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Rapid Detection of *Raffaelea lauricola* Directly from Host Plant and Beetle Vector Tissues Using Loop-Mediated Isothermal Amplification

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Abstract

Since its introduction in 2002, laurel wilt disease has devastated indigenous lauraceous species in the southeastern United States. The causal agent is a fungal pathogen, *Raffaelea lauricola*, which, after being introduced into the xylem of trees by its vector beetle, *Xyleborus glabratus*, results in a fatal vascular wilt. Rapid detection and accurate diagnosis of infections is paramount to the successful implementation of disease management strategies. Current management operations to prevent the spread of laurel wilt disease are largely delayed by time-consuming laboratory procedures to confirm the diagnosis. In order to greatly speed up the operations, we developed a loop-mediated isothermal amplification (LAMP) species-specific assay that targets the β-tubulin gene region of *R. lauricola*, and allows for the rapid detection of the pathogen directly from host plant and beetle tissues. The assay is capable of amplifying as little as 0.5 pg of fungal DNA and as few as 50 conidia. The assay is also capable of detecting *R. lauricola* directly from wood tissue of artificially inoculated redbay saplings as early as 10 and 12 days postinoculation, when testing high-quality and crude DNA extracts, respectively. Finally, crude DNA extracts of individual adult female *X. glabratus* beetles were assayed and the pathogen was detected from all specimens. This assay greatly reduces the time required to confirm a laurel wilt diagnosis and, because LAMP technology is well suited to provide point-of-care testing, it has the potential to expedite and facilitate implementation of management operations in response to disease outbreaks.

Keywords: crude DNA, detection rapid response, LAMP, laurel wilt disease, *Persea borbonia*, *Sassafras albidum*, early *Xyleborus glabratus*

Nonnative plant diseases have the ability to drastically alter the composition of forests worldwide, and early detection is key to implementing a rapid management response and contain their spread (Aglietti et al. 2019; Luchi et al. 2020). Laurel wilt disease is a deadly, invasive vascular disease afflicting all lauraceous species in the southeastern United States, including ecologically important forest species such as redbay (*Persea borbonia* (L.) Spreng) and sassafras (*Sassafras albidum* (Nutt.) Nees), the threatened pondberry (*Lindera melissifolia* (Walter) Blume) and the endangered pondspice (*Litsea aestivalis* (L.) Fernald), and the economically important crop avocado (*Persea americana*) (Fraedrich et al. 2008, 2011; Ploetz et al. 2017a). The causal agent of laurel wilt disease is *Raffaelea lauricola* (T. C. Harr., Fraedrich & Aghayeva), a mycangial fungus and nutritional symbiont of *Xyleborus glabratus* Eichoff, the redbay ambrosia beetle (Harrington et al. 2008). Both the fungus and the beetle vector are indigenous to southeastern Asia, where they are primarily secondary pests and are not known to cause a vascular wilt in healthy native hosts (Fraedrich et al. 2008; Harrington et al. 2011; Shih et al. 2018). In their nonnative range, however, they are readily capable of quickly infecting, and ultimately killing, healthy plants.

Since its first detection in 2002 near Savannah, GA, laurel wilt disease has spread rapidly through the southeastern coastal plain and beyond, causing the deaths of millions of trees (Hughes et al. 2017), with the potential to spread even further wherever susceptible lauraceous hosts can be found (Gramling 2010). The rapid spread of laurel wilt disease to redbay in Texas (Menard et al. 2016), sassafras in Arkansas (Olatinwo et al. 2016) and North Carolina (Mayfield et al. 2019), and, very recently, sassafras in Kentucky and Tennessee (Loyd et al. 2020) highlights the need for improved monitoring, rapid diagnostics, and aggressive management of this destructive, invasive disease. Additionally, laurel wilt disease is a direct threat to the commercial avocado industry in Florida, which is valued at $30 million annually (Pisani et al. 2015), and is a potential threat to avocado production in other areas such as Mexico and California. Avocado plantations in Myanmar have recently developed symptoms of laurel wilt disease, indicating the first instance of the disease outside of the southeastern United States (Ploetz et al. 2016).

The introduction of *R. lauricola* into the xylem elicits the abundant production of tyloses, which are ineffective for preventing the systemic movement of the fungus (Hughes et al. 2015; Inch et al. 2012). However, the combined effects of the breakdown of host cells, the presence of fungal spores, and the production of tyloses ultimately block the transport of water and nutrients from roots, resulting in the loss of hydraulic conductivity and turgor pressure in vascular tissue (Inch and Ploetz 2011; Pearce 1996). Symptoms of laurel wilt disease include leaf wilt, vivid brown to black sapwood discoloration, and, eventually, host death in as fast as a few months (Fraedrich et al. 2008). These symptoms, however, are fairly common to wilt diseases and abiotic stressors (Dimond 1970) and, thus, a more definitive verification in addition to a visual diagnosis is required.

Currently, laurel wilt disease is managed through detection of symptomatic host plants, laboratory confirmation of the diagnosis, and removal of infected material, as well as through monitoring of the beetle vector and movement restrictions for wood products (Dreaden et al. 2014b; Hughes et al. 2015). Early detection of the disease is a crucial step for its successful management (National Invasive Species Council 2016) but current strategies relying on morphological and molecular confirmation of the diagnosis are time consuming. In particular, the confirmation of laurel wilt disease relies on external laboratories and time-intensive procedures: specifically, isolation of a pure culture of the fungus, which can take up to a week, followed by

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DNA extraction and species-specific PCR amplification (Dreaden et al. 2014b). Moreover, no PCR-based molecular method is available to reliably confirm the presence of *R. lauricola* directly in the host tissues, emphasizing the need for alternative approaches that can reliably and rapidly detect *R. lauricola*, possibly directly in the field, from plant tissues and insect vectors.

