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Techniques

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 and combined these with loop-mediated amplification (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 microneedles, and LAMP assays were run in tubes (with hydroxynaphthol blue) on a heat block or on a newly designed microfluidic slide 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, whereas *Phytophthora 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 microneedle extraction followed by LAMP on a microfluidic chip for rapid diagnosis of four important tomato pathogens.

Keywords: bacterial pathogens, fungal pathogens, oomycetes, pathogen detection

Crop pests and diseases cause between 20 and 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 presymptomatic 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, recombinase polymerase amplification, 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 *P. infestans*, bacterial spot caused by *Xanthomonas perforans* Jones et al., and tomato spotted wilt virus caused by TSWV (Supplementary Fig. S1).

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Tomatoes, both processing and fresh market, are the second most consumed vegetable in the United States behind potato and are valued at over 1.85 billion dollars. North Carolina ranks sixth in the nation in tomato production and grows more than 4,000 acres (Anonymous 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., Debreczeni et al. 2011; Lees et al. 2019; Roberts et al. 2000), LAMP (e.g., Hansen et al. 2016; Khan et al. 2017; Lees et al. 2019), recombinase polymerase amplification (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 recombinase polymerase amplification 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. Although 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 North Carolina (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 (i) develop LAMP assays for the detection of *Alternaria* spp. and *X. perforans* and validate their specificity and sensitivity; (ii) test the LAMP for detection of *A. linariae*, *X. perforans*, *P. infestans*, or TSWV on inoculated tomato assays as disease progresses over time using MNs; and (iii) develop and test a microfluidic chip for running LAMP assays on a smartphone device.

Materials and 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 (Supplementary Table S1). For the purposes of inoculations, testing, and validating the LAMP assays both in vitro and in vivo, we selected four plant pathogens: *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°C for 15 to 30 min in a water bath. After incubation, one volume (~300 µl) of chloroform/isoamyl alcohol (24:1) 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-24 × 2 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 3 M sodium acetate (pH 8.0) and two 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 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 of 10% polyvinyl alcohol and spun in an Eppendorf 5810R centrifuge using a high-speed A-2-DWP-AT plate rotor (Eppendorf) at 4,200 rpm for 25 min 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 10 s 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 (Supplementary Table S2). New primers were designed to target *A. linariae* and *X. perforans* using PrimerExplorer version 5 (Eiken Chemical, Tokyo, Japan). We selected the β-*tubulin* (β-*tub*) gene for the development of *Alternaria* spp. LAMP primers (GenBank Accession 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 a hypothetical protein (Araújo et al. 2012) and used the subsequent sequence data to design LAMP primers for *X. perforans* (Supplementary Table S2). Primer candidates were bioinformatically evaluated against sequences of closely related species for species specificity and to evaluate the position of informative single-nucleotide polymorphisms. The primer sets selected for further testing showed a larger number of single-nucleotide polymorphisms 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 Supplementary Figure S2.

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 (Supplementary Table S1). 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), *Ralstonia solanacearum* (RS), and five strains of *X. perforans* (XP) (Supplementary Table S1). 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 (Honeywell Fluka, Charlotte, NC). Positive reactions using hydroxynaphthol blue 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 (10 mM Tris-HCl, 1 mM 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 was 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 10× isothermal amplification buffer (New England Biolabs, Ipswich, MA), 1.25 µl of 100 mM magnesium sulfate (New England Biolabs), 3.5 µl of dNTPs (10 mM each) (Apex Bioresearch Products, Genesee Scientific, El Cajon, CA), 2 µl of 5 M 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 hydroxynaphthol blue, 1.25 µl of 20× EvaGreen, 1 µl of 8 U/µl Bst 2 WarmStart DNA polymerase (New England Biolabs), 0.5 µl of reverse transcriptase 15 U/µl (New England Biolabs) (for TSWV detection only), and 1 µl of DNA (Supplementary Table S3). LAMP reactions were performed on a Bio-Rad CFX96 real-time machine or on a Bio-Rad T100 conventional thermal cycler with a hold at 65°C for 30 min followed by a deactivation step at 80°C for 5 min.

