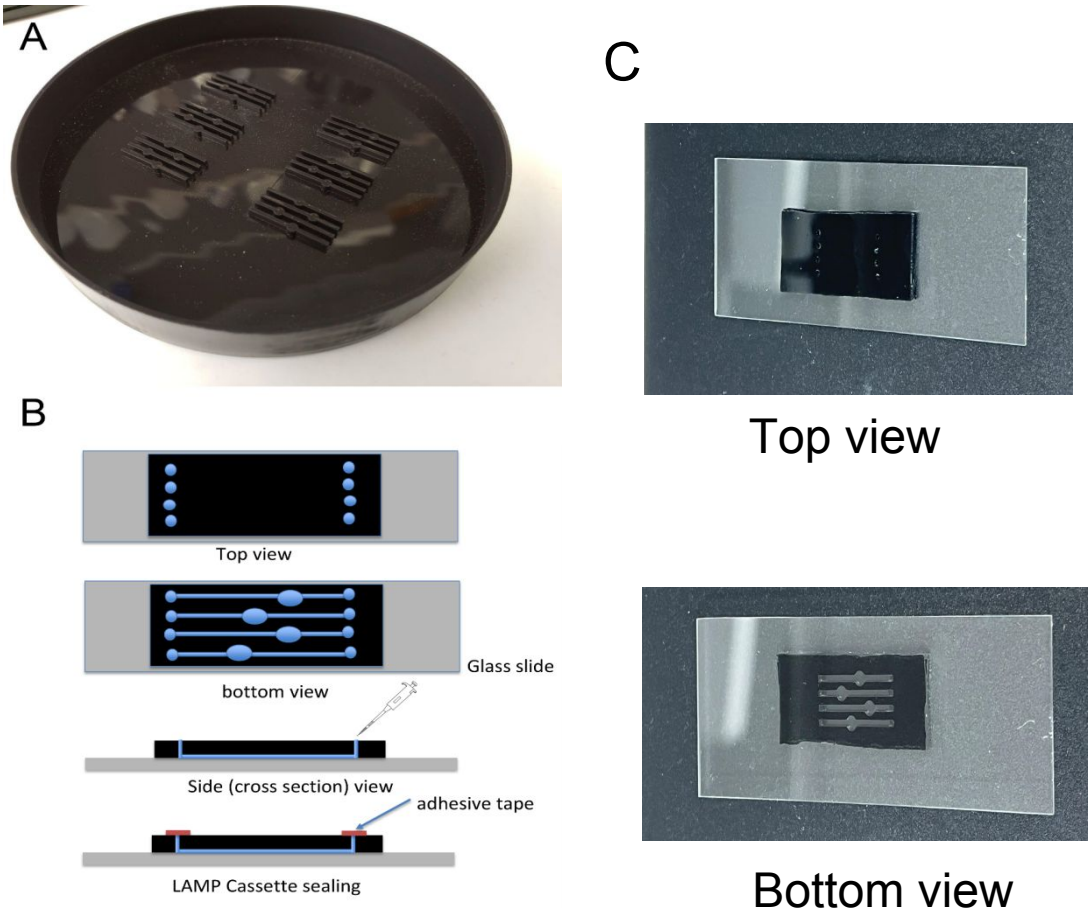
[a]RFU above a fixed threshold value of 76.6 were positive.