Loop-mediated isothermal amplification (LAMP) is a molecular technique that facilitates rapid detection of target DNA sequences (Notomi et al. 2000) in an hour or less compared with days or weeks associated with conventional laboratory confirmation (Le and Vu 2017). LAMP utilizes multiple primers that bind to distinct sections of target DNA, making the assay highly specific and sensitive (Notomi et al. 2000). Furthermore, in contrast to conventional PCR, its polymerase enzyme is less sensitive to reaction inhibitors, allowing for the use of a crude DNA template (Kaneko et al. 2007; Kogovšek et al. 2015; Poon et al. 2006). LAMP reactions are isothermal, thereby removing the requirement for a bulky, laboratory-bound, power-intensive thermocycler (Notomi et al. 2000) and allowing for the use of small, field-portable instrumentation (Niessen 2015); recent examples of portable PCR thermocyclers were reported

Table 1. Fungal isolates used in this study to validate the specificity of the loop-mediated isothermal amplification (LAMP) primers

Species	Isolation host	Original location	Collector	Isolate name	Year[a]	LAMP result
Raffaelea lauricola	*Lindera benzoin*	South Carolina	S. Fraedrich	FS-0017	2007	+
	Persea borbonia	South Carolina	S. Fraedrich	FS-0001	2004	+
	…	Texas	S. Fraedrich	FS-0002	2014	+
	…	South Carolina	S. Fraedrich	FS-0008	2004	+
	…	South Carolina	S. Fraedrich	FS-0009	2007	+
	…	South Carolina	S. Fraedrich	FS-0010	2007	+
	…	Mississippi	S. Fraedrich	FS-0011	2015	+
	…	Texas	S. Fraedrich	FS-0015	2014	+
	…	Texas	S. Fraedrich	FS-0016	2014	+
	…	Georgia	S. Fraedrich	FS-0018	2007	+
	…	Georgia	S. Fraedrich	FS-0019	2007	+
	…	Georgia	S. Fraedrich	FS-0020	2007	+
	…	Georgia	S. Fraedrich	FS-0021	2007	+
	…	Georgia	T. Harrington	C2246	2005	+
	…	Georgia	T. Harrington	C2245	2005	+
	…	Georgia	T. Harrington	C4073	…	+
	…	Georgia	T. Harrington	C2208	…	+
	Sassafras albidum	Alabama	S. Fraedrich	FS-0003	2015	+
	…	Georgia	S. Fraedrich	FS-0012	2016	+
	…	Georgia	S. Fraedrich	FS-0013	2016	+
	…	Georgia	S. Fraedrich	FS-0014	2014	+
	…	North Carolina	S. Fraedrich	CV2018003	2018	+
	…	North Carolina	S. Fraedrich	CV2018004	2018	+
	…	Alabama	T. Harrington	C2953	2012	+
	…	Georgia	T. Harrington	C2212	…	+
	Xyleborus glabratus	South Carolina	S. Fraedrich	FS-0004	2007	+
	…	South Carolina	S. Fraedrich	FS-0005	2007	+
	…	South Carolina	S. Fraedrich	FS-0006	2007	+
	…	South Carolina	S. Fraedrich	FS-0007	2007	+
R. aguacate	…	Florida	J. Smith/T. Dreaden	PL1004	…	–
R. albimanens	*Platypus externedentatus*	South Africa	T. Harrington	C2223	1969	–
R. amasae	*Amasa concitatus*	Taiwan	T. Harrington	C2750	2000	–
R. ambrosiae	*P. cylindrus*	England	T. Harrington	C2225	1963	–
R. arxii	*X. volvulus*	Florida	D. Carrillo/L. Cruz	Ph 24-3 MEA	2015	–
R. brunnea	…	Florida	J. Hulcr	CV2018026	2018	–
R. canadensis	*P. wilsonii*	Canada	T. Harrington	C2233	1966	–
R. ellipticospora	*X. glabratus*	South Carolina	T. Harrington	C2395	…	–
R. fusca	…	Florida	D. Carrillo/L. Cruz	AH3-M2	2016	–
R. gnathotrichi	*Gnathotrichus retusus*	Colorado	T. Harrington	C2219	1965	–
R. montetyi	*P. cylindrus*	France	T. Harrington	C2221	1993	–
R. quercivora	*P. quercivorus*	Japan	T. Harrington	C2526	…	–
R. santoroi	*P. mutatus*	Argentina	T. Harrington	C2748	1966	–
R. sp. PL1001	*X. volvulus*	Florida	D. Carrillo/L. Cruz	Ph 24-2 MEA	2015	–
R. subalba	*X. bispinatus*	Florida	D. Carrillo/L. Cruz	AFH M1	2016	–
	X. volvulus	Florida	D. Carrillo/L. Cruz	A2-1 P	2016	–
R. subfusca	*X. bispinatus*	Florida	D. Carrillo/L. Cruz	A M2 G	2016	–
	X. volvulus	Florida	D. Carrillo/L. Cruz	A3-1M	2016	–
R. sulcati	*G. sulcatus*	Canada	T. Harrington	C2234	1970	–
R. sulphurea	*Xyleborinus saxeseni*	Kansas	T. Harrington	C593	1967	–
R. tritirachium	*Quercus* sp.	Pennsylvania	T. Harrington	C2222	…	–
R. xyleborina	*Xyleborus glabratus*	Florida	D. Carrillo/L. Cruz	R. PL6099	2016	–
Leptographium profanum	*Hylobius pales*	Georgia	M. Buland	CV2017305	2017	–
Ophiostoma ips	*H. porculus*	Georgia	M. Buland	CV2017308	2017	–
Nigrospora oryzae	*Pinus taeda*	Georgia	A. Niyas	CV2018018	2017	–
Phytophthora cinnamomi	*Castanea dentata*	Georgia	A. Niyas	CV2017095	2018	–

[a] Year of isolation.