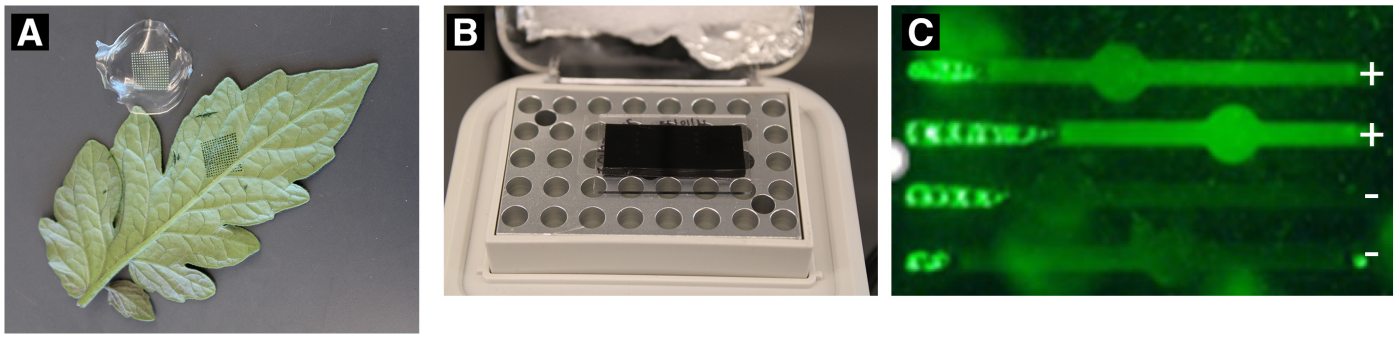

Fig. 1. Process for running an in-field loop-mediated isothermal amplification (LAMP). **A,** DNA extraction from a tomato leaf with a microneedle patch. **B,** Running the PDMS microfluidic LAMP chip on a heating block. **C,** Microfluidic chip with positive fluorescence reaction observed in chip after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

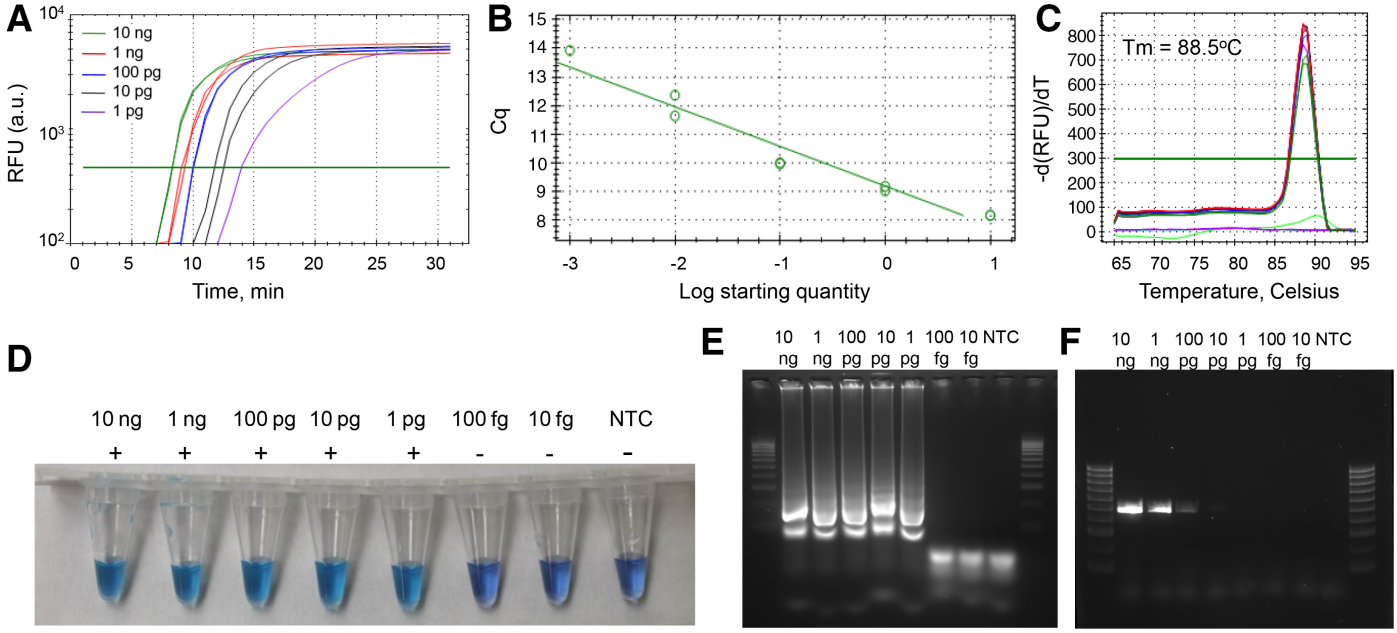

Fig. 2. *Alternaria* spp. loop-mediated isothermal amplification (LAMP) reactions using **A to E,** *A. linariae* run for 30 min. A, Real-time (rt) amplification at different DNA concentrations; B, standard curve for DNA dilution series; C, melting peak temperature for LAMP amplicon; D, colorimetric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

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 were 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, 3-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, 2-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 noninoculated control group. We conducted these experiments twice with *X. perforans*, *P. infestans*, *A. linariae*, and TSWV infections.