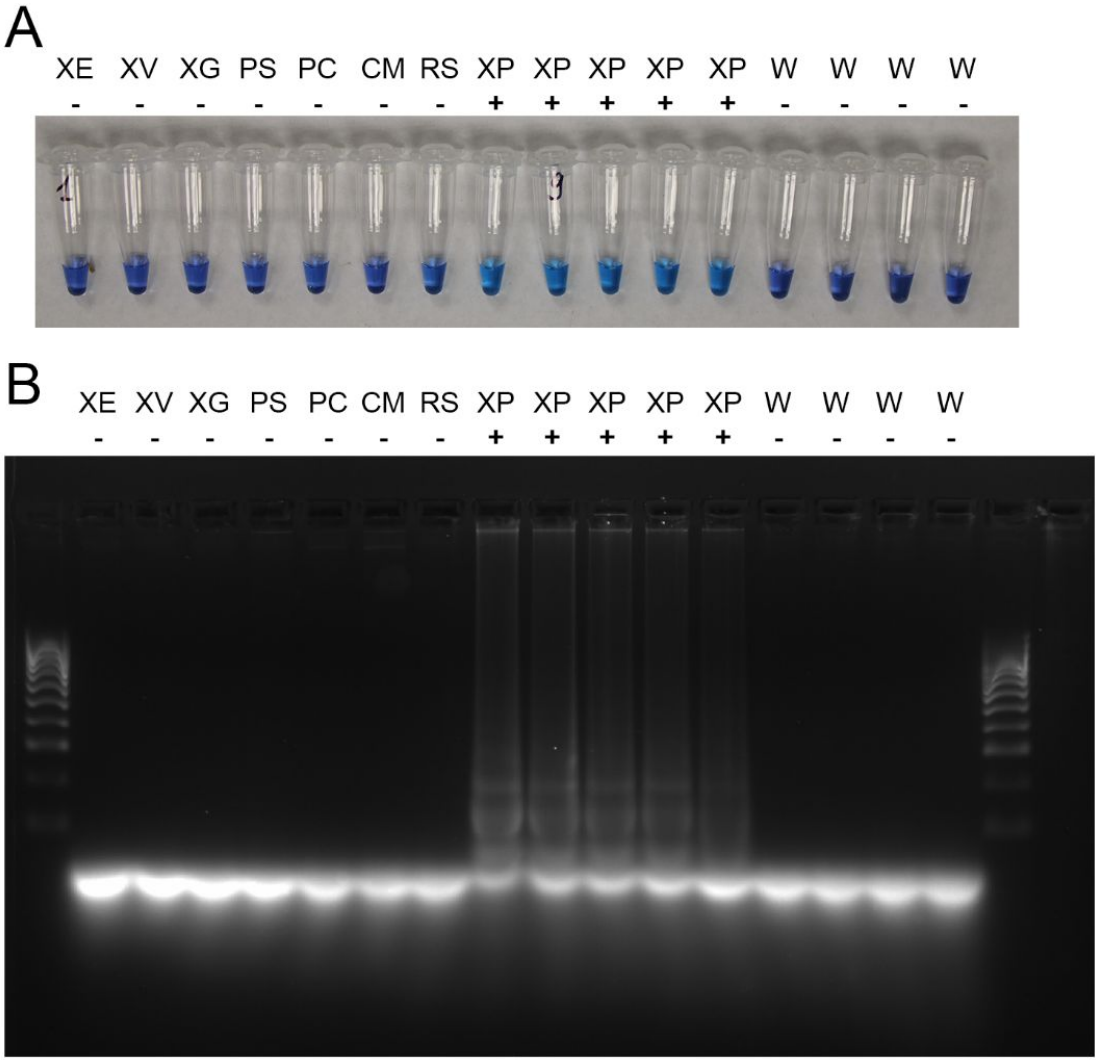
Supplemental Fig. 1. Inoculation experiments with *Alternaria linariae*, *Xanthomonas perforans*, *Phytophthora infestans* and *TSWV* on tomato were sampled over time and used for microneedle extractions and LAMP tests. For *A. linariae*, *X. perforans*, and *P. infestans*, examples of infection at 2, 4, and 7 days after inoculation are shown. Whole plants were inoculated including four control plants (left) and TSWV inoculated plants (right) shown at 7 days after inoculation. Symptoms on TSWV inoculated tomato included necrotic lesions and stunting compared to the non-inoculated control plants.



