Development of a recombinase polymerase amplification assay with qualitative end-point detection for *Geosmithia morbida*, the causal agent of thousand cankers disease in walnut

By

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ABSTRACT

Geosmithia morbida, the fungus that causes thousand cankers disease, forms localized cankers on the trunk and branches of various walnut species following inoculation of spores carried by the phloem-feeding walnut twig beetle (*Pityophthorus juglandis*). Diagnosis currently requires expertise in fungal isolation and morphological identification or laboratory PCR. A molecular diagnostic technique using crude tissue extracts and interpreted in-field will simplify diagnoses. We developed a qualitative recombinase polymerase amplification (RPA) assay for *G. morbida* in which dual-labeled translation elongation factor 1-alpha (*TEF1-α*) amplicons are detected with a lateral flow strip. A consensus sequence for the *TEF1-α* gene was generated for *G. morbida* and RPA parameters established. The selected primer/probe combination successfully identifies all tested isolates of *G. morbida* at DNA concentrations of 100 pg/µL. One isolate was chosen for serial dilution and was identified at concentrations as low as 100 fg/µL. The assay has also identified the closely related, nonpathogenic species *Geosmithia fassatiae* when DNA is purified and in a high concentration, albeit with reduced sensitivity. Furthermore, the assay can be used successfully to test crude extracts from lesioned walnut bark.

INTRODUCTION

Thousand cankers disease (TCD) is a fungal disease complex caused by *G. morbida* (Ascomycota:Hypocreales) and its vector, the walnut twig beetle (WTB, *Pityophthorus juglandis* (Coleoptera:Scolytinae)). The known host range of the WTB is restricted to walnuts and butternuts in the genus *Juglans,* and wingnuts in the genus *Pterocarya* (Hishinuma et al. 2016). The host range of *G. morbida* is the same as its vector *P. juglandis*. The current known distribution of *P. juglandis* and *G. morbida* includes eighteen states in the U.S., including nine

western states and nine eastern states (Cranshaw 2010; Fisher et al. 2013; Grant et al. 2011; Hadziabdic et al. 2013; Moore et al. 2019; Seybold et al. 2016; Tisserat et al. 2011). Thousand cankers disease also occurs in Europe on *Juglans nigra* and *Juglans regia* (Montecchio et al. 2016; Moricca et al. 2019). The USDA Forest Service (FS) and the USDA APHIS Plant Protection and Quarantine (PPQ) have funded a WTB trap network since 2012 in order to elucidate TCD distribution in the U.S. and monitor for new introductions (USDA 2019). Quarantines have been enacted in many states within the native range of *J. nigra* in the eastern U.S. in order to prevent TCD introductions through imported plant materials (Jackson 2019).

Infection of trees by *G. morbida* is initiated when adult WTB bore through the outer bark of a host plant and inoculate the phloem with conidia that are passively carried on the cuticular surface of beetles (Kolarik et al. 2011; Tisserat et al. 2009). *G. morbida* mycelium colonizes the phloem directly surrounding *P. juglandis* galleries, but the infection does not become systemic (Utley et al. 2012). The pathogen sporulates in *P. juglandis* galleries, and emerging WTB introduce conidia to new sites. Symptoms of TCD usually begin with flagging, characterized by wilted and yellow leaves on heavily affected branches, followed by branch dieback. Epicormic branches will often sprout from the trunk (Seybold and Lacan 2015). Outer bark may appear to be stained and sunken where a canker is present. Under the outer bark, necrotic, brown, and clearly delineated cankers can be seen surrounding WTB galleries. When a large number of localized infections occur due to mass beetle attacks, cankers can coalesce resulting in branch girdling. Heavy WTB attack and associated *G. morbida* colonization can lead to tree death if enough crown dieback or trunk girdling occurs. Tree mortality can be seen as soon as three to four years after disease symptoms are first noticed (Hadziabdic et al. 2014; Kolarik et al. 2011).

The presence of *G. morbida* and WTB in the native range of *J. nigra* (eastern black walnut) is worrisome because *J. nigra* is particularly susceptible to TCD (Utley et al. 2012). The standing volume of eastern black walnut in the United States was valued at $539 billion in 2009 due to the high quality timber provided by *J. nigra* (Newton and Fowler 2009). In California, TCD is commonly found in English walnut (*J. regia*) orchards. Although *J. regia* is less susceptible to TCD than *J. nigra*, TCD may still cause or contribute to tree decline, and there is evidence that the Paradox hybrid rootstock ('*Juglans hindsii* x *J. regia'*) is more susceptible than either *J. hindsii* or *J. regia* alone (Nguyen 2015; Utley et al. 2012). Paradox hybrids are the most frequently used rootstocks in the California walnut industry, accounting for over 70% of the rootstocks in orchards (Pramuk 2016). Thus, there is concern that TCD poses a threat to the English walnut industry, which had a $1.29 billion farm gate revenue in 2019 (CDFA 2021) .

Diagnosis of TCD by fungal isolation and morphological identification can be confounded by the presence of morphologically similar, nonpathogenic *Geosmithia* species and other fungi. For instance, *Geosmithia lavendula* has also been found to be carried by WTB, but is nonpathogenic on excised walnut branches (Roubtsova et al. 2015). Furthermore, we and others found that some of the walnut pathogens can be co-isolated from lesions with *G. morbida* such as *Fusarium solani* or *Botryosphaeria dothidea* (McDermott-Kubeczko 2016). Due to the slow growth rate of *G. morbida* and the lack of *G. morbida*-specific media, such pathogens can frequently overtake a culture plate, complicating axenic culturing and identification. Early detection remains challenging. Pheromone-baited traps are utilized to monitor for *P. juglandis*, but must be in close proximity to infested walnut trees (Seybold et al. 2013). Thousand cankers disease can be difficult to identify; walnut crown symptoms can resemble other abiotic or biotic stressors/diseases, and are not apparent until the tree has long been infested with many beetles,

requiring a high local population. Beetle entrance and emergence holes are very small (~0.4 mm diameter), and disease symptoms are not always visible at the bark surface (USDA-FS-PPQ 2019).

In 2018, a diagnostic assay was published that uses PCR to amplify microsatellite loci of both *G. morbida* and *P. juglandis* (Oren et al. 2018). The protocol was designed for purified DNA extracts of drill cores, bypassing the need for fungal isolation. However, diagnosis still requires purified DNA samples, a thermocycler, and electrophoresis. Sensitivity was not reported, but was sufficient to detect extracted and purified pathogen DNA from 150 mg of bark tissue, utilizing 1 µL of DNA extract. A molecular technique for direct analysis of crude extracts of infected walnut tissue that reduces the need for expensive laboratory equipment will simplify diagnoses, providing another option for diagnostic laboratories with potential for in-field use.

Recombinase polymerase amplification (RPA) is a nucleic acid amplification technique that is rapid and incubated at an isothermal temperature (commonly 37°C to 42°C). RPA is more tolerant than PCR to the presence of some common inhibitors, making crude DNA extracts more suitable for *G. morbida* detection using RPA (Daher et al. 2016; Li et al. 2019; Moore and Jaykus 2017). RPA has been applied to the detection of various filamentous plant pathogens including fungi (Burkhardt et al. 2019; Burkhardt et al. 2018; Lau et al. 2016; Reyes Gaige et al. 2018; Sakai et al. 2014) and *Phytophthora* species (Dai et al. 2019; Miles et al. 2015; Munawar et al. 2019; Si Ammour et al. 2017). Aside from filamentous plant pathogens, RPA assays have been designed to target plant viruses, bacteria, and phytoplasmas, as well as various animal pathogens (Donoso and Valenzuela 2018; Li et al. 2019). Given RPA's success with a variety of plant and animal pathogens, we believed this assay format would work well for *G. morbida* detection.

