Development of a Portable LAMP Assay for Detection of *Neofabraea perennans* in Commercial Apple Fruit

David A. Enicks,¹ Rachel A. Bomberger,² and Achour Amiri^{1,†}

¹ Washington State University, Tree Fruit Research and Education Center, Wenatchee, WA 98801

² Washington State University, Department of Plant Pathology, Pullman, WA 99164

Abstract

Bull's eye rot (BER) is a major economic postharvest disease of apple and pear that can be caused by four *Neofabraea* species: *N. perennans*, *N. alba*, *N. malicorticis*, and *N. kienholzii*. In Central Washington, BER is predominantly caused by *N. perennans*. The fungus infects fruit preharvest, and because of the dry growing season in the region, infections remain latent with symptoms expressed only after 3 to 4 months of storage, when BER incidences as high as 20% can be seen, especially in rainy seasons and on susceptible cultivars. To ensure early and efficient infection detections before BER symptoms become visible at point-of-care locations, a portable diagnostic tool based on loop-mediated isothermal amplification (LAMP) was developed using the β -tubulin gene. The LAMP assay was optimized and tested for specificity and sensitivity using DNA extracted from pure cultures of *N. perennans* and seven other fungal species. The results showed that the selected LAMP primer set was specific to *N. perennans* and highly sensitive as it detected DNA concentrations as low as 0.001 ng/ μ l after only

10 min. The assay was validated for *N. perennans* detection on artificially inoculated apples using a portable thermocycler, Genie II, without the need for DNA extraction. The LAMP assay detected *N. perennans* on apples inoculated with spore suspensions 3 weeks prior to harvest at concentrations of 10^3 spores/ml or higher. The assay was further validated using commercial Piñata apples from organic and conventional orchards, demonstrating the ability of this technique to amplify *N. perennans* from asymptomatic fruit in a commercial setting 3 months before commercial maturity. The LAMP assay developed for *N. perennans* detection can be easily expanded to detect the other BER causal species. LAMP has potential to be used in orchards and at point-of-care facilities to better inform on BER management at different fruit growth stages, and it has potential to be utilized to better understand the epidemiology of *Neofabraea* spp.

Keywords: bull's eye rot, tree fruits, pathogen detection, latent infections

Washington State leads the U.S. apple and pear production with more than 50% of the planted area and about 65 and 50% of the apple and pear fresh fruit production, respectively (USDA-NAAS 2017). The semiarid climate in the growing regions of Central Washington and the Pacific Northwest (PNW) are ideal to grow fruit, because the disease pressure is usually low except for fire blight and powdery mildew. Once harvested, apples and pears are stored at low temperatures ranging between 0 and 4°C in regular or controlled saturated (>95% humidity) atmospheres for up to 12 months. During this long storage period, postharvest decays can lead to significant revenue reduction for both the packer and the grower. Nearly 100 fungal species have been reported to cause decay on apple and pear (Rosenberger and Xiao 2014). However, major losses are caused by *Penicillium* spp. (blue mold), *Botrytis cinerea* (gray mold), *Neofabraea* spp. (bull's eye rot), *Phacidiopycnis* spp. (speck rot), and *Mucor piriformis* (Mucor rot) (Amiri and Bompeix 2005; Spotts et al. 1999; Xiao and Kim 2008). In recent surveys conducted on 160 grower lots in Washington State, blue mold, gray mold, bull's eye rot, speck rot,

and Mucor rot accounted for 45, 25, 10, 8, and 10% of total decayed fruit, respectively (Amiri and Ali 2016). Besides decaying fruit, some of the abovementioned pathogens, such as *Neofabraea* spp. and *Phacidiopycnis* spp., are quarantine pathogens in numerous countries where apples and pears from the PNW are exported to, and this has led in the past to a significant reduction in the access of PNW packers to key international markets (Warner 2014).