Pathogen inoculum preparation

The 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 an optical density 0.07 (Spectronic 21, Bausch & Lomb, Laval, Canada), which corresponds to 10^8 CFUs/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 of $CaCO_3$, 100 ml of V8 juice, 20 g of Difco Bacto Agar [BD Diagnostics, East Rutherford, NJ], and 1 liter of dH_2O) using a cell spreader and leaving the plate exposed for 24 to 72 h. The plate was then brushed with 2 ml of sterile water, and the water was collected. Conidia density was calculated under a microscope with a hemacytometer, and conidia were diluted to 2,000 conidia/ml. For *P. infestans* experiments, an isolate of *P. infestans* (isolate NC14-1, US-23 genotype) was maintained on detached tomato leaves (cultivar Mountain Fresh Plus). A leaf with active *P. infestans* sporulation was vortexed in 10 ml of distilled water to harvest sporangia, and 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 to 10 ml of sodium sulfite (63 mg per 50 ml of 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 (Supplementary Table S4). 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 (Shymanovich 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, 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. An 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 (dai). 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 to 15% compared with control), 2 = significant stunting (20 to 25% compared with control) and smaller young leaves, 3 = strong stunting (25 to 50% compared with control) and yellowish leaves, 4 = severe stunting (50 to 75% compared with control) and some necrosis, and 5 = dwarf, stunting (>75% compared with control), and 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 of 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, IA) via manufacturer instructions, and 2 µl was used per reaction. Two microliters of molecular-grade water was used for no-template controls (NTCs). LAMP results were visualized either by gel electrophoresis with 5 µl of LAMP product on a 2% agarose gel with 1 M TAE buffer at 130 V, 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 noninoculated 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 deionized 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 with traditional PCR testing. Conventional PCR tests were performed by using previously described PCR primers for *A. linariae*

and *X. perforans* (Supplementary Table S2). For 25-µl reactions, we used 2.5 µl of 10× PCR buffer (Apex Bioresearch Products, Genesee Scientific), 1.25 µl of dNTPs (2 mM each) (Apex Bioresearch Products, Genesee Scientific), 1 µl of each forward and reverse 10 µM primer, 0.9 µl of $MgCl_2$ (50 mM) (Apex Bioresearch Products, Genesee Scientific), 0.125 µl of BSA (20 mg/ml) (Thermo Scientific, Waltham, MA), 0.1 µl Taq (5 U/µl) (Apex Bioresearch Products, Genesee Scientific), and 16.125 µl of molecular-grade water. For each reaction, we added 2 µl of MN-extracted DNA. Gel electrophoresis was run with 5 µl of PCR product on 1% agarose gel with 1 M TAE buffer at 130 V. For *A. linariae*, a 483-bp band was detected, whereas 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 four-celled reaction chip run on a heating slide and analyzed using a smartphone camera (Paul et al. 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 (Morrisville, NC) (Supplementary Fig. S3A). Six microfluidic chips can be made simultaneously with the 3D-printed mold. Each chip consists of four microfluidic channels (Supplementary Fig. S3B), and the length, width, and height of each channel were 16, 0.8, and 2 mm, respectively. To fabricate the PDMS microfluidic chip, Sylgard elastomer, a curing agent, and charcoal powder were mixed in a weight ratio of 100:10:1 and vacuumed for 15 min 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 (Supplementary Fig. S3C).

LAMP reactions and imaging of microfluidic chip slides

Each of the four pathogen LAMP reactions were evaluated on PDMS chips heated on an AmplifyRP heat block (Agdia, 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 (1 ng/µl), 2 = MN extraction from an infected tomato leaf, 3 = MN extraction from a healthy control tomato leaf, and 4 = NTC (Fig. 1C). A standard LAMP master mix was prepared as described previously. Two microliters 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 (Supplementary Fig. S3B). After loading reagents, the backside of the PDMS chip was sealed with a piece of PCR film. The loaded microfluidic chip was heated in one of two ways: (i) on an AmplifyRP heat block surface at 65°C for 30 min (Fig. 1B) or (ii) 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 with 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 20 no-template reactions (NTCs) in five chips and measured the RFU of each reaction after 30 min. 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 an 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 and 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 and F). Specificity tests indicated that although 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 (Supplementary Table S1).

X. perforans LAMP assay

Likewise, the same detection limit of 1 pg of DNA was observed for *X. perforans* after a 30-min LAMP reaction at 65°C (Fig. 3A and 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 (Supplementary Table S1; Supplementary Figs. S4 and S5).