RPA utilizes a recombinase enzyme, single-stranded DNA binding (SSB) proteins, and a strand-displacing DNA polymerase so that a thermocycler is not necessary to amplify specific genomic regions (Piepenburg et al. 2006). To initiate the reaction, recombinase enzymes form complexes with primers and pair the primers to homologous sites in duplex template DNA. SSBs bind to the displaced strand to stabilize the newly formed D-loop. The orientation of primers and probes for RPA reactions are shown in **Figure 1**. The strand displacing polymerase then extends the new strand from the annealed primer (Piepenburg et al. 2006). Sequences, lengths, and reaction concentrations of primers and probes all need to be tested and optimized to maximize sensitivity and selectivity**.**

Similar to PCR, RPA formats can be modified in many ways for different applications. This paper focuses on the Acceler8® format (Agdia, Inc., Elkhart, IN), which we have designed for qualitative end-point detection of the presence of *G. morbida* DNA by a sandwich assay on lateral flow strips. It can be applied to crude extracts of infected bark tissue to provide a positive or negative test result. This format utilizes three nucleotides: an unlabeled forward primer, a 5' antigen (biotin)-labeled reverse primer, and a 5' antigen (FAM)-labeled forward probe which binds 3' of the forward probe. The probe also has a 3' C3 spacer to block polymerase extension and an abasic THF residue placed 15-20 nucleotides 5' of the C3 spacer. The probe is cut by Endonuclease IV (nfo) at the THF residue, releasing the C3 spacer and allowing for polymerase extension. The final amplicon is dual-labeled with biotin and FAM which is utilized in a lateral flow strip sandwich assay to provide qualitative end-point detection of pathogen DNA.

A second format, the XRT assay (Agdia, Inc.) provides real-time amplification results similar to real-time PCR. Pathogen DNA concentration can be determined using of a standard curve. The XRT assay requires DNA purification because phenolics can interfere with fluorescence.

Unlike the Acceler8® format, the XRT format is not applicable to crude tissue extracts or in-field applications. Like Acceler8®, XRT uses three nucleotides: an unlabeled forward primer, a reverse primer (can be antigen (biotin)-labeled if desired), and a forward probe which binds 3' of the forward primer. The probe contains an internal abasic THF residue flanked on the 5' side with a fluorophore (dT-FAM) and on the 3' side with a quencher (dT-Q). The final amplicon is labeled with a fluorophore allowing for fluorescence measurement by a fluorometer, and may be labeled with a non-fluorescent antigen such as biotin if the assay is also intended to be used in the Acceler8® format. Comparable formats to Acceler8 and XRT are offered by TwistDx, Inc.

The objective of this study was to develop an RPA assay for diagnosis of *G. morbida* for use in the laboratory or field. We report development of an RPA assay in the Acceler8 format that targets the translation elongation factor 1-alpha (*TEF1-α*) gene to discriminate *G. morbida* from other fungi commonly associated with walnut bark cankers and the WTB, including other *Geosmithia* species and pathogens of walnut. We were able to detect as little as 100 femtograms of purified *G. morbida* DNA. Although the application of the primers and probe to the XRT format is also described, the XRT format was only utilized to optimize primer and probe concentrations and did not perform well enough to be used in diagnostics.

MATERIALS AND METHODS

Nucleotide Design. Partial sequences of the *TEF1-α* gene were generated for 21 *G. morbida* isolates and were aligned using Molecular Evolutionary Genetics Analysis (MEGA) version 6.0. Primers and probes were designed manually. All combinations of forward primers, reverse primers, and probes were screened against DNA extracts of *G. morbida*, *G. fassatiae*, *G. lavendula, J. regia*, and walnut isolates of *F. solani* and *B. dothidea*, and against water as a

negative control. All DNA extracts were at a concentration of 1 µM. Primers and probes were designed according to the AmplifyRP® Discovery Kits Assay Design Help Book guidelines (Agdia, Inc., Elkart, IN, USA). All primers and probes were purchased from Integrated DNA Technologies, Inc., Coralville, IA, USA. The probe and biotin-labeled reverse primers were dual HPLC-purified and the unlabeled forward primers were purified using standard desalting. All other reaction components were kept at concentrations recommended in the AmplifyRP® Discovery Kits Assay Design Help Book.

XRT Reaction Protocol. AmplifyRP® XRT Discovery Kits (Agdia, Inc.; Catalog # XCS 99200/0048) were used according to the reaction protocol provided with the kits. For each reaction, a rehydration solution is prepared that contains 14.75 µL of rehydration buffer (Agdia, Inc.), 1.05 µL of each primer solution (2 µM in nuclease-free water), 0.3 µL of the XRT probe solution (8 µM in nuclease-free water), 1.00 µL of purified template DNA solution, and 4.6 µL of nuclease-free water (Ambion Inc., Austin, TX, USA,). The rehydration solution (22.75 µL) is added to a desiccated XRT reaction pellet (Agdia, Inc.). Magnesium acetate solution (1.25 µL of 120 mM stock; Agdia, Inc.) is added to the cap of each reaction tube. To start the reactions, the tubes are quickly spun using a Phenix Quickspin with a rotator designed for 0.2 mL PCR tubes and incubated for 4 min in a thermocycler or digital dry block heater at 39°C. Tubes are removed from the heat block, gently vortexed so as to avoid bubbles, and spun down using a Phenix QuickSpin. Tubes are then inserted into an isothermal fluorometer or real-time PCR detection system at 39°C to monitor fluorescence from amplification. Although the fluorometer used in this assay was a Real-Time PCR thermocycler, Agdia® offers a portable isothermal fluorometer called the AmpliFire® Isothermal Fluorometer. Other real-time fluorometers may be used as

long as it detects fluorescence at the emission wavelength of the fluorophore used in the probe. Reactions are not allowed to incubate for more than 30 minutes, and onset of amplification usually begins within 20 min if the reaction is positive.

The fluorometer used in this assay is a Bio-Rad CFX96 Real-Time PCR Detection System. The protocol is set to incubate the reaction vials at 39°C for 2 sec followed by a plate read, and this is repeated 120 times. Including plate reads, the protocol takes around 40 min, but data collected past 30 min is not included in analyses since false positives are more likely to occur past this time point.

Acceler8 Reaction Protocol. AmplifyRP® Acceler8® Discovery Kits (Agdia, Inc.; Catalog #ACS 98800) were used according to the reaction protocol provided with the kits. For each reaction, a rehydration solution is prepared that contains 5.9 µL of rehydration buffer (Agdia Inc.; Catalog #ACC 00119), 0.42 µL of each primer solution (2 µM in nuclease-free water), 0.12 µL of the Acceler8® probe solution (8 µM in nuclease-free water), 1.00 µL of the template DNA solution, and 1.64 µL of nuclease-free water (Ambion Inc., Austin, TX, USA, Catalog #AM9938). The 9.5 µL rehydration solution is added to a desiccated reaction pellet (Agdia, Inc.). Magnesium acetate solution (0.5 µL of 120 mM stock; Agdia, Inc.) is added to the cap of each reaction tube. To start the reactions, the tubes are quickly spun using a Phenix QuickSpin and incubated for 4 min in a thermocycler or digital dry block heater set to 39°C. Tubes are removed from the heat block, gently vortexed to avoid bubbles, spun using a Phenix QuickSpin, and inserted back into the heat block.

For crude tissue extracts, Acceler8® RPA assays were incubated for 30 min including the initial 4-min incubation period. Amplicon detection chambers are interpreted by the number of

lines that appear on the enclosed lateral flow strip. One of the lines is a control and should always appear. If a line appears below the control line, then the reaction is positive for the presence of dual-labeled amplicons.