Bull's eye rot (BER) is a continuous threat to apple and pear storability in the PNW and in many other growing regions. Recent surveys from Washington State showed that BER accounted for 8 to 10% of overall decayed fruit and 40% of the surveyed orchards had BER at frequencies ranging between 1 and 75% (Amiri and Ali 2016). In the neighboring Canadian Province of British Columbia, BER incidence as high as 40% was reported previously on the highly susceptible cultivar Golden Delicious (Sholberg and Haag 1996). In Europe, several studies showed variable BER incidences between 30 and 64% in countries such as Norway, Denmark, Germany and the Czech Republic (Grantina-Ievina 2015; Pešicová et al. 2017). In the United Kingdom, BER used to be the largest (50%) source of apple decay in the 1960s but decreased recently to 15% (Kingsnorth et al. 2017), likely due to the planting of less susceptible cultivars. In the U.S. PNW, BER can be caused by four *Neofabraea* species: *N. perennans*, *N. malicorticis*, *N. alba*, and *N. kienholzii* (Soto-Alvear et al. 2013; Spotts et al. 2009). *N. perennans* is the most predominant species causing BER on pome fruit in the PNW, and it is generally more frequent East of the Cascade Mountain regions, characterized by a semiarid climate and where most commercial apple and pear orchards are planted. *N. malicorticis* is predominant in the wetter western region of the PNW, whereas *N. alba* has been reported to be more frequent in Oregon (Spotts et al. 2009). *N. kienholzii* is the latest species reported on pome fruit in Central Washington State and North Oregon (Spotts et al. 2009) and was previously reported in Portugal, Nova Scotia, and Australia (Cunnington 2004; de Jong et al. 2001). *Neofabraea* species can cause anthracnose (*N. malicorticis*) and perennial (*N. perennans*) cankers on which the fungus overwinters as acervuli from which

[†]Corresponding author: A. Amiri; a.amiri@wsu.edu

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conidia are released when conditions become favorable in the spring and summer (Rosenberger and Xiao 2014). Besides cankers, *Neofabraea* species can also overwinter in mummified fruits, pruned wood, leaf litter, and grass around the orchard (Köhl et al. 2018). Inoculum of *Neofabraea* spp. may also come from alternate hosts such as western crabapple (*Malus fusca*), thought to be the original host of anthracnose canker (Aguilar et al. 2017). The primary and secondary inoculums are water-splashed to limbs and fruits to cause infections that can occur between petal fall and harvest, when the fruit are more susceptible.

Although all four *Neofabraea* species are capable of causing BER, their symptoms are difficult to distinguish from each other based on morphological traits (Gariépy et al. 2003). The similarities and overlap in characteristics on artificial media and fruit symptoms make morphological characterization of these species a difficult task because the fungus grows slowly and requires several weeks for sporulation. To aid with detection of *Neofabraea* spp., assays such as multiplex PCR and intersimple sequence repeat PCR assays have been developed. Previous molecular characterization efforts to distinguish *Neofabraea* spp. from their close relatives in the *Dermea* and *Pezicula* clades have been focused on sequencing the ribosomal small subunit gene (18S rDNA), the ribosomal large subunit (5.8S rDNA LSU), and the internal transcribed spacer (ITS) regions 1 and 2 (Abeln et al. 2000). More recently, the β -tubulin and the translation elongation factor 1 genes proved useful in distinguishing *Dermea* from *Pezicula* (Pešicová et al. 2017). The β -tubulin gene is a powerful marker for *Neofabraea* species characterization because the polymorphism within this gene allows the development of species-specific primers (Gariépy et al. 2003, Michalecka et al. 2016). However, besides varying degrees of specificity and failure to differentiate between all *Neofabraea* species (Michalecka et al. 2016), the traditional PCR-based assays are laboratory-dependent and require extensive protocols, expensive equipment, and trained personnel.