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 (Supplementary Table S5). The color of positive reactions changed to sky blue, whereas 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 (Supplementary Fig. S6). 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 9 out of 10 infected samples (90% detection rate), whereas only one sample was positive by PCR (10% detection rate) (Supplementary Fig. S6A to C). *X. perforans* was detected in all 10 samples by LAMP assay, and the detection rate was 100%, whereas the detection rates by PCR were lower (70%) (Supplementary Fig. S6D to F).

Detection of target pathogens in whole-plant inoculation assays

Initial symptoms of early blight on tomato were first observed 3 dai, and by day 7, lesions covered up to 25 to 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 to 2 mm on day 4, and some reached up to 3 to 4 mm by day 7 (Supplementary Fig. S1). 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). *A. linariae* was detected in six out of eight inoculated plants (75%) by LAMP 7 dai (Fig. 4E). Positive LAMP results were obtained from lesions more than 2 mm in diameter. No positive LAMP reactions were observed in either the control plants or the NTC LAMPs.

Disease symptoms caused by *X. perforans* on tomato plants developed rapidly over time in our experiments (Fig. 4B; Supplementary Fig. S1). 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, the 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, 4, and 7 dai, respectively (Fig. 4F).

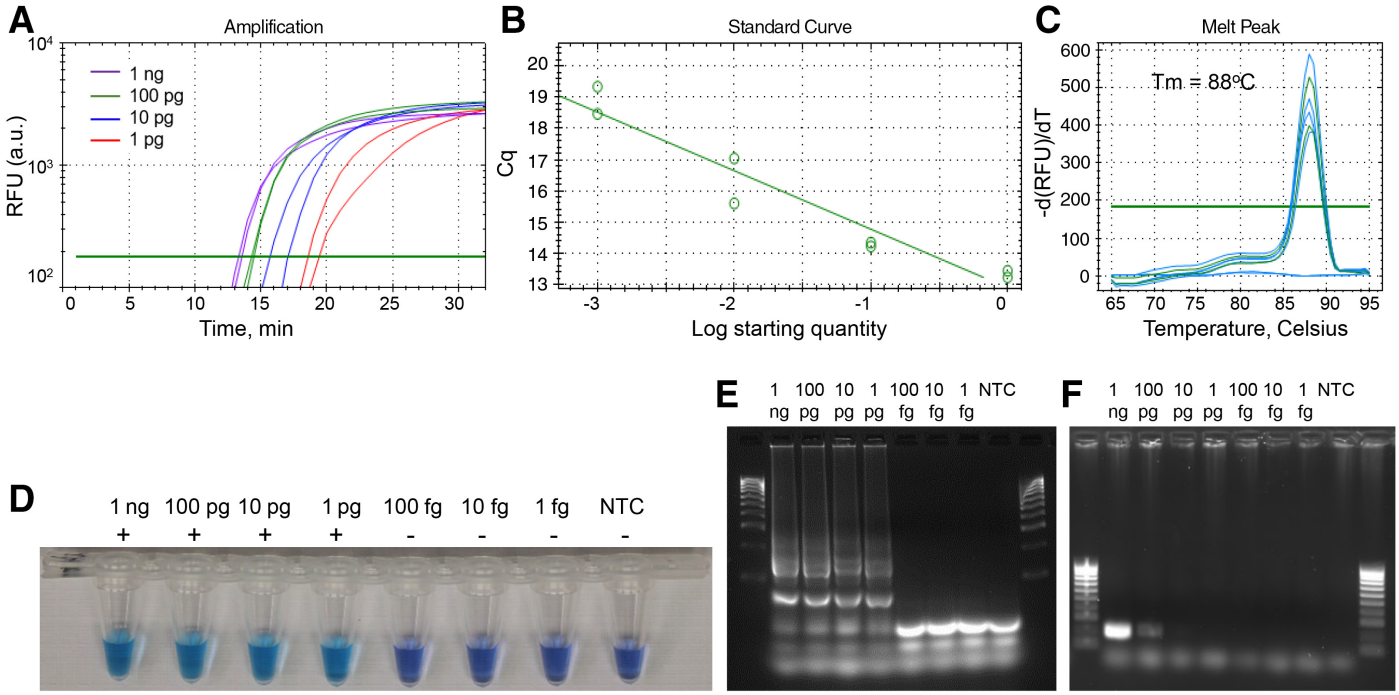

Fig. 3. A to E, *Xanthomonas perforans* loop-mediated isothermal amplification (LAMP) reactions run for 30 min. A, Real-time amplification at different DNA concentrations; B, standard curve for DNA dilution series; C, melting peak temperature for LAMP amplicon; D, colorimetric reactions for DNA dilution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