Inoculation of Walnut Branches. *Juglans regia* cv. 'Chandler' branches (around 12 years old) were collected from mature walnut trees in an orchard on the UC Davis campus and were cut into 36 cm segments. All cut ends of the branches were sealed with paraffin wax. Segments used in experiments were between 4 cm and 8 cm in diameter. Branches were artificially inoculated by removing a portion of the bark down to the sapwood with a #3 cork borer (7.5 mm diameter) and inserting into the excised area a piece of *G. morbida*-colonized potato dextrose agar (PDA) removed from the margin of a 14-day-old culture with the same-sized cork borer. Each branch was inoculated in three locations; the outer inoculation sites were 9 cm from the cut ends and the center inoculation point was equidistant from the two outer inoculation sites. The inoculation sites were wrapped in Parafilm and branches were kept on the lab bench at room temperature (~23°C) for 3 weeks to allow for canker development.

Crude Tissue Extraction. General extract buffer 2 (GEB2, Agdia, Inc.; Catalog #ACC 00130), general extract buffer 3 (GEB3, Agdia Inc.; Catalog #ACC 00360), general extract buffer 4 (GEB4, Agdia Inc.; Catalog #ACC 00380), and a 0.2N NaOH buffer were screened for crude extraction efficacy. The excised tissue was placed in a mesh sample bag (Agdia Inc.; Catalog #ACC 00930) with the buffer at a 1:100 mass/volume ratio and pulverized with a pestle. After maceration, the particles were allowed to settle and 1 µL of the macerate was used in an RPA reaction. For crude extracts, the assay was incubated for 30 min.

Assay Development and Optimization. Initially, five forward and five reverse primers for the *TEF1α* gene were screened as described in the AmplifyRP® Discovery Kits Assay Design Help Book to determine the optimum primer pair. The final primer pair and probe sequences are shown in **Table 1.** The Acceler8® probe was labeled at the 5' end with FAM (carboxy fluorescein) and at the 3' end with an extension-blocking C3 spacer, and an abasic nucleotide replaced an internal nucleotide in the probe. Forward and reverse primers were designed to flank the probe, and the primer in the opposite orientation to the probe was labeled at the 5' end with biotin.

For primer concentration optimization and application to real-time fluorescence measurement using the XRT format, the probe was redesigned by internally attaching the FAM to a dT base 5' of the abasic d-Spacer and attaching a black hole quencher to a dT base 3' of the abasic d-Spacer, as shown in **Table 1**. Nucleotide concentrations were optimized to 2 µM for primers and 8 µM for the probe using a BioRad CFX96 instrument. These optimized concentrations were also applied to the Acceler8® format. An example of reactions using different primer concentrations is shown in **Figure 2.**

Assay Sensitivity and Selectivity. To assess the cross reactivity of the assay, we tested purified DNA of *G. fassatiae* (2 isolates), *G. lavendula* (3 isolates), *B. dothidea* (1 isolate), and *F. solani* (1 isolate). To evaluate the lower limit of detection, 1 ng μL^{-1} of *G. morbida* DNA was prepared using a NanoDrop™ One Microvolume UV-Vis Spectrophotometer and was serially diluted down to 1 fg μL^{-1}. For purified DNA, Acceler8® RPA assays were incubated for 20 min total. Although 20 min is a sufficient incubation period for crude extracts, incubating for 30 min provides a stronger response without losing specificity.

Detecting *G. morbida in situ* in Cankers. Twelve *G. morbida* isolates were cultured and inoculated in excised *J. regia* branches as described earlier. The selected isolates, the hosts they were isolated from, and the location of the source materials are presented in **Table 2**. Branches were also inoculated with other species associated with branch cankers or *P. juglandis*, including *B. dothidea*, *F. solani*, and *G. lavendula* (**Fig. 3**). After a 3-week incubation period, cankers were sampled and the tissue tested using the Acceler8 format. To test infected walnut tissue for *G. morbida* DNA, a canker was selected, and a portion of the secondary phloem was excised at the canker margin. The sampled tissue contained both lesioned and healthy tissue (**Fig. 3F**). Because bark is a novel tissue type for RPA, we screened three extraction buffers to determine which buffer would be most effective. Of the four extraction buffers tested, only GEB2 provided positive results from crude tissue extracts of branches inoculated with *G. morbida* (**Fig. 4**).

RESULTS

Assay Sensitivity and Specificity. Figure 5 shows specificity of the Acceler8® assay for *G. morbida* over closely related *G. fassatiae* and pathogens commonly associated with branch cankers using purified DNA of each species as template. Isolates of *Geosmithia fassatiae* occasionally displayed a barely perceptible band, while *F. solani* and *B. dothidea* consistently tested negative. However, *G. fassatiae* has never been isolated from *Juglans* species or *P. juglandis,* and only showed low-intensity positive results with high concentrations of purified DNA. *Geosmithia lavendula*, while occasionally isolated from *P. juglandis*, did not test positive in the RPA assay. Using the XRT format, *G. morbida* displayed amplification while *G. fassatiae, G. lavendula, F. solani,* and *B. dothidea* did not (**Fig. 6; Table 3**). However, even a single isolate would give mixed positive and negative results with multiple repetitions and was unreliable,

unlike the Acceler8® assay. The Acceler8® assay detected purified *G. morbida* DNA down to 100 fg in the reaction mix (**Fig. 7)**. In the XRT assay, DNA diluted down to 10 fg/µL began to be amplified at around cycle 120 (**Fig. 8**), which aligns well with a graph of Ct vs Log([template DNA]) (**Fig. 9**). However, this amplification occurred past the 30 min mark, at which point false positives are more likely to occur.

Detecting *G. morbida in situ* in Cankers. Various ratios of bark mass to GEB2 volume were tested, including 1:10, 1:25, 1:50, 1:75, 1:100, 1:200, and 1:400. Both 1:100 and 1:200 ratios provided strong positive results for infected tissue, while other ratios displayed no or less intense positive results for infected tissue. For more concentrated extracts, this is likely because of inhibitors in the bark such as phenolics. 1:100 and 1:200 ratios had sufficient DNA to provide sufficient amplification while inhibitors were at low enough concentrations to not inhibit amplification. 1:400 m/v extracts were too dilute to provide as strong of results as 1:100 and 1:200 extracts. Branch cankers that developed from each of the twelve isolates of *G. morbida* all tested positive in the RPA assay following the GEB2 extraction protocol (**Fig. 10**). Branches inoculated with isolates of *B. dothidea, F. solani,* or *G. lavendula* tested negative (**Fig. 11)**.

DISCUSSION

RPA is undergoing rapid acceptance within the isothermal amplification market (Li et al. 2019). RPA assays have been designed for a wide variety of nucleic acid targets, including ssDNA, dsDNA, and cDNA from reverse transcription of RNA and miRNA (Lobato and O'Sullivan 2018) . Target organisms include bacteria, virus, protozoa, fungi, animals and plants (Li et al. 2019). In this study, we have shown that RPA can be used to detect the TCD pathogen, *G.*

morbida, with high sensitvity and specificity and in lesioned walnut bark tissue. To our knowledge, this is the first example of an RPA assay developed to detect a specific pathogen in the bark of a woody plant.

In this assay, we chose the translation elongation factor 1-alpha (*TEF1-α*) gene as the diagnostic target because it is a good marker for phylogenetically distinct species (Ghosh et al. 2015; Kashyap et al. 2017; Knutsen et al. 2004; Kristensen et al. 2005; Roger et al. 1999; Stielow et al. 2015). We also attempted to develop a diagnostic primer set for the ITS 5.8S region, but did not achieve species specificity. Because of this, we focused on the *TEF1-α* gene for developing species-specific primers. Nucleic acid purification is necessary when using plant tissue as a sample in PCR, because plant tissue is rich in polyphenolics and polysaccharides which can inhibit *Taq* Polymerase (Koonjul et al. 1999). Compared to PCR, RPA is more tolerant to inhibitors found in crude biological samples such as milk, stool samples, human serum, and tick pool homogenate (Henson and French 1993; Shahin et al. 2018) . For this reason, RPA assays have been designed which use crude extracts from wood (Cha et al. 2020) and roots (Doan et al. 2014; Subbotin 2019; Valasevich and Schneider 2017) as the DNA template.