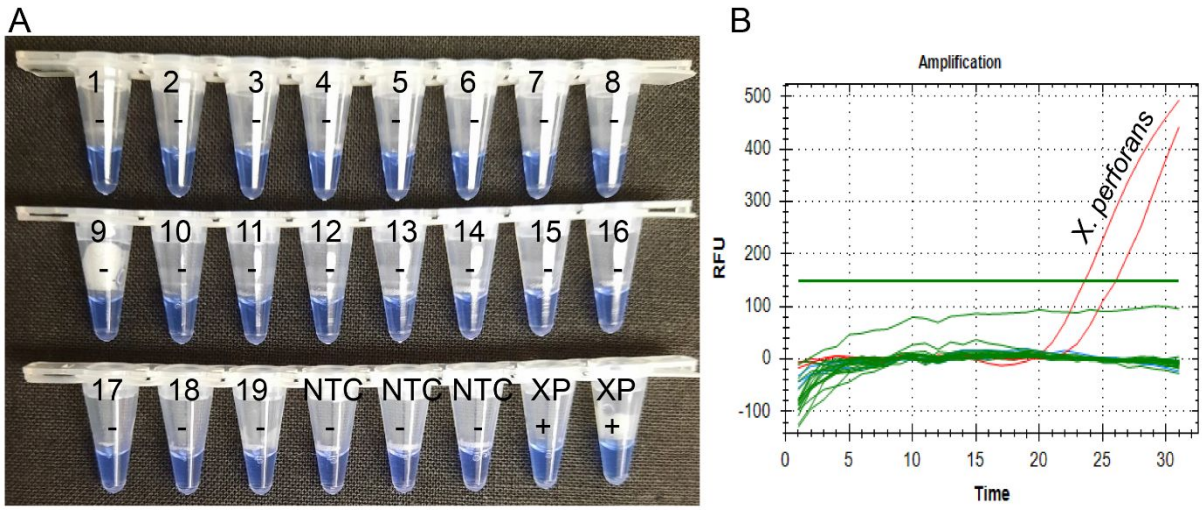
Supplemental Fig. 2. Binding sites for LAMP primers for **A**, *Alternaria* spp. and **B,** *Xanthomonas perforans,* respectively. For *Alternaria* spp., LAMP amplifies a portion of the *B tubulin* gene. For *X. perforans*, LAMP amplifies a hypothetical protein in genome XPE_07070 at positions 1612629-1613216.



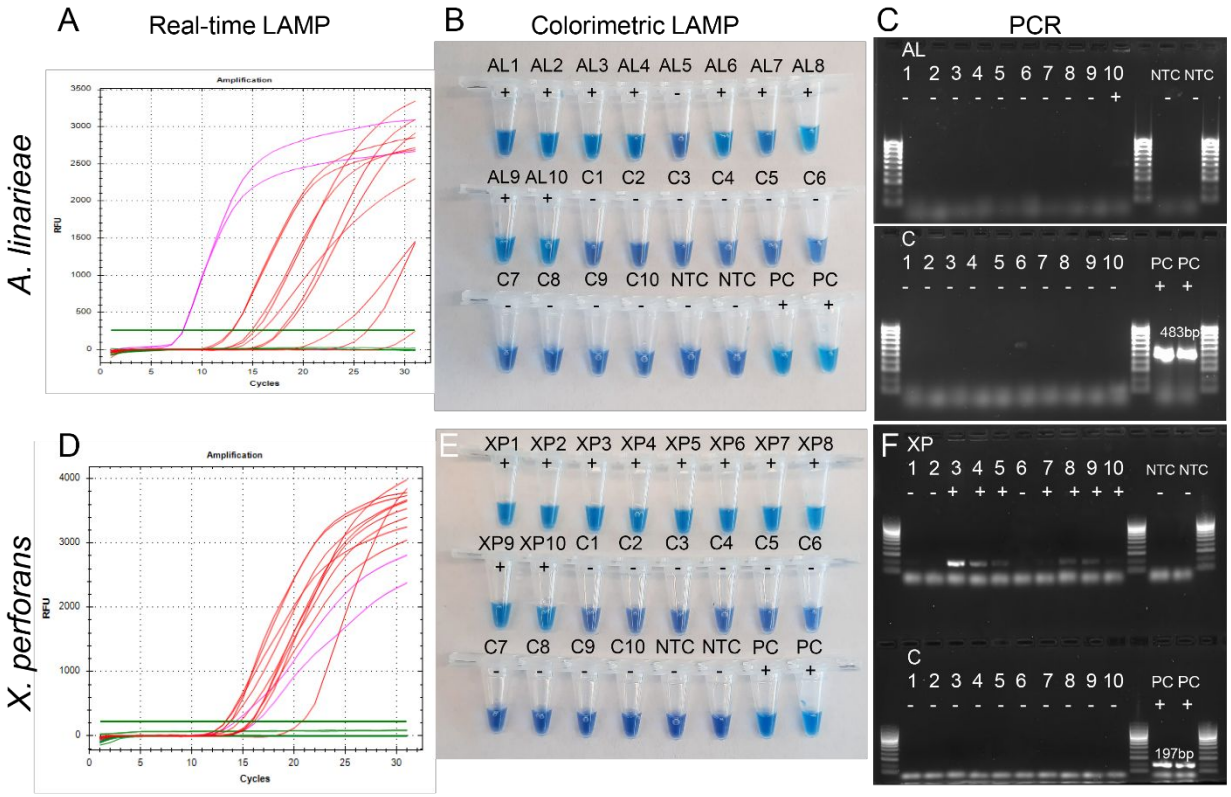
Supplemental Fig. 3: Preparation of the microfluidic chip: **A**, Pouring PDMS into a 3D printed microneedle mold; **B**, Schematic illustrations of the PDMS chip prepared using the mold with top, bottom and side views of the chip; **C**, Top and bottom view of the PDMS microfluidic chip



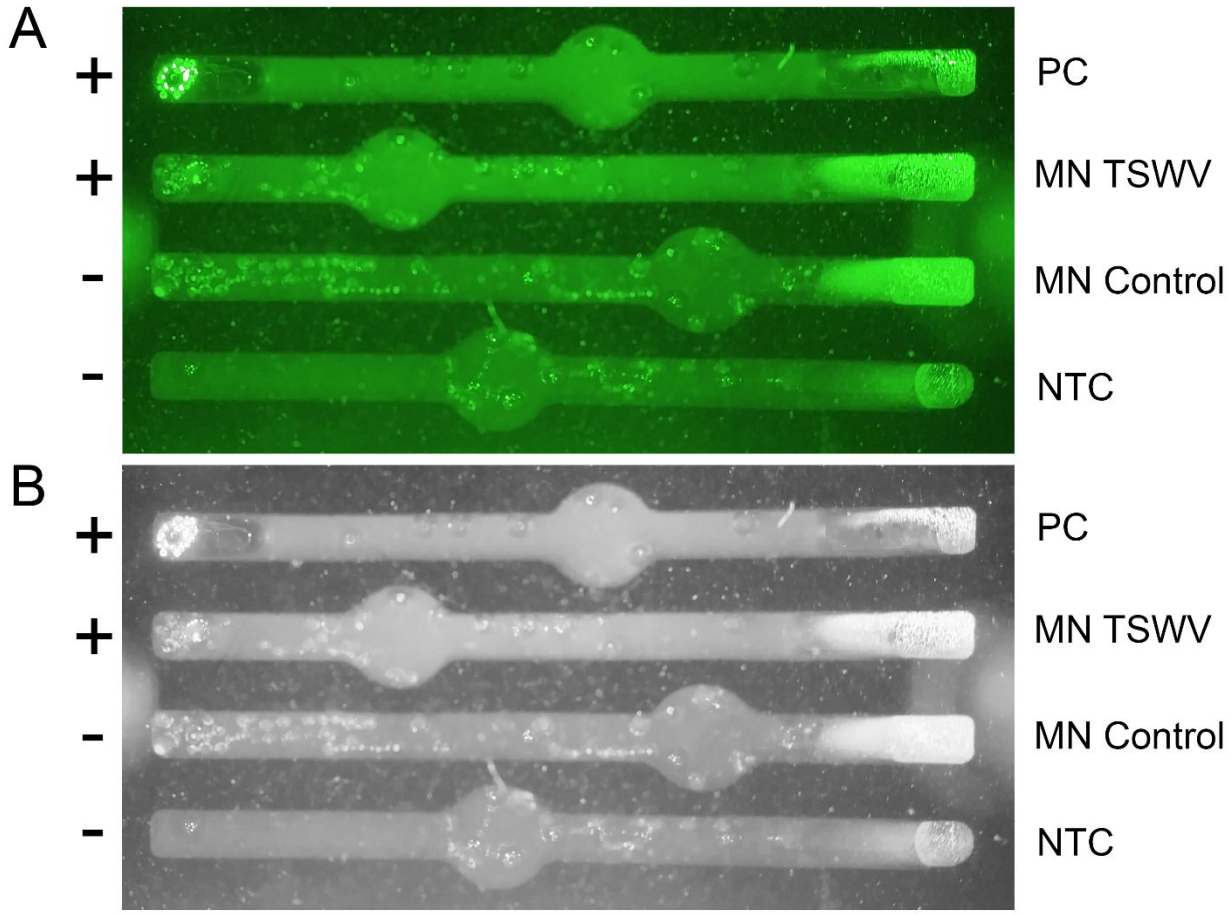
Supplemental Fig. 4. *Xanthomonas perforans* specificity tests LAMP reactions run for 30 min with different bacterial species (see **Supplemental Table 1**) found on tomatoes. **A,** Colorimetric reactions in tubes. Positive reactions for *X. perforans* are light blue while negative reactions remain purple or dark blue; and **B**, gel electrophoresis of LAMP products. Species abbreviations are; XE , *X. euvesicatoria*, XV, *X. vesicatoria*, XG, *X. gardeneri,* PS, *Pseudomonas syringae ,* PC, *Pectobacterium carotovorum ,* CM, *Clavibacter michiganensis ,* RS, *Ralstonia solanacearum,* XP, *X. perforans* (isolates 15-0018, 19-027, 19-031, 19-041A, NC-71)*,* or W, no template water control.