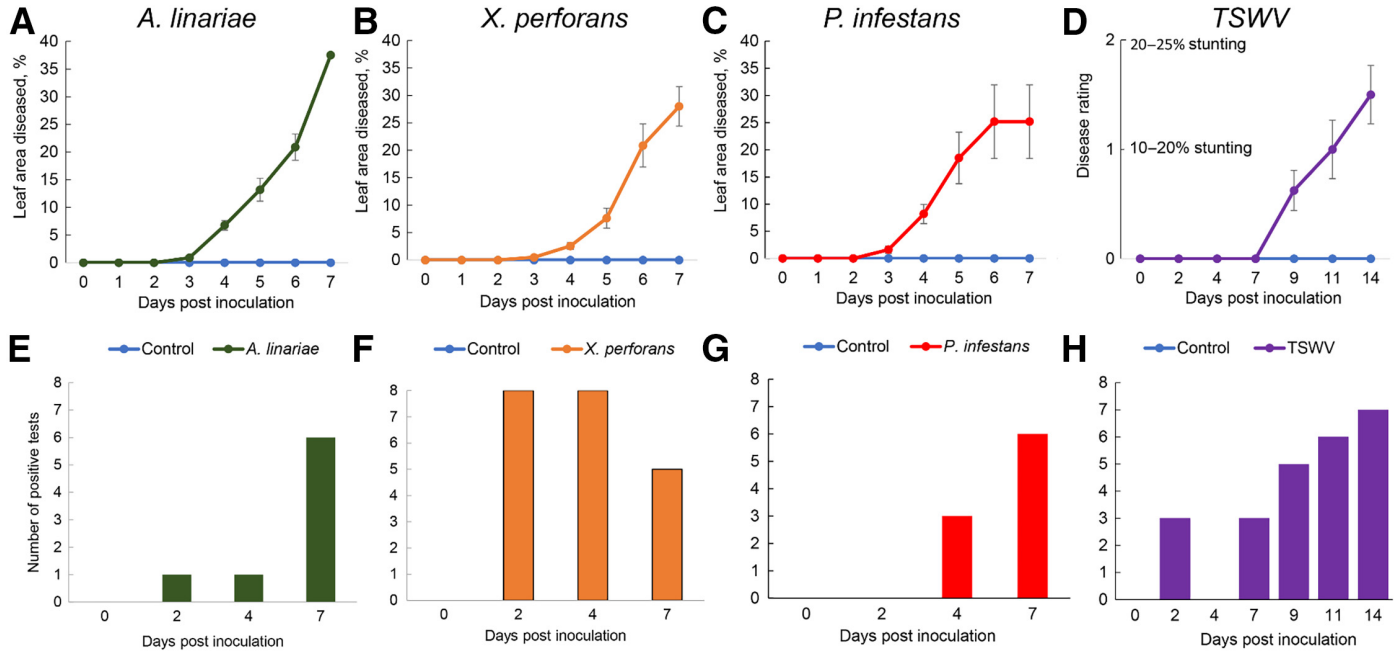

Fig. 4. Disease progress curves from tomato plant inoculation assays for **A,** *Alternaria linariae*; **B,** *Xanthomonas perforans*; **C,** *Phytophthora infestans*; and **D,** *Tomato spotted wilt virus* (TSWV). A to D show the mean disease severity ± SE of midpoints from each rating. Loop-mediated isothermal amplification (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.

Disease symptoms in *P. infestans*-inoculated tomato developed very rapidly (Supplementary Fig. S1). Symptoms were first observed 3 dai, and lesions reached up to 20 to 35% of total leaf area within the next 4 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 (Supplementary Fig. S1). 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 noninoculated leaves were negative.

Disease symptoms caused by TSWV were evaluated by the degree of stunting of the inoculated plants when compared with 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 (Supplementary Fig. S1), and five out of eight inoculated plants had 10 to 15% stunting compared with the control plants by 9 dai (Fig. 4D). Plant growth was reduced by 15 to 25% by 14 dai in seven of eight inoculated plants compared with the control plants, and the AUDPC was 71.1. TSWV was detected by real-time LAMP 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 real-time LAMP at 9, 11, and 14 dai, respectively, and the pathogen was detected in all seven plants with symptoms by 14 dai (Fig. 4H). The real-time LAMP assay detected TSWV as early as 2 days prior to visible symptoms. All reactions from NTCs and noninoculated plants were negative, indicating that 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 microfluidic slides on a heat block. We also detected *P. infestans* by running the LAMP assay in the microfluidic slides on a heating slide attached to an electric plug or 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 (Supplementary Fig. S7). Positive reactions displayed a solid green color (Supplementary Fig. S7A) and appeared white on the non-fluorescent original image (Supplementary Fig. S7B). 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 (Supplementary Table S6).