Despite its higher tolerance to common PCR inhibitors, RPA reactions can still be inhibited. For example, high concentrations of urine and SDS in the reaction can inhibit amplification (Li et al. 2019). Walnut bark extract also appeared to have RPA inhibitors. Using walnut bark extract in GEB2 buffer at a 1:10 m/v ratio as the DNA template did not provide positive results. Positive results can be obtained from a 1:100 m/v ratio or a 1:200 m/v dilution performs even better. Although it is not known what specific inhibitors may be in walnut bark, phenolic compounds are likely too concentrated in 1:10 m/v extracts for effective amplification (Tanase et al. 2019).

Further dilutions of 1:400 m/v resulted in decreased intensity of positive responses.The assay can be applied to qualitative endpoint detection using a lateral flow strip, as well as real-time fluorescence measurement, although the XRT probe was primarily used to optimize primer and probe concentrations in our study. Off-target species commonly associated with walnut bark cankers and *P. juglandis* did not test positive in the assay, but purified *G. fassatiae* DNA at a concentration of 1 ng/µL or higher would occassionally produce a faint positive line in the Acceler8® format. However, this should not affect the viability of this assay in-field as *G. fassatiae* has never been found in association with walnut cankers or *P. juglandis*. Furthermore, *G. fassatiae* did not provide any false positive results when crude GEB2 extracts were used. This assay should simplify diagnosis of TCD, as it can be run on crude tissue extracts without a thermocycler. There is an approximate cost of $12.23 per sample by using the AmplifyRP® Acceler8® Discovery Kit and AmplifyRP® Amplicon Detection Chambers from Agdia, and primers and probes from Integrated DNA Technologies. This compares with the PCR method targeting a species-specific region of the β-tubulin gene developed by Oren and colleagues with a reported cost of $10.76 per sample (Oren et al. 2018).

The Acceler8® assay could likely be used in-field or in inspection environments to provide rapid results without having to isolate and identify the pathogen from bark tissue based on morphology or by using a molecular diagnostic with purified DNA. We did not determine how canker age may influence test results, but this likely depends on viable pathogen being present in the excised tissue. Some trapped beetles have also tested positive in the Acceler8® format after crude extraction following the same protocol as with excised bark tissue, which may allow for monitoring trapped beetles for presence of *G. morbida* inoculum. However, beetles were not cultured for confirming presence of viable *G. morbida* and the inoculum load required for a

beetle to test positive using this assay is unknown. Spore load assays from 2014 suggest that average spore counts on a beetle can be as high as 226 (Hishinuma 2017).

The RPA assay described here will expedite diagnoses of bark cankers in walnut trees. Diagnosis by culturing *G. morbida* and morphological identification can be bypassed, and the assay requires less equipment than diagnosis using PCR amplification of target DNA extracted from bark tissue. Though the assay is specific when using crude extracts, further optimization is still in process in order to increase specificity when using purified DNA for long incubation times. The development of an RPA for *G. morbida* reported here adds to an expanding catalogue of isothermal assays for rapid detection of diverse plant pathogens (Lau et al. 2016; Miles et al. 2015; Tomlinson and Boonham 2008; Tomlinson et al. 2010a; Tomlinson et al. 2010b).

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Table 1. Sequences of primers and probes targeting *TEF1α* gene of *Geosmithia morbida*.

Oligonucleotide	Oligonucleotide Sequence
Unlabeled forward primer	CGTCGAACCCGGCAAGTCGACCACCGTGAGTCTT
Acceler8® probe	6-FAM/TCTACCAGTGCGGTGGTATCGACAAGCGTAC/idSp/ ATCGAGAAGTTCGAGAAG/3SpC3
XRT probe	TCTACCAGTGCGGTGGTATCGACAAGCG/i6FAMK/AC/idSp/A/iBHQ-1Dt/CGAGAAGTTCGAGAAG/3SpC3
Biotin-labeled reverse primer	GCAGTGCAGACAGGCAGCGGGGTAATGGACCCATGAG/Biotin

Table 2. *Geosmithia morbida*, *G. fassatiae*, *and G. lavendula* isolates and other fungi tested using the Acceler8® RPA format. Isolates are from a wide range of locations and sources.

Species	Isolate No.	Date of Isolate Collection	Plant Host or Insect Source	Location
Geosmithia morbida	GM-2	09/09/2009	*J. hindsii*	Sutter Co., CA
"	GM-5	11/18/2009	*J. regia*	San Benito Co., CA
"	GM-8	10/05/2009	*J. hindsii x J. regia*	Sutter Co., CA
"	GM-53	08/10/2012	*Pterocarya stenoptera*	Solano Co., CA
"	GM-74	08/29/2012	*J. californica*	Solano Co., CA
"	GM-115	10/07/2012	*Pityophthorus juglandis*	Solano Co., CA
"	GM-124	06/26/2013	*J.nigra*	Tennessee
"	GM-14	07/30/2010	*J. nigra*	Tennessee
"	GM-33	02/14/1011	*J. californica*	Solano Co., CA
"	GM-34	02/14/1011	*J. hindsii*	Solano Co., CA
"	GM-46	11/02/2011	*J.regia*	San Joaquin Co., CA
"	GM-91	08/23/2012	*J. hindsii x J. regia*	Solano Co., CA
Geosmithia fassatiae	GF-1	11/01/2012	Oak (*Quercus* sp.)	Phaff Yeast Collection, UC Davis
"	GF-2	11/01/2012	Cerambicyd larvae (lower gut)	"
Geosmithia lavendula	GL-1	05/15/2014	*P. juglandis*	California
"	GL-2	08/22/2014	*P. juglandis*	California
"	GL-3	08/23/2014	*P. juglandis*	California
Fusarium solani	FS-1	10/15/2015	*J. regia*	Yolo Co., CA
Botryosphaeria dothidea	BD-1	10/15/2015	*J. regia*	Yolo Co., CA

Table 3. Relative Fluorescent Units (RFUs) from the AmplifyRP® XRT assay tested on five *Geosmithia morbida* isolates (GM1-GM5), *Geosmithia fassatiae, Geosmithia lavendula, Botryosphaeria dothidea*, and *Fusarium solani*.

Sample	**End RFU**	**Call**
GM1	98.14	(+) Positive
GM2	107.42	(+) Positive
GM3	139.72	(+) Positive
GM4	114.00	(+) Positive
GM5	128.30	(+) Positive
G. fassatiae	4.84	(-) Negative
G. lavendula	3.01	(-) Negative
B. dothidea	5.94	(-) Negative
F. solani	5.12	(-) Negative
H_2O	2.95	(-) Negative

Figure 1. Orientations of primers and probes designed for Acceler8® and XRT RPA formats. (A) Orientation and features of primers and probe designed for the Acceler8 RPA format. **(B)** Orientation and features of the primers and probe designed for the XRT+ RPA format.