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by Hole and Nfon (2019) and Thomas et al. (2019). Finally, LAMP is also rapid, being capable of amplifying target DNA to detectable levels in as little time as 20 min (Villari et al. 2017), and visualization of results can be performed in real time using fluorescent assimilating probes (Jenkins et al. 2011; Kubota et al. 2011). LAMP is commonly used for point-of-care testing and rapid diagnosis confirmation because of its portability and quick and accurate diagnostic capabilities (Niessen 2015). Previous research has shown that LAMP is capable of detecting fungal pathogens under a wide range of conditions in both clinical and agricultural settings (Endo et al. 2004; King et al. 2018; Niessen and Vogel 2010; Villari et al. 2017). However, the technique has only recently been used in forestry settings to detect pathogens directly from host plant tissue (Aglietti et al. 2019; Dai et al. 2019; Sillo et al. 2018) and insect vectors (Villari et al. 2013), and to detect phytoparasitic nematodes (Meng et al. 2018).

The aim of this study was to develop a LAMP assay for the rapid and accurate molecular detection of the laurel wilt disease pathogen directly from host tissues. Specifically, our objectives were to (i) develop and validate an *R. lauricola* species-specific LAMP assay, (ii) determine the earliest time after host infection that the assay can detect *R. lauricola* in host sapwood, and (iii) test the assay's suitability for amplification of the target species directly within host plant tissue using both high-quality and crude DNA, and from beetle tissues using crude DNA.

Materials and Methods

Outline of the experiment. To fulfill objective (i), the LAMP assay was first developed and validated using high-quality DNA from pure fungal isolates. To fulfill objectives (ii) and (iii), a time-course experiment with artificially inoculated redbay saplings was performed, and plant tissues collected from the experiment were tested with the LAMP assay using both high-quality and crude DNA extracts. In addition, to fulfill objective (iii), crude extracts from female *X. glabratus* specimens were tested.

Fungal isolates and DNA extraction. Fungal isolates used for the development and validation of the LAMP assay are listed in Table 1. Different types of extraction procedures were used for the different components of the study. High-quality DNA from both fungal mycelia and plant tissues was extracted using the Qiagen DNeasy plant mini kit (Qiagen, Germantown, MD, U.S.A.) as per the manufacturer's protocol. Mycelia of the pure fungal isolates were collected from fresh cultures grown on potato dextrose agar (Becton, Dickinson and Company, Sparks, MD, U.S.A.) plates covered with a sterile cellophane disk. All samples were ground in liquid nitrogen prior to DNA extraction. Presence of DNA in each sample was assessed by gel electrophoresis, and approximate DNA concentrations were quantified using a Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.).

To extract crude DNA from plant tissues, a flame-sterilized scalpel was used to shave off 15 to 20 mg of wood tissue after debarking, which was then placed into a 1.5-ml microcentrifuge tube along with 300 µl of prepared 5% Chelex 100 solution (Bio-Rad, Hercules, CA, U.S.A.). To extract crude DNA from *X. glabratus*, whole beetles were manually ground with a sterile micropestle in a 1.5-ml microcentrifuge tube with 50 µl of 5% Chelex 100 solution. Crude extraction samples were placed into a floating tube rack, then boiled for 5 min in a water-filled beaker placed on a hot plate, vortexed for 15 s, boiled for an additional 5 min, and vortexed again for 15 s. Before testing, which occurred on the same day as extraction, samples were centrifuged at 3,884 relative centrigual force in a minicentrifuge (VWR, Radnor, PA, U.S.A.) for 30 s and only the supernatant was used in the LAMP reaction. Chelex is an ion-exchange resin that chelates cations such as magnesium ions, which are cofactors for DNA degradative enzymes, thus protecting DNA from degradation and reducing contaminants, and it is commonly used for simple DNA extraction protocols (Walsh et al. 1991). Q:2