Supplemental Fig. 5. Specificity tests for *Xanthomonas perforans* LAMP reactions run for 30 min using different fungal and or oomycete species (**Supplemental Table 1**) found on tomatoes were tested. **A**, Tubes show colorimetric reactions, and **B,** real-time LAMP results indicate positive rtLAMP samples for *X. perforans* only (red lines). Other fungal species and isolates tested are listed numerically as follows: 1, *Alternaria linariae* JD1B; 2, *Fusarium sambucinum* LRSJ27-LY; 3, *Fusarium solani* Caco-2; 4, *Fusarium sp.*F5; 5, *Helminthosporium solani* 12-SS2; 6, *Helminthosporium solani* 19-SS-2TI; 7, *Pythium aphanidermatum* 1; 8, *Pythium aphanidermatum* 2; 9, *Rhizoctonia solani* R-109; 10, *Rhizoctonia solani* AG3NC1; 11, *Sclerotium rolfsii* SR-DD-5; 12, *Sclerotium rolfsii* SR-DD-10; 13, *Sclerotinia sclerotiorum* SS-DD-1 14, *Sclerotinia sclerotiorum* TI; 15, *Verticillium albo-atrum* 462; 16, *Verticillium candidae* 21; 17, *Phytophthora cinnamomi* P10; 18, *Phytophthora infestans* NC 14-1; 19, *Alternaria alternata* 19-068.