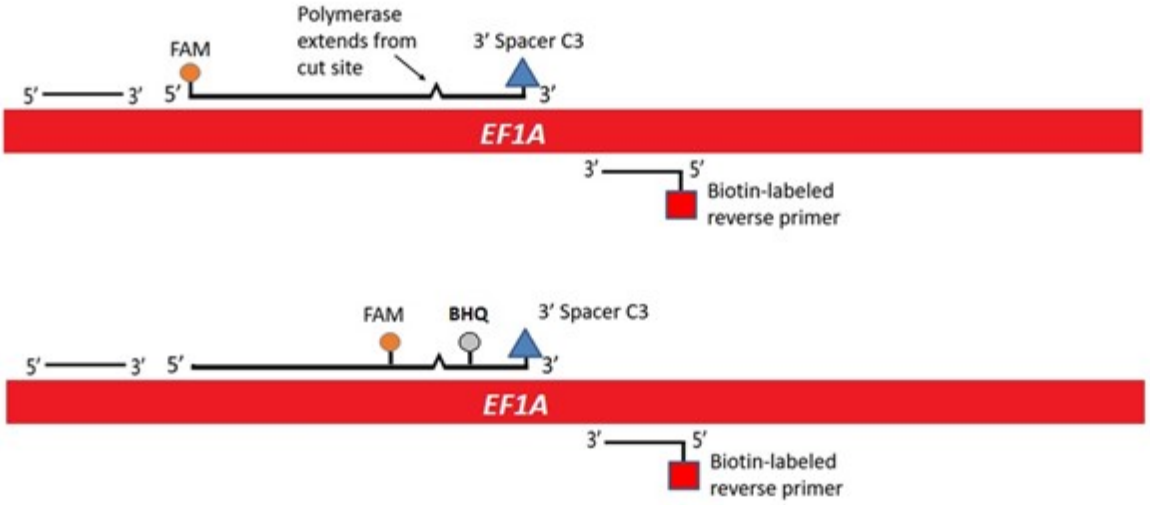

Figure 2. Example of XRT amplification curves of 1 ng/µL *G. morbida* DNA at varying primer concentrations. All tested primer concentrations result in amplification, but primers at 2 ng/µL resulted in the most rapid amplification.

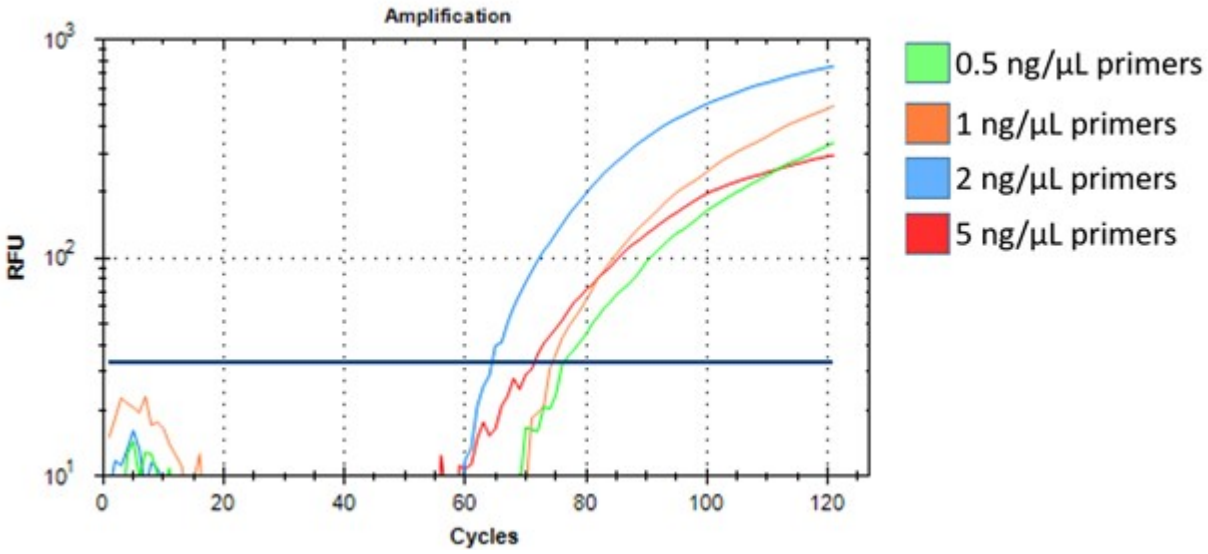

Figure 3. *Juglans regia* branches inoculated with fungi, including species associated with walnut cankers. (A) *F. solani* **(B)** *G. morbida* **(C)** *G. lavendula* **(D)** *G. fassatiae* **(E)** *B. dothidea*. *Geosmithia fassatiae* is not pathogenic in walnut. **(F)** Sampling of a *G. morbida* canker for testing lesion tissue in Acceler8® assays. Approximately 5 x 5 mm samples are excised on the canker margin and contain both lesioned and healthy tissue.

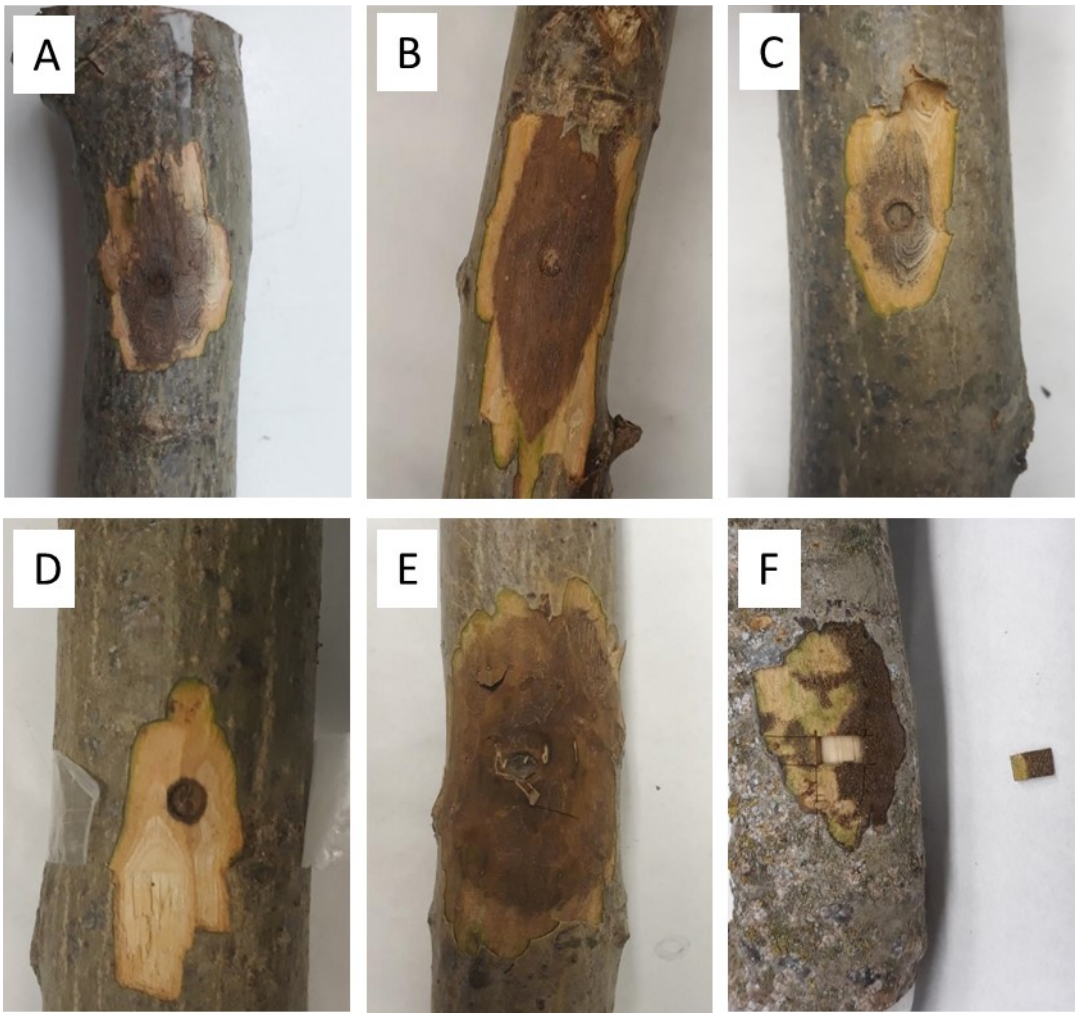

Figure 4. Acceler8® RPA testing the efficacy of various crude extraction buffers with *G. morbida* DNA as a positive control. A positive result is indicated by a (+) above the lateral flow strip cartridge, and a negative result is indicated by a (-). The template solution used in the reaction is indicated underneath each lateral flow strip. Arrows indicate positive reaction.