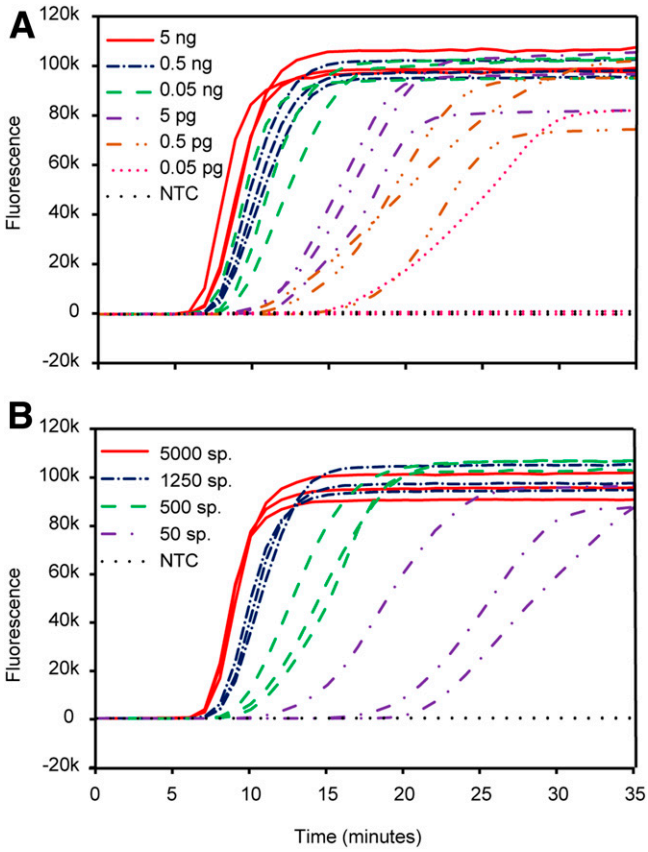

Fig. 1. Sensitivity test of the β-tubulin loop-mediated isothermal amplification assay using **A,** high-quality DNA of *Raffaelea lauricola* mycelium and **B,** crude DNA of *R. lauricola* conidia suspensions. Each reaction was run in three replicates. NTC = no-template control and sp. = spores.

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Table 2. Loop-mediated isothermal amplification (LAMP) primer set and probes used for the detection of *Raffaelea lauricola*

Primer or probe	Sequence (5′–3′)
LAMP primer set[a]	
BT-F3	GTGT ATGT GTCC CTGC TGAA
BT-B3	GCTC GAGA TCGA CGAG GA
BT-FIP	CTCA TGCG CTCA AGCT GGAG CTGC CTTC TCTA ACGT GATG C
BT-BIP	CCAG CCAC TGGG CAAA GTGA AACG TACT TGTT GCCA GAGG
BT-F Loop	CCGA CGTG CCAT TGTA CCT
Assimilating probe[b]	
BT-FAM, fluorescent strand	FAM-ACGC TGAG GACC CGGA TGCG AATG CGGA TGCG GATG CCGA TACC AAAA ACGA GCAA GCAT CT
BT-Q, quench strand	TCGG CATC CGCA TCCG CATT CGCA TCCG GGTC CTCA GCGT-BHQ

[a] Primers are designed against the β-tubulin (BT) gene region.
[b] Assimilating probe was designed as per Kubota et al. (2011). Underlined fragment of BT-FAM acts as backward loop primer. FAM = 6-carboxyfluorescein and BHQ = Black Hole Quencher-1 (Biosearch Technologies, Novato, CA, U.S.A.).

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Development of an *R. lauricola* species-specific LAMP assay. Two different LAMP primer sets were designed, separately targeting the *R. lauricola* β-tubulin (BT) gene region (GenBank accession number KJ909302) (Dreaden et al. 2014a) and the CHK microsatellite region (GenBank accession number KF381410), the latter of which is currently used for PCR-based identification of *R. lauricola* (Dreaden et al. 2014b). BLAST analysis of the BT gene sequences from *R. lauricola* was performed using the NCBI database (Zhang et al. 2000). The sequences of the 70 most similar species in terms of E-value and percent identity, which included all available *Raffaelea* spp. and other Ophiostomataceae species, were aligned together with the host species sequences in order to select an appropriately divergent region of the *R. lauricola* BT gene for primer design. Sequence alignment was performed using ClustalW in MEGA version 7 software (Kumar et al. 2016). The CHK microsatellite is taxon specific to *R. lauricola* (Dreaden et al. 2014b). Primers were then designed using PrimerExplorer (v. 4.0: Eiken Chemical Co., Tokyo, Japan), as per Notomi et al. (2000) and Nagamine et al. (2002), and a 6-carboxy-fluorescein (FAM) fluorescent assimilating probe and associated quencher were designed against the backward loop of the amplicon, as per Kubota et al. (2011). However, preliminary testing showed that the CHK primers cross-reacted with redbay host DNA, and were excluded from further analysis. Primers, probes, and quencher were synthesized by Integrated DNA Technologies, Inc. (IDT, Coralville, Iowa, U.S.A.). Sequences of the BT primer set are reported in Table 2.

LAMP reactions were performed as per Villari et al. (2017), with the addition of a loop primer to speed up the reaction. Loop primers are complementary to single-stranded loop regions and facilitate additional binding sites for the polymerase, thus increasing the speed of the reaction (Nagamine et al. 2002). Each 25-μl reaction contained 15 μl of 1× no-dye Isothermal Master Mix (Optigene, Horsham, U.K.), 2.8 μM internal primers BT-FIP and BT-BIP, 0.28 μM external primers BT-F3 and BT-B3, 0.8 μM F-Loop primer, 0.092 μM assimilating probe fluorescent (FAM) strand, 0.184 μM quencher strand, 1.3 μl of molecular-grade water (Thermo Fisher Scientific), and 5 μl of template. Reactions were carried out in 0.2-ml optically clear PCR eight-tube strips (Thermo Fisher Scientific) on a StepOnePlus Real-Time PCR system (Thermo Fisher Scientific) and monitored in real time. Reactions were programmed with the following conditions: 65°C for 60 1-min cycles with fluorescence reading every minute, followed by a denaturing step at 85°C for 5 min to halt the reaction and deactivate the polymerase. Each sample was tested in triplicate and a no-template control was included in each run, while high-quality DNA from a known *R. lauricola* isolate (i.e., FS-0001, FS-0004, or FS-0006 to FS-0017) was used as a positive control. A result was considered positive if two of three replicates resulted in amplification.