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 min 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 infect tomato. The LAMP assay we developed was initially targeted for *A. linariae*, but over the course of the study, we found that the LAMP assay also amplified two other important *Alternaria* species that are also known to cause early blight on tomato: *A. alternata* and *A. solani* (Adhikari et al. 2021). Although 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., Liu et al. 2022; Yang et al. 2019), to the best of our knowledge, no LAMP assay has been developed that detects all three. However, it should be noted that although 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. Although *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) and *X. gardneri* (Stehlíková et al. 2020, and, more recently, duplex LAMP for simultaneous detection of *X. euvesicatoria* and *X. vesicatoria* (Beran et al. 2023) were reported. In those reports, the authors tested their methods with BioRanger, a portable device that allows for real-time detection of two fluorophores. Like other LAMP assays for *Xanthomonas* spp. detection, our new assay has high specificity and sensitivity. Compared with a conventional PCR, our LAMP assay was 100 times more sensitive and allowed for detection to levels as low as 1 pg of target DNA. Moreover, our assay can detect *X. perforans* with 100% accuracy from presymptomatic 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* (2 mm 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 5 min 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 (Supplementary Fig. S3B). 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 the back

of a truck. However, further improvements to the slide heater on the smartphone 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 nontemplate 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. Although 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. 2018; 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 MN 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 smartphone. 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 North Carolina tomato fields.

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Literature Cited

Adhikari, T. B., Muzhinji, N., Halterman, D., and Louws, F. J. 2021. Genetic diversity and population structure of *Alternaria* species from tomato and potato in North Carolina and Wisconsin. Sci. Rep. 11:17024.

Anonymous. 2022. North Carolina Agricultural Statistics. 2022. North Carolina Department of Agriculture & Consumer Services.

Araújo, E. R., Costa, J. R., Ferreira, M. A. S. V., and Quezado-Duval, A. M. 2012. Simultaneous detection and identification of the *Xanthomonas* species complex associated with tomato bacterial spot using species-specific primers and multiplex PCR. J. Appl. Microbiol. 113:1479-1490.

Beran, P., Stehlikova, D., Cohen, S. P., Rost, M., Beranova, K., and Curn, V. 2023. Utilization of a new hundred-genomes pipeline to design a rapid duplex LAMP detection assay for *Xanthomonas euvesicatoria* and *X. vesicatoria* in tomato. Plant Dis. 107:1822-1828.

Brittain, I. B. 2018. Towards early automated detection of pre-symptomatic pathogen risk: Mitigating the impact of airborne fungal plant pathogens. PhD thesis. University of Nottingham, Nottingham, U.K.

Canton, H. 2021. Food and Agriculture Organization of the United Nations—FAO. Pages 297-305 in: The Europa Directory of International Organizations 2021. Routledge, London, U.K.

Clark, K. J., Anchieta, A. G., da Silva, M. B., Kandel, S. L., Choi, Y.-J., Martin, F. N., Correll, J. C., Van Denyze, A., Brummer, E. C., and Klosterman, S. J. 2022. Early detection of the spinach downy mildew pathogen in leaves by recombinase polymerase amplification. Plant Dis. 106:1793-1802.

Dai, T., Yang, X., Hu, T., Li, Z., Xu, Y., and Lu, C. 2019. A novel LAMP assay for the detection of *Phytophthora cinnamomi* utilizing a new target gene identified from genome sequences. Plant Dis. 103:3101-3107.

Debreczeni, D. E., Ruiz-Ruiz, S., Aramburu, J., López, C., Belliure, B., Galipienso, L., Soler, S., and Rubio, L. 2011. Detection, discrimination, and absolute quantitation of *Tomato spotted wilt virus* isolates using real-time RT-PCR with TaqMan®MGB probes. J. Virol. Methods 176:32-37.

de Mendiburu, F. 2021. agricolae: Statistical procedures for agricultural research. R package version 1.3-5. https://CRAN.R-project.org/package=agricolae

Edin, E. 2012. Species specific primers for identification of *Alternaria solani*, in combination with analysis of the F129L substitution associates with loss of sensitivity toward strobilurins. Crop Prot. 38:72-73.

Fenu, G., and Malloci, F. M. 2021. Forecasting plant and crop disease: An explorative study on current algorithms. Big Data Cogn. Comput. 5:2.

Fry, W. 2008. *Phytophthora infestans*: The plant (and *R* gene) destroyer. Mol. Plant Pathol. 9:385-402.

Gold, K. M., Townsend, P. A., Chlus, A., Herrmann, I., Couture, J. J., Larson, E. R., and Gevens, A. J. 2020. Hyperspectral measurements enable pre-symptomatic detection and differentiation of contrasting physiological effects of late blight and early blight in potato. Remote Sens. 12:286.