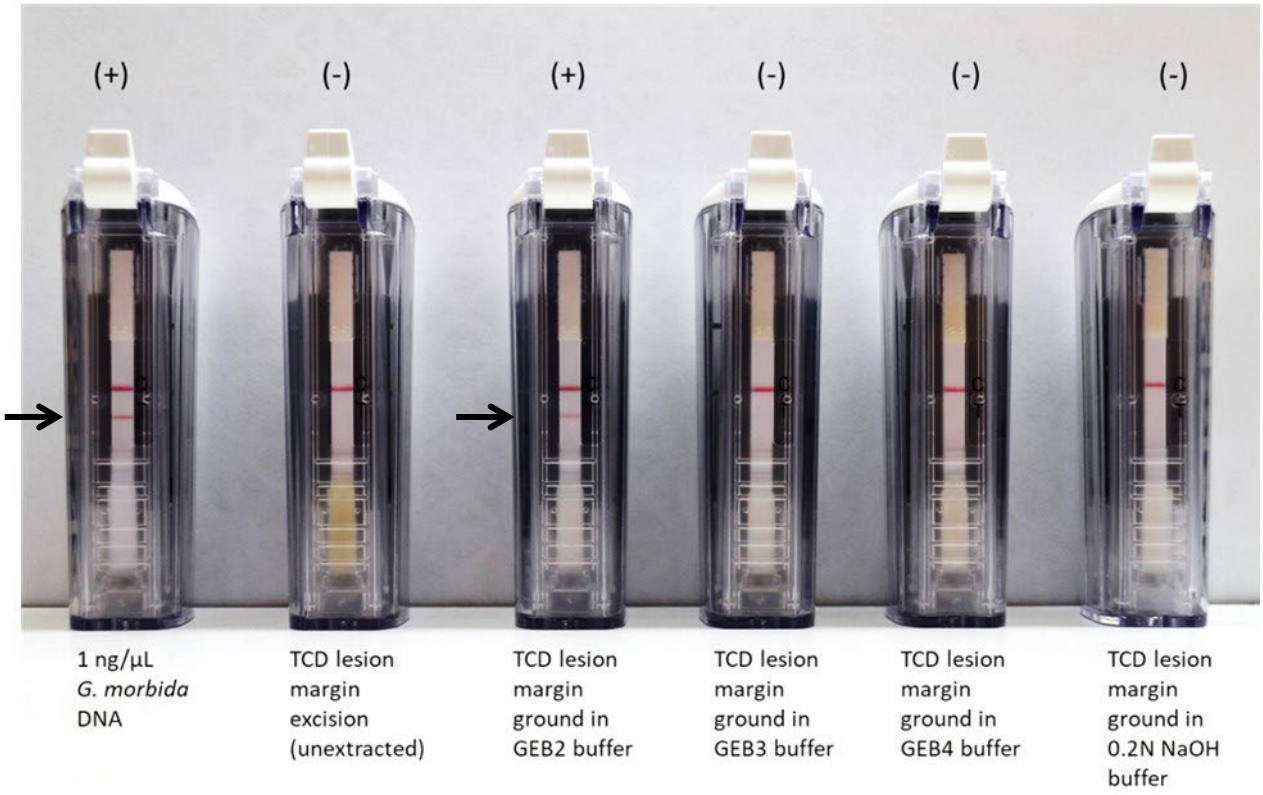

Figure 5. Recombinase polymerase amplification assays with *G. morbida*, *G. fassatiae*, and the walnut pathogens *Fusarium solani* and *Botryosphaeria dothidea*. Acceler8 assay lateral flow format using purified DNA for each species at a template concentration of 1ng/µl. Arrow indicates band confirming positive reaction for *G. morbida*.

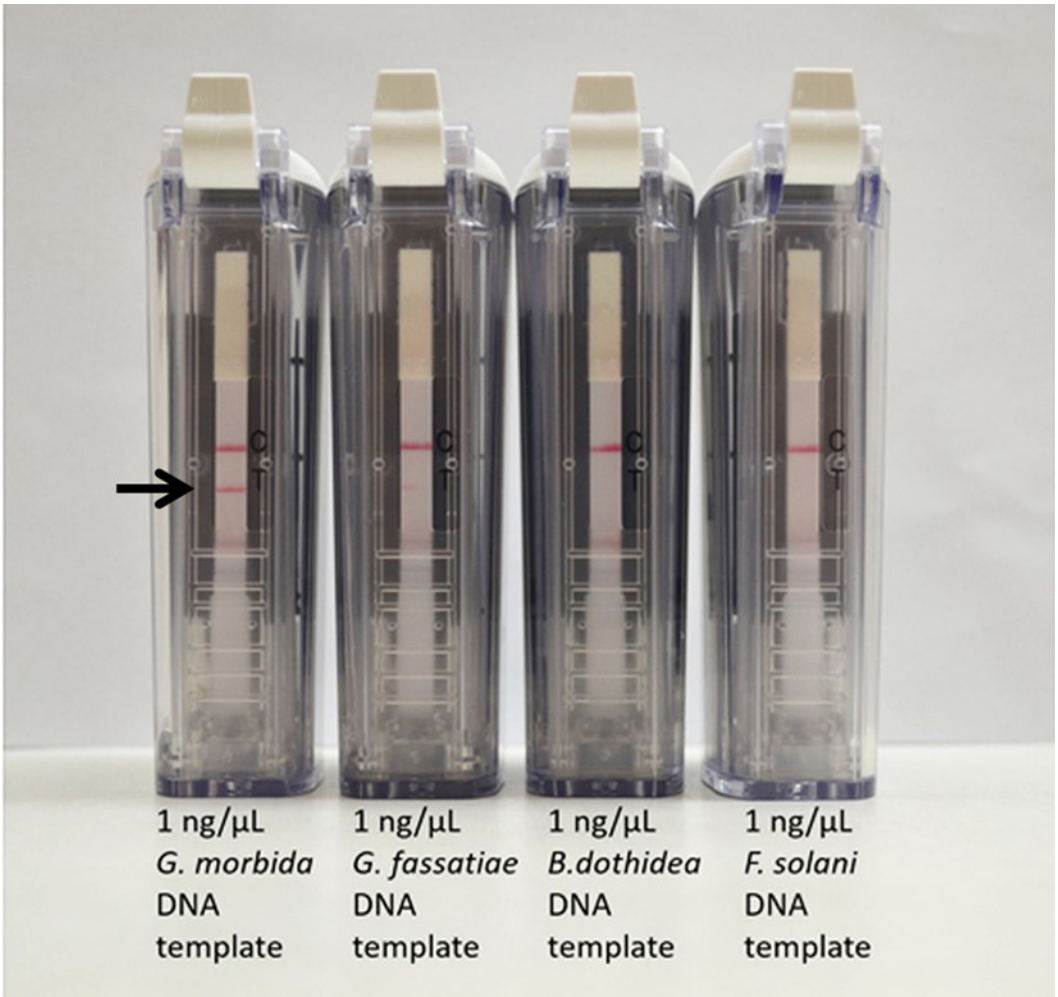

Figure 6. XRT amplification curves of various *G. morbida* isolates, closely related *Geosmithia* species, and fungal pathogens commonly associated with walnut cankers.