Table 3. Results of the time-course experiment

Sample ID[a]	dpi[b]	Presence of leaf wilt	Presence of wood discoloration	Positive fungal isolation	LAMP with high-quality DNA[c]	LAMP with Crude DNA[c]
R1	2	–	–	–	0/3	0/3
R2	…	–	–	–	0/3	0/3
R3	…	–	–	–	0/3	0/3
C1	…	–	–	–	0/3	0/3
C2	…	–	–	–	0/3	0/3
C3	…	–	–	–	0/3	0/3
R4	4	–	–	+	0/3	0/3
R5	…	–	–	+	0/3	0/3
R6	…	–	–	–	0/3	0/3
C4	…	–	–	–	0/3	0/3
C5	…	–	–	–	0/3	0/3
C6	…	–	–	–	0/3	0/3
R7	6	–	–	+	0/3	0/3
R8	…	–	–	–	0/3	0/3
R9	…	–	–	–	0/3	0/3
C7	…	–	–	–	0/3	0/3
C8	…	–	–	–	0/3	0/3
C9	…	–	–	–	0/3	0/3
R10	8	–	–	+	0/3	0/3
R11	…	–	–	+	0/3	0/3
R12	…	–	–	–	0/3	0/3
C10	…	–	–	–	0/3	0/3
C11	…	–	–	–	0/3	0/3
C12	…	–	–	–	0/3	0/3
R13	10	–	–	+	1/3	0/3
R14	…	–	–	+	**2/3**	0/3
R15	…	–	–	+	**2/3**	0/3
C13	…	–	–	–	0/3	0/3
C14	…	–	–	–	0/3	0/3
C15	…	–	–	–	0/3	0/3
R16	12	–	+	+	**3/3**	**3/3**
R17	…	–	+	+	**3/3**	**2/3**
R18	…	+	+	+	**3/3**	**3/3**
C16	…	–	–	–	0/3	0/3
C17	…	–	–	–	0/3	0/3
C18	…	–	–	–	0/3	0/3

[a] Sample names starting with R indicate inoculated samples and sample names starting with C indicate control samples.
[b] Days postinoculation.
[c] Loop-mediated isothermal amplification (LAMP) results are reported as number of positive reactions of three technical replicates. Positive observations are highlighted in bold.

After verifying that the BT primer set was not cross-reacting with host plant DNA, specificity of the LAMP assay was tested with high-quality DNA extracted from the isolates described in Table 1. Assay sensitivity was determined by testing a 1-ng to 0.01-pg serial dilution of *R. lauricola* isolate FS-0009 DNA. Additionally, to estimate a more biologically relevant sensitivity threshold, dilutions of *R. lauricola* spore suspensions were tested. Spores of isolate FS-0001 were collected by covering the surface of plates of colonized malt extract agar (VWR) amended with cycloheximide at 200 ppm (Sigma-Aldrich, St. Louis, MO, U.S.A.) and streptomycin at 100 ppm (Sigma-Aldrich) (CSMA) (Harrington and Fraedrich 2010) with 10 ml of molecular-grade water, and gently scraping the agar surface and mycelium with a sterilely gloved finger or a sterile glass spreader. The suspension was then collected and filtered through sterile cheesecloth. Conidia concentration was estimated under magnification using a hemacytometer (Thermo Fisher Scientific) and adjusted to 1,000, 250, 100, and 10 conidia/µl. Spore suspension dilutions were then boiled for 5 min and vortexed for 15 s prior to testing with the LAMP assay as previously described.

Time-course experiment. To determine the earliest time after host infection that the LAMP assay is capable of detecting *R. lauricola* within plant tissues, 36 redbay saplings (2 to 3 years old) were placed in a walk-in growth chamber (Conviron Model GR48; Controlled Environments Ltd., Winnipeg, Manitoba, Canada) in August 2018. Saplings were grown at 25°C on a day and night cycle of 14 and 10 h, respectively, at ambient humidity for 2 weeks before being subjected to the following treatments: 18 saplings (average height 117.78 ± 4.04 cm standard error; diameter 12.28 ± 0.41 mm standard error) were randomly selected to receive an *R. lauricola* spore suspension, while the remaining 18 were used as negative controls (height 116.89 ± 2.97 cm standard error; diameter 12.79 ± 0.35 mm standard error). Two holes at a 45° inclination were drilled with a 2.5-mm flame-sterilized bit into the opposite sides of each sapling's stem, 5 to 7 cm above the soil line. Each drilled hole was inoculated with 10 µl (20 µl per plant) of either spore suspension or sterile water, before being wrapped in Parafilm (Bemis Company, Inc., Neenah, WI, U.S.A.). The spore suspension was prepared following the procedure previously described, and diluted to 5,000 conidia/µl so that treatment saplings received, in total, 100,000 conidia. Beginning 2 days after inoculation and continuing at 2-day intervals for up to 12 days after inoculation, three inoculated and three control saplings were randomly selected and destructively sampled by obtaining a 10-cm stem segment at 50 cm above the inoculation point using flame-sterilized pruning shears. Saplings were also visually inspected for the presence of wilting or vascular streaking at each sampling time. Collected stem segments were stored at −20°C until extraction and testing. We initially planned to include sampling and analysis of leaf samples as well but preliminary testing showed inconsistency in the results; hence, leaves were dropped from further testing. An additional 10-cm stem segment was also taken at 60 cm above the inoculation point to confirm colonization by *R. lauricola* via plating, as described below. High-quality DNA was extracted from each stem segment with a Qiagen DNeasy plant mini kit and crude DNA extracts were obtained with the 5% Chelex 100 extraction method as previously described. LAMP assays were performed as previously described.