Supplemental Fig. 6. Probability of detection using microneedle (MN) extracted DNA from 10 tomato leaves inoculated with *A. linariae* or *X. perforans* using either rtLAMP (**A,D)**, colorimetric LAMP **(B,E**) or PCR **(C,F**). For rtLAMP **(A, D)** positive samples are shown in red and positive pathogen controls in pink. For colorimetric LAMP (**B,E**) positives are light blue and negatives are purple/dark blue, and for PCR **(C,F)** amplicon sizes are 483 bp for *A. linariae* and 197 bp for *X. perforans,* respectively. Real-time and colorimetric LAMPs were positive for 90% of *A. linariae*-infected leaves, and 100% of the *X. perforans*-infected leaves. In contrast, 10 % and 70% were positive by PCR for *A. linariae* and *X. perforans*, respectively.



Supplemental Fig. 7. Images of TSWV LAMP reactions on a microfluidic slide taken with a smartphone-based device in green (**A**) or grayscale (**B**) for four reactions. The lanes for each image, from top to bottom are: positive control of TSWV RNA (PC), MN extracted TSWV from an infected leaf (MN TSWV); a negative control MN rinsate with buffer only (MN Control) or no template control (NTC), **A**, Florescence images from reactions; **B**. Grayscale images from the same four reactions. Positive reactions florescence green or appear solid gray on grayscale images. Negative reactions are transparent in green and grayscale images.