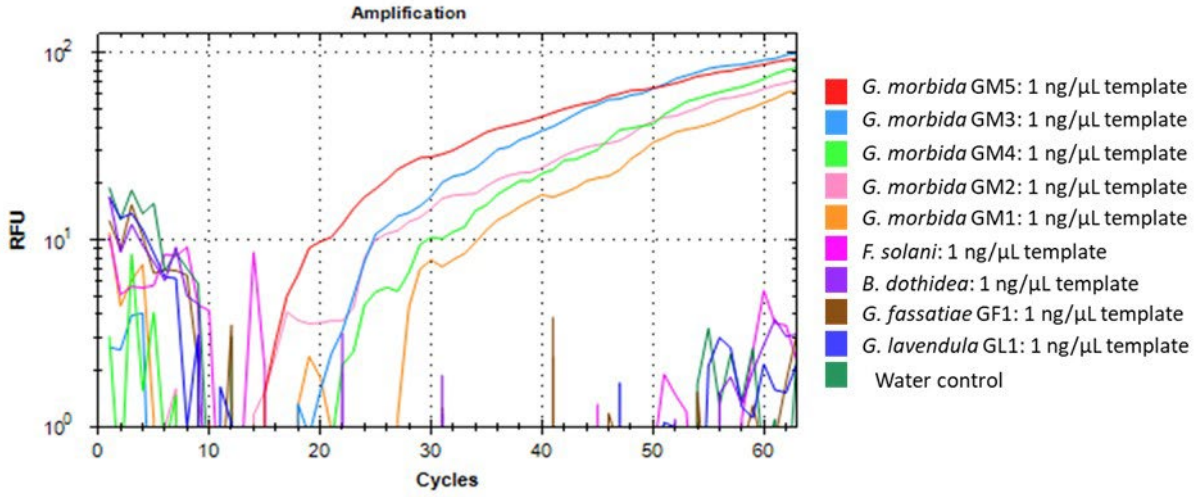

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Figure 7. RPA assay with serially diluted *G. morbida* DNA showing positive results down to 100 fg/µL. Positive tests are indicated by development of a red line at the test position (arrow). At 100 fg/µL, near the lower limit of detection for the assay, the positive band is not as strong as samples with 1 ng/µL (see Fig. 2).

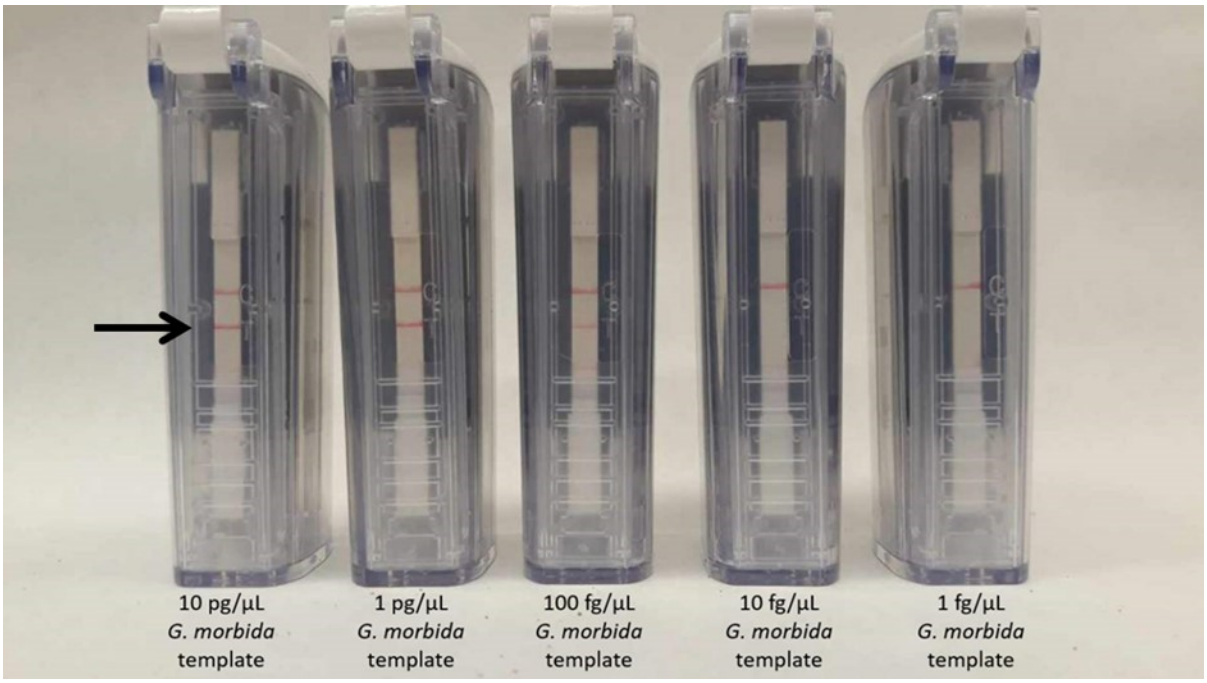


Figure 8. XRT amplification curves of *G. morbida* DNA at serially diluted template concentrations.

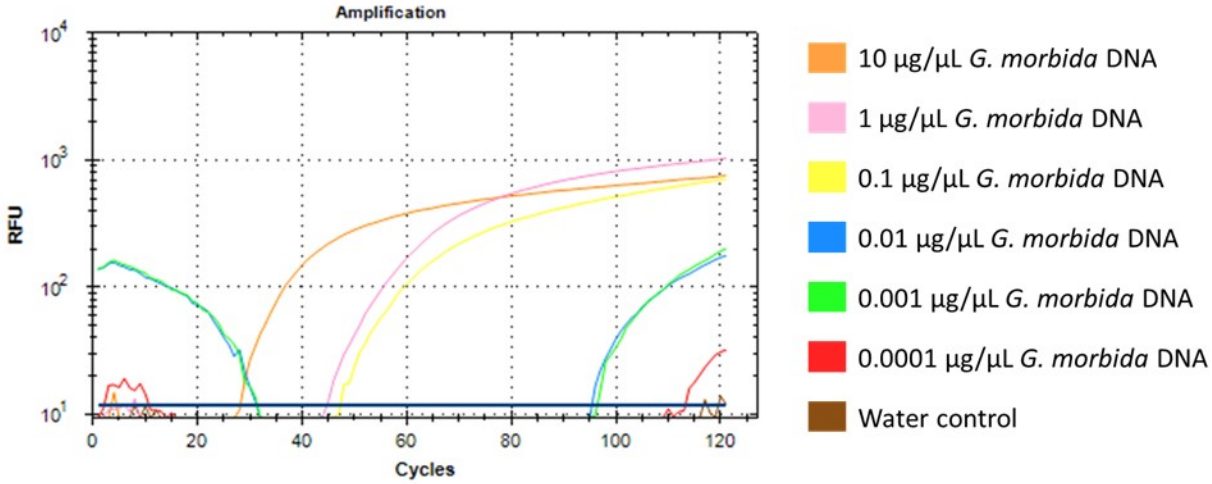


Figure 9. Plot of RT-RPA reactions of *G. morbida* DNA at serially diluted concentrations (Cq vs. Log([template DNA])). DNA concentrations as low as 0.0001 ng/µL display amplification, but the r^2 value is below 0.95, indicating that this method should not be used to quantify the amount of template DNA present in the sample.