Processing of the samples to determine *R. lauricola* colonization of stems consisted of debarking samples, rinsing with 95% EtOH, flame sterilization, and plating onto CSMA plates. Plates were then wrapped in Parafilm and incubated at 25°C on a day and night cycle of 14 and 10 h, respectively, in a Precision Model FU019ARW2 Dual Program Illuminated Incubator (Thermo Fisher Scientific) until *R. lauricola* growth was observed or a minimum of 2 weeks had passed.

Capability of the LAMP assay to detect *R. lauricola* DNA in *X. glabratus* beetles. To test the capability of the LAMP assay to detect *R. lauricola* directly from the insect vector, 11 female *X. glabratus* beetles were collected from infected sassafras bolts at the Conway Cemetery Historic State park in Lafayette County, AR and assayed individually. Each beetle was stored separately in a 1.5-ml centrifuge tube and kept at −20°C until extraction. Crude DNA extracts from beetles were obtained with the 5% Chelex 100 extraction method and LAMP was performed as previously described, with the exception that only 1 µl was used in the reaction to avoid excessive background noise.

Results

Specificity of BT LAMP assay to *R. lauricola*. The BT LAMP assay tested positive for all *R. lauricola* isolates evaluated from both beetles and plants, regardless of their geographic origin (Table 1). No amplification was observed when the assay was tested against 20 other *Raffaelea* spp., more distantly related fungal and oomycete species (Table 1), or redbay host DNA.

Sensitivity of BT LAMP assay. Using serial dilutions of *R. lauricola* DNA (Fig. 1A), all technical replicates amplified within 16 min at levels as low as 0.5 pg of DNA. Only one of three technical replicates amplified with 0.05 pg of DNA. When tested with *R. lauricola* spore suspensions (Fig. 1B), all technical replicates with 500 to 5,000 conidia amplified within 12 min, and those with 50 conidia amplified within 20 min.

Time-course experiment. The LAMP assay detected *R. lauricola* in redbay xylem as early as 10 days postinoculation (dpi), with all technical replicates for two of three samples testing positive using high-quality DNA (Table 3). However, no amplification was observed in 10 dpi samples using crude DNA (Table 3). By 12 dpi, the LAMP assay successfully amplified all three inoculated replicate saplings with both high-quality (Fig. 2A) and crude (Fig. 2B) DNA extracts. In the test with crude DNA extracts, amplification was delayed by approximately 10 min for the majority of the reactions but all successful reactions occurred within 40 min (Fig. 2B). For one of the samples (i.e., R17), only two of three technical replicates

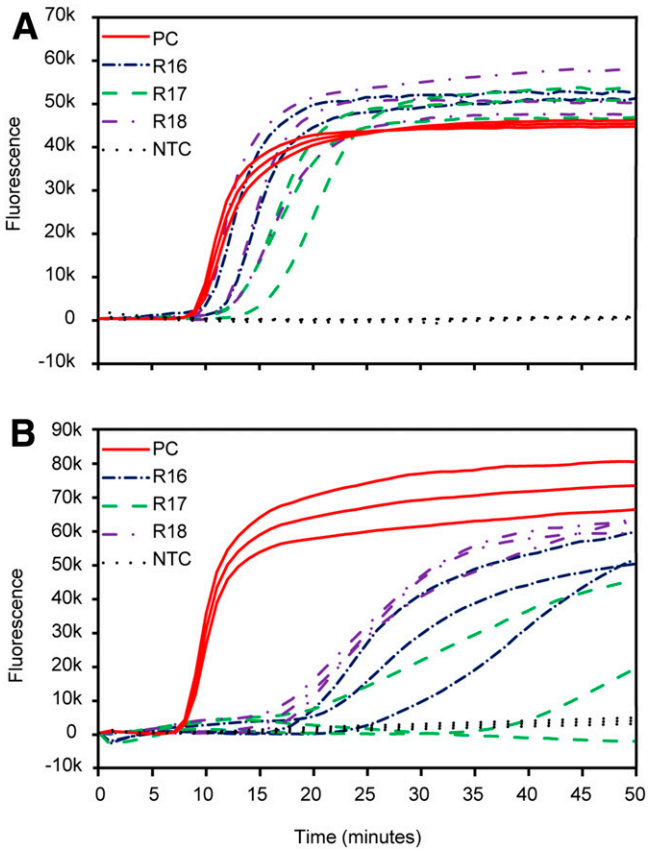

Fig. 2. Results of the β-tubulin loop-mediated isothermal amplification assay on redbay saplings inoculated with *Raffaelea lauricola* and sampled 12 days postinoculation. Template of the reaction was either **A,** high-quality kit-extracted DNA or **B,** 5% Chelex crude extracted DNA. Each reaction was run in three replicates. PC = positive control and NTC = no-template control.

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successfully amplified. No amplification was observed for any sample earlier than 10 dpi. All control samples tested negative for both high-quality or crude DNA extracts.