One method that revolutionized the discipline of disease diagnostics since it was first developed in 2000 is loop-mediated isothermal amplification (LAMP) (Notomi et al. 2000). Several aspects make LAMP a valuable tool; first, the reaction can amplify DNA under isothermal conditions such as those provided by a water bath or heat block thanks to the *Bst* DNA polymerase. Next, LAMP has higher specificity and sensitivity compared with conventional PCR because it utilizes three primer pairs. The reaction time is considerably shorter with test to be completed in under 20 min to an hour maximum (Notomi et al. 2000). Additional advantages of LAMP include easy diagnosis that can be achieved without the need for electrophoresis required for traditional PCR assays. The accumulation of magnesium pyrophosphate in the LAMP reaction causes turbidity, which can be used to visually assess the success of the assay (Mori et al. 2004). Other methods such centrifugation (Hadersdorfer et al. 2011; Mori et al. 2001), the use of turbidimeters (Mori et al. 2013), and color indicator dyes (Denschlag et al. 2013; Goto et al. 2009; Soli et al. 2013; Ward and Harper 2012) have been successfully incorporated into LAMP assays to aid interpretation. Thus far, LAMP has been effectively used to detect different types of pathogens in different systems including viruses and phytoplasmas (Best et al. 2018; Tomlinson et al. 2010; Varga and James 2006), bacteria (Temple and Johnson 2011), oomycetes (Feng et al. 2019), several other fungi (Kaczmarek et al. 2019; Zhang et al. 2016), and monitoring resistance to fungicides (Duan et al. 2014; Hu et al. 2017; Vielba-Fernández et al. 2019). The development of laboratory-independent portable devices such as Genie II and III (OptiGene, Horsham, U.K.) and BioRanger (Diagenetix, Manoa, HI) among others has rendered detection at points of care possible in a relatively efficient way. Besides the relevancy of developing a LAMP assay for *Neofabraea* detection for point-of-care diagnostics (i.e., in orchards and packinghouses), the interest in obtaining knowledge about the epidemiology and the presence of epiphytic and latent infections in asymptomatic fruit before or at harvest will help determine the key focal points for BER management and fruit storage and packing. The objectives of this study were to (i) develop and optimize a LAMP assay for *N. perennans* detection

and test its sensitivity and specificity against the other *Neofabraea* species and other pathogens of pome fruit and (ii) validate its use for detection in artificially and naturally inoculated apples from orchards and storage rooms using a portable device to help with management and packing of fruit.

Materials and Methods

Fungal isolates, DNA extraction, and species characterization.

Four isolates of *N. perennans*, five isolates of *N. kienholzii*, one isolate of each of *N. alba* and *N. malicorticis*, and four isolates of the most common apple fruit fungal contaminants (i.e., *Alternaria alternata*, *Botrytis cinerea*, *Nectria galligena*, and *Phacidium washingtonensis*) (Table 1) were used to develop and optimize the LAMP assay in the lab. These isolates were stored as mycelial plugs in 20% glycerol at -80°C in the Tree Fruit Research and Extension Center-Pathology Lab in Wenatchee, Washington. Isolates were grown as needed on potato dextrose agar (PDA) at 22°C for 2 to 4 weeks for mycelium production and on Roma tomato agar (Cameldi et al. 2016) for 7 to 10 days at 22°C for conidial production.

For DNA extraction, the isolates were first grown on PDA for 14 days at 22°C and then on potato dextrose broth (24 g/liter) in 50-ml flasks inoculated with three plugs taken from growing margins of each PDA colony and incubated on a rotary shaker at 100 rpm for 7 days. The mycelia were centrifuged for 20 min at 6,000 rpm, washed twice with sterile distilled water, and used for DNA extraction with the FastDNA kit (MP Biomedicals, Solon, OH) following the manufacturer's protocol. The concentration and the quality of the extracted DNA were assessed using a spectrophotometer, and 100 ng/ μl dilutions were made and stored at -20°C . The identity of the *Neofabraea* isolates was confirmed using a multiplex PCR assay based on the β -tubulin gene (Michalecka et al. 2016) with the primers shown in Table 2. The identity of *A. alternata*, *B. cinerea*, *N. galligena*, and *P. washingtonensis* isolates was confirmed by sequencing the ITS regions using the ITS1 and ITS4 primers (White et al. 1990).

LAMP primers design. The primers were designed in Primer-Explorer program version 5 (<https://primerexplorer.jp/e/>) using *N. perennans* partial CDS sequence reference of the β -tubulin gene with the GenBank accession number AF281476.1. The primers were checked for polymorphism against sequences of *N. alba*, *N. kienholzii*, and *N. malicorticis* to ensure at least four nucleotides of the primers were different from the β -tubulin sequences of the three other species. During the design of LAMP primers, parameters were set to select primers with a ΔG of the 3' ends of each primer lower than -4 kcal/mol to improve annealing and avoid hairpins. Eight sets, of six primers each, were designed for *N. perennans*. Preliminary reactions with the eight sets showed that primer set 22 (Table 2) was the most adequate and was, therefore, used for all subsequent reactions.

LAMP reaction and optimization of parameters. LAMP reactions were conducted in a 25- μl volume including nuclease-free H_2O , 1 $\times$ isothermal buffer, 10 mM MgSO_4 , 0.8 M betaine, 10 mM dNTPs, FIP/BIP, LF