Gosavi, G., Yan, F., Ren, B., Kuang, Y., Yan, D., Zhou, X., and Zhou, H. 2020. Applications of CRISPR technology in studying plant-pathogen interactions: Overview and perspective. Phytopathol. Res. 2:21.

Hansen, Z. R., Knaus, B. J., Tabima, J. F., Press, C. M., Judelson, H. S., Grünwald, N. J., and Smart, C. D. 2016. Loop-mediated isothermal amplification for detection of the tomato and potato late blight pathogen, *Phytophthora infestans*. J. Appl. Microbiol. 120:1010-1020.

Khan, M., Li, B., Jiang, Y., Weng, Q., and Chen, Q. 2017. Evaluation of different PCR-based assays and LAMP method for rapid detection of *Phytophthora infestans* by targeting the *Ypt1* gene. Front. Microbiol. 8:01920.

Khan, M., Wang, R., Li, B., Liu, P., Weng, Q., and Chen, Q. 2018. Comparative evaluation of the LAMP assay and PCR-based assays for the rapid detection of *Alternaria solani*. Front. Microbiol. 9:02089.

Kumar, S., Singh, R., Kashyap, P. L., and Srivastava, A. K. 2013. Rapid detection and quantification of *Alternaria solani* in tomato. Sci. Hortic. 151:184-189.

Lahre, K., Shekasteband, R., Meadows, I., Whitfield, A. E., and Rotenberg, D. 2023. First report of resistance-breaking variants of tomato spotted wilt virus (TSWV) infecting tomatoes with the *Sw-5* tospovirus-resistance gene in North Carolina. Plant Dis. 107:2271.

Larrea-Sarmiento, A., Dhakal, U., Boluk, G., Fatdal, L., Alvarez, A., Strayer-Scherer, A., Paret, M., Jones, J., Jenkins, D., and Arif, M. 2018. Development of a genome-informed loop-mediated isothermal amplification assay for rapid and specific detection of *Xanthomonas euvesicatoria*. Sci. Rep. 8: 14298.

Lee, H.-J., Cho, I.-S., Ju, H.-J., and Jeong, R.-D. 2021. Rapid and visual detection of tomato spotted wilt virus using recombinase polymerase amplification combined with lateral flow strips. Mol. Cell. Probes 57:101727.

Lees, A. K., Roberts, D. M., Lynott, J., Sullivan, L., and Brierley, J. L. 2019. Real-time PCR and LAMP assays for the detection of spores of *Alternaria solani* and sporangia of *Phytophthora infestans* to inform disease risk forecasting. Plant Dis. 103:3172-3180.

Leiminger, J., Bäßler, E., Knappe, C., Bahnweg, G., and Hausladen, H. 2015. Quantification of disease progression of *Alternaria* spp. on potato using real-time PCR. Eur. J. Plant Pathol. 141:295-309.

Liu, B., Li, Z., Du, J., Zhang, W., Che, X., Zhang, Z., Chen, P., Wang, Y., Li, Y., Wang, S., and Ding, X. 2022. Loop-mediated isothermal amplification (LAMP) for the rapid and sensitive detection of *Alternaria alternata* (Fr.) Keissl in apple Alternaria blotch disease with *Aapg-1* encoding the endopolygalacturonase. Pathogens 11:1221.

May, K. J., and Ristaino, J. B. 2004. Identity of the mtDNA haplotype(s) of *Phytophthora infestans* in historical specimens from the Irish potato famine. Mycol. Res. 108:471-479.

Ocenar, J., Arizala, D., Boluk, G., Dhakal, U., Gunarathne, S., Paudel, S., Dobhal, S., and Arif, M. 2019. Development of a robust, field-deployable loop-mediated isothermal amplification (LAMP) assay for specific detection of potato pathogen *Dickeya dianthicola* targeting a unique genomic region. PLoS One 14:e0218868.

Paul, R., Ostermann, E., Chen, Y., Saville, A. C., Yang, Y., Gu, Z., Whitfield, A. E., Ristaino, J. B., and Wei, Q. 2021. Integrated microneedle-smartphone nucleic acid amplification platform for in-field diagnosis of plant diseases. Biosens. Bioelectron. 187:113312.

Paul, R., Saville, A. C., Hansel, J. C., Ye, Y., Ball, C., Williams, A., Chang, X., Chen, G., Gu, Z., Ristaino, J. B., and Wei, Q. 2019. Extraction of plant DNA by microneedle patch for rapid detection of plant diseases. ACS Nano 13:6540-6549.

R Core Team. 2022. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Riley, D., Sparks, A., Jr., Srinivasan, R., Kennedy, G., Fonsah, G., Scott, J., and Olson, S. 2018. Thrips: Biology, ecology, and management. Pages 49-71 in: Sustainable Management of Arthropod Pests of Tomato. W., Wakil, G. E., Brust, and T. M., Perring, eds. Academic Press, London, U.K.