Full crown wilt was not observed in any of the saplings inoculated with *R. lauricola* for the duration of the experiment. External symptoms of laurel wilt disease were first evident at 12 dpi but were observed in only one of the three inoculated saplings, whose leaves had just begun to wilt and discolor (Table 3). At this time, all inoculated saplings exhibited internal symptoms at the 50-cm sampling height that appeared primarily as a faint sapwood discoloration (Table 3; Fig. 3). All control samples remained asymptomatic and showed no evidence of either wilting or vascular discoloration.

In inoculated redbay saplings, *R. lauricola* was recovered from samples collected 60 cm above the inoculation point as early as 4 dpi (Table 3). *R. lauricola* was not recovered from any control saplings.

Detecting *R. lauricola* DNA in *X. glabratus* beetles. Amplification was observed for all technical replicates from all 11 female *X. glabratus* adult beetles tested with the LAMP assay. Amplification occurred in less than 10 min when using crude DNA extracts from beetles and was only delayed approximately 2 min compared with a high-quality fungal DNA positive control (Fig. 4).

Discussion

This study describes the development of the first LAMP assay for the detection of *R. lauricola*, the causal agent of laurel wilt disease, directly form host plant tissues and using crude DNA extracts. The BT LAMP assay is species specific and capable of detecting *R. lauricola* DNA regardless of its origin, showing no cross-reaction with other closely related *Raffaelea* spp. or redbay host DNA. The assay is sensitive and capable of detecting target DNA at levels as low as 0.5 pg in less than 16 min, and fungal conidia at levels as low as 50 conidia within 20 min. Moreover, the BT LAMP assay can successfully detect *R. lauricola* associated with *X. glabratus* females with minimal DNA extraction processing and reaction times of less than 10 min. The ability of the BT assay to detect as little as 50 conidia is well within the range typically found within a single *X. glabratus* female, which may harbor between 1,000 and 30,000 conidia (Harrington and Fraedrich 2010). This potentially makes the assay useful for laurel wilt disease pathogen detection from other ambrosia beetle species which are known to harbor *R. lauricola* but at much lower quantities such as *X. bispinatus* (Ploetz et al. 2017b). Additionally, thanks to the capability of detecting *R. lauricola* directly from the beetle vector, the assay provides an alternative method to confirm the presence of the pathogen in an area (i.e., by testing trapped insects). This could be particularly useful in those instances when *X. glabratus* beetles are trapped during routine monitoring but no symptomatic trees have been found.

The ability to detect *R. lauricola* within host plant tissues is crucial for the rapid detection of laurel wilt disease. Similarly, developing a crude DNA extraction protocol to enable direct testing of host tissue samples with LAMP assays is a major step for "in-field" diagnosis. Our results show that the assay is capable of detecting the pathogen 50 cm above the inoculation point in artificially inoculated saplings as soon as 10 dpi if using high-quality DNA and as early as 12 dpi if

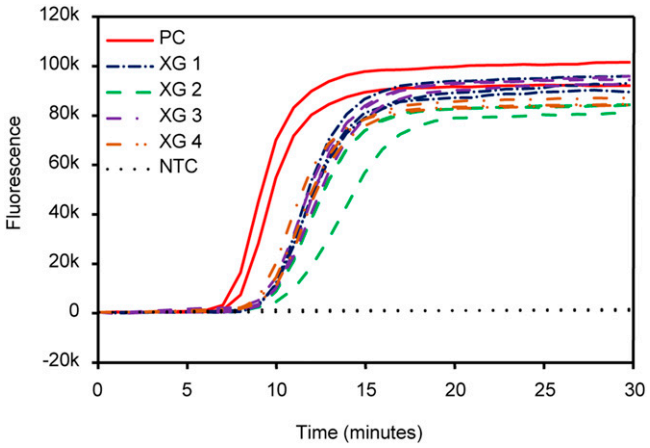

Fig. 4. Results of a β-tubulin loop-mediated isothermal amplification assay test on four adult *Xyleborus glabratus* female beetles. Each beetle was tested individually. PC = positive control, XG = *X. glabratus*, and NTC = no-template control.

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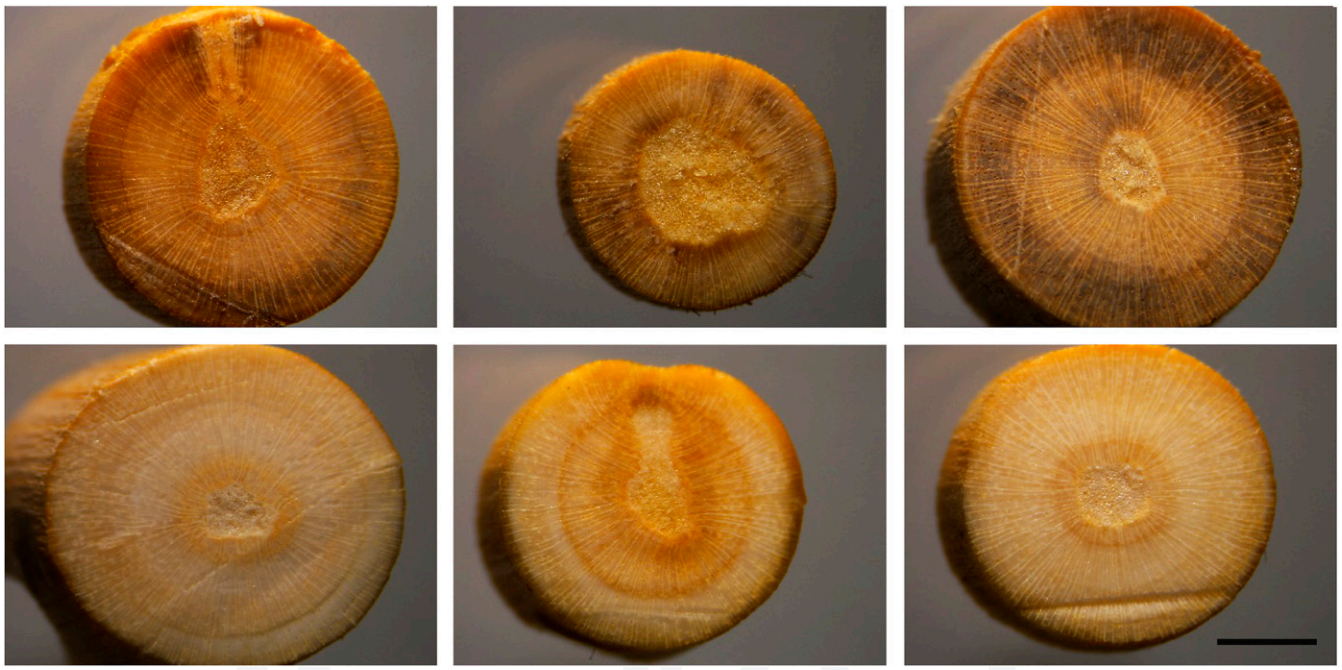