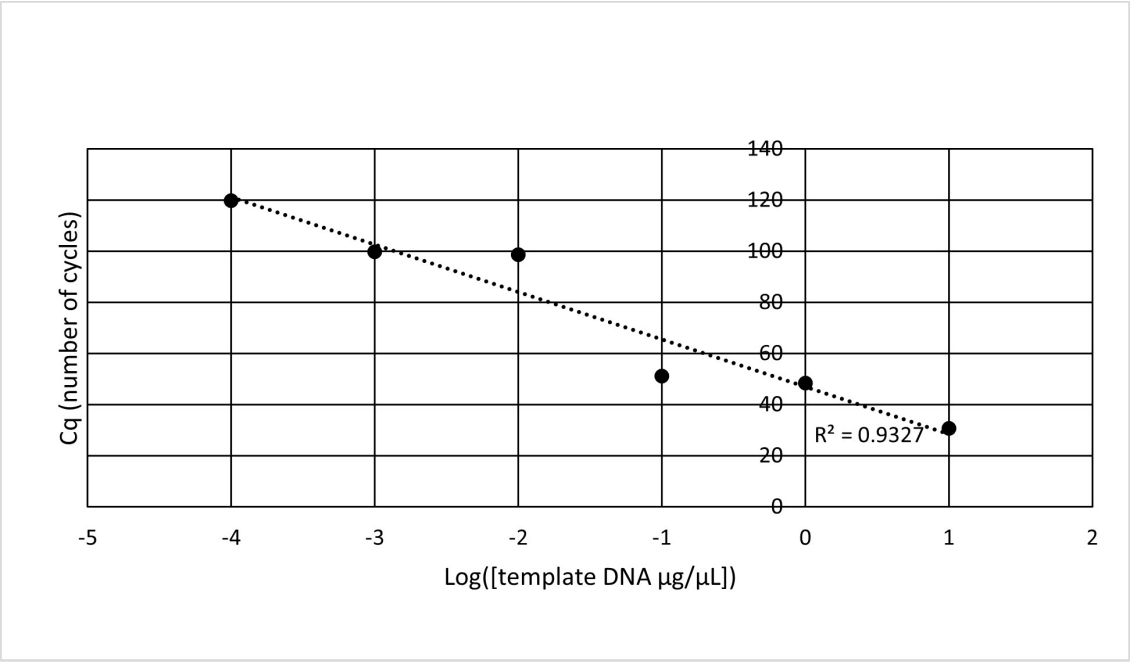


Figure 10. RPA on excised lesion margins ground in GEB2 buffer from *J. regia* branches inoculated with various *G. morbida* isolates. Underneath each lateral flow strip, the *Geosmithia* isolate used is indicated. A positive result is indicated by a (+) above the lateral flow strip.

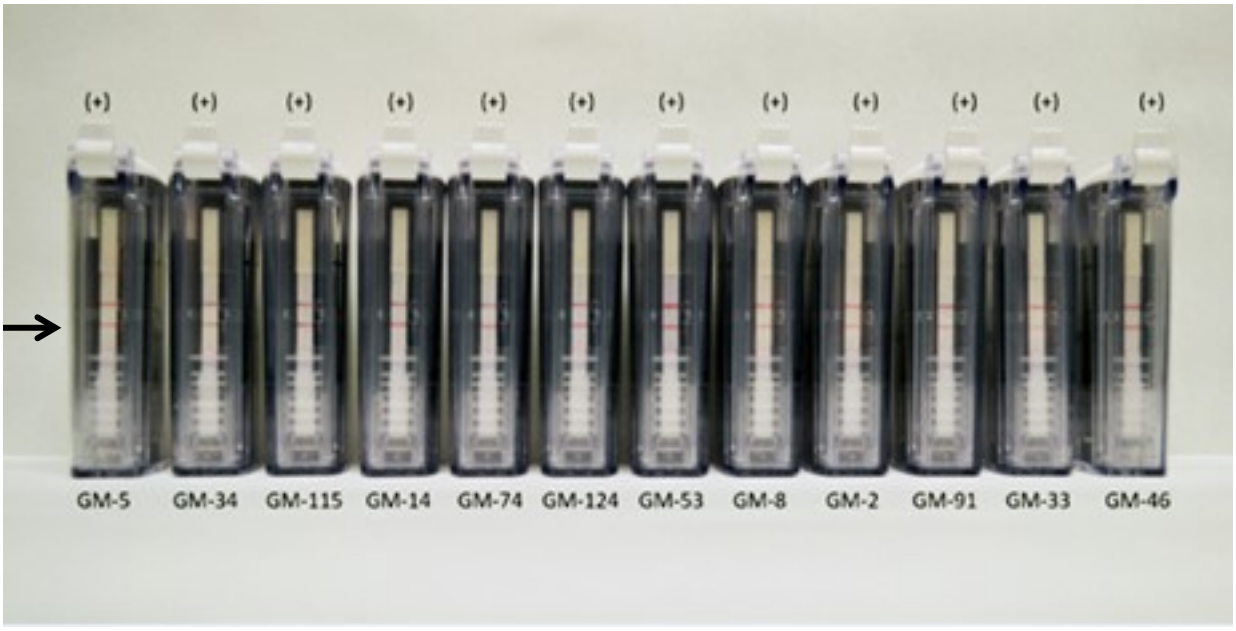
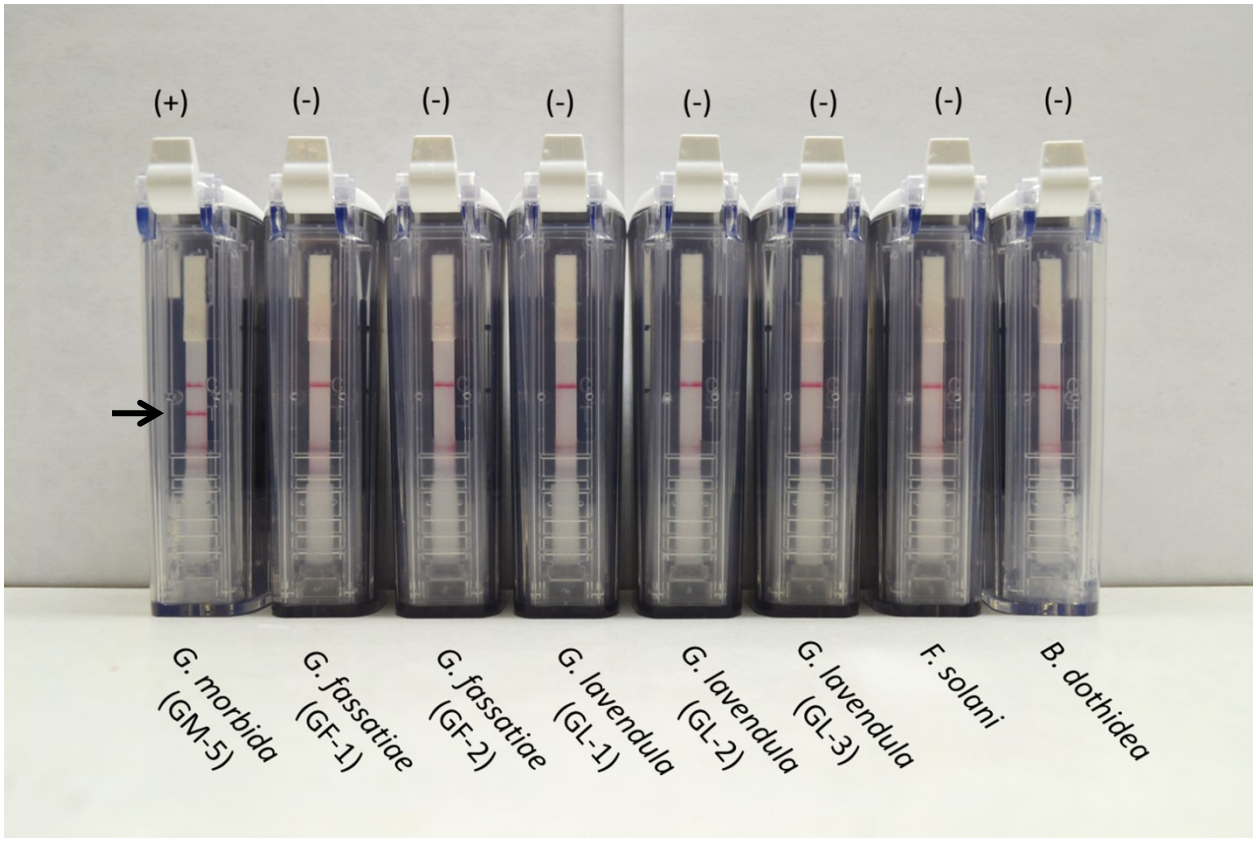


Figure 11. RPA results using direct GEB2 extracts of *J. regia* bark inoculated with *Geosmithia* species and fungi commonly associated with walnut branch cankers. Arrow indicates positive band.



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