Ristaino, J. B., Anderson, P. K., Bebber, D. P., Brauman, K. A., Cunniffe, N. J., Fedoroff, N. V., Finegold, C., Garrett, K. A., Gilligan, C. A., Jones, C. M., Martin, M. D., MacDonald, G. K., Neenan, P., Records, A., Schmale, D. G., Tateosian, L., and Wei, Q. 2021. The persistent threat of emerging plant disease pandemics to global food security. Proc. Natl. Acad. Sci. U.S.A. 118:e2022239118.

Ristaino, J. B., Saville, A. C., Paul, R., Cooper, D. C., and Wei, Q. 2020. Detection of *Phytophthora infestans* by loop-mediated isothermal amplification, real-time LAMP, and droplet digital PCR. Plant Dis. 104:708-716.

Roberts, C. A., Dietzgen, R. G., Heelan, L. A., and Maclean, D. J. 2000. Real-time RT-PCR fluorescent detection of tomato spotted wilt virus. J. Virol. Methods 88:1-8.

Saravitz, C. H., and Chiera, J. 2019. NCSU Phytotron procedural manual for controlled-environment research at the Southeastern Plant Environmental Laboratory. Tech. Bull. 244.

Savary, S., Willocquet, L., Pethybridge, S. J., Esker, P., McRoberts, N., and Nelson, A. 2019. The global burden of pathogens and pests on major food crops. Nat. Ecol. Evol. 3:430-439.

Saville, A., Graham, K., Grünwald, N. J., Myers, K., Fry, W. E., and Ristaino, J. B. 2015. Fungicide sensitivity of U.S. genotypes of *Phytophthora infestans* to six oomycete-targeted compounds. Plant Dis. 99: 659-666.

Shymanovich, T., Saville, A. C., Mohammad, N., Wei, Q., Rasmussen, D., Lahre, K. A., Rotenberg, D., Whitfield, A. E., and Ristaino, J. B. 2024. Disease progress and detection of a California resistance-breaking strain of tomato spotted wilt virus in tomato with LAMP and CRISPR-Cas12a assays. PhytoFrontiers 4:50-60.

Stehlíková, D., Beran, P., Cohen, S. P., and Čurn, V. 2020. Development of real-time and colorimetric loop mediated isothermal amplification assay for detection of *Xanthomonas gardneri*. Microorganisms 8:1301.

Strayer, A. L., Jeyaprakash, A., Minsavage, G. V., Timilsina, S., Vallad, G. E. Jones, J. B., and Paret, M. L. 2016. A multiplex real-time PCR assay differentiates four *Xanthomonas* species associated with bacterial spot of tomato. Plant Dis. 100:1660-1668.

Strayer-Scherer, A., Jones, J. B., and Paret, M. L. 2019. Recombinase polymerase amplification assay for field detection of tomato bacterial spot pathogens. Phytopathology 109:690-700.

Trout, C. L., Ristaino, J. B., Madritch, M., and Wangsomboondee, T. 1997. Rapid detection of *Phytophthora infestans* in late blight-infected potato and tomato using PCR. Plant Dis. 81:1042-1048.

Yang, X., Qi, Y.-J., Al-Attala, M. N., Gao, Z.-H., Yi, X.-K., Zhang, A.-F., Zang, H.-Y., Gu, C.-Y., Gao, T.-C., and Chen, Y. 2019. Rapid detection of *Alternaria* species involved in pear black spot using loop-mediated isothermal amplification. Plant Dis. 103:3002-3008.

Yu, J., Shen, D., Dai, T., Lu, X., Xu, H., and Dou, D. 2019. Rapid and equipment-free detection of *Phytophthora capsici* using lateral flow strip-based recombinase polymerase amplification assay. Lett. Appl. Microbiol. 69:64-70.

Zhang, W., Jiao, Y., Ding, C., Shen, L., Li, Y., Yu, Y., Huang, K., Li, B., Wang, F., and Yang, J. 2021. Rapid detection of tomato spotted wilt virus with Cas13a in tomato and *Frankliniella occidentalis*. Front. Microbiol. 12:745173.

Zhao, Y., Chen, F., Li, Q., Wang, L., and Fan, C. 2015. Isothermal amplification of nucleic acids. Chem. Rev. 115:12491-12545.

Zhou, C., Bucklew, V. G., Edwards, P. S., Zhang, C., Yang, J., Ryan, P. J., Hughes, D. P., Qu, X., and Liu, Z. 2023. Portable diffuse reflectance spectroscopy of potato leaves for pre-symptomatic detection of late blight disease. Appl. Spectrosc. 77:491-499.