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Fig. 3. Redbay stem sections 12 days postinoculation with either *Raffaelea lauricola* (top row; three different biological replicates) or sterile water (bottom row; three different biological replicates), sampled 50 cm above the inoculation point. Inoculated samples exhibit a faint discoloration of the wood, while control samples show no discoloration. Scale bar = 2 mm.

Proof Only

using crude DNA, which coincides with the development of the first external and internal symptoms in plants. It is notable that our assay was capable of detecting *R. lauricola* in two experimental saplings that had yet to exhibit external symptoms of laurel wilt disease. This is particularly relevant given that, in disease monitoring operations, field personnel will most likely sample wilting or otherwise externally symptomatic trees, hence minimizing the risk of false negatives. Additional experimentation will be required to determine whether the assay can detect *R. lauricola* before the appearance of visual symptoms in larger, more mature trees, or if other tissue types are appropriate for testing in more advanced cases of the disease.

When using crude DNA extracts, amplification commenced slightly later compared with high-quality DNA extracts. However, the delayed response time associated with the rapid, crude DNA extraction protocol is inconsequential when compared with the time required to culture *R. lauricola* and perform a high-quality DNA extraction from pure-culture mycelium for a traditional PCR-based confirmation. Because the reaction efficiency decreases when using crude DNA extracts or when tests are performed near the lower limit of detection, we suggest that all reactions be run in triplicate, and that a sample be considered positive only when at least two of the three technical replicates test positive (Villari et al. 2013, 2017).

Previous research in this disease system has shown that *R. lauricola* can be recovered from infected wood tissue at various stem heights several days after laboratory inoculations (Fraedrich et al. 2015). In our time-course experiment, we were able to reisolate *R. lauricola* at 60 cm above the inoculation point as soon as 4 dpi, which is 6 days before we could detect the pathogen with the LAMP assay. It should be noted, however, that the number of reproductively viable units (i.e., conidia) needed to successfully culture *R. lauricola* from infected wood (in theory, as low as one viable spore) is significantly less than the number of spores that were detectable with the LAMP assay (approximately 50).

The current standard procedure to process a suspected laurel wilt disease sample and provide a confirmation of the diagnosis relies on an external laboratory performing fungal isolations from symptomatic tissues followed by DNA extraction and traditional PCR (Hughes et al. 2015). This laboratory-based process can provide a response in approximately 1 week (Dreaden et al. 2014b). However, because the *R. lauricola*-specific PCR primers used in the traditional assay do not produce consistent amplification directly from diseased wood tissues (Dreaden et al. 2014b), the time required for diagnosis confirmation can often be extended beyond a week when there are difficulties isolating the pathogen. The LAMP assay developed in this study addresses both the time constraint and the issue of testing directly from the host plant or insect vector while maintaining high specificity and sensitivity. This demonstrates that the time required to confirm laurel wilt disease can be significantly reduced, speeding up the implementation of management strategies that aim to slow the spread of laurel wilt disease.

Future work will focus on the validation of the LAMP assay for field implementation by utilization of portable LAMP devices. The use of portable devices is crucial in the field of forestry where remote distances, large areas, and finite laboratory capabilities limit the utilization of traditional techniques. Additionally, as an alternative to the use of fluorescent probes and, hence, the need for portable LAMP devices, further work could also investigate the possibility of using colorimetric methods for the visualization of the LAMP reaction results, as described by Goto et al. (2009), even though the pH variability in plant tissue crude extracts might limit their applicability. Finally, efforts should be directed toward possible strategies to reduce the risk of cross contamination, which is particularly high in LAMP reactions due to both the high sensitivity of the technique and the stability of amplification products. For instance, reaction visualization methods requiring the opening of the vials after the reaction should be avoided, while the use of preassembled reaction mixtures, which limit the handling of the reagents, should be favored when available.

The implementation of the LAMP assay developed in this study would enable forest and orchard managers to confirm the presence of laurel wilt disease directly in-field in as little as an hour compared with a week or more required for existing procedures. This assay facilitates the recently proposed framework for effective early warning and rapid response to successfully mitigate the impact of invasive pathogens of forest ecosystems (Aglietti et al. 2019; Luchi et al. 2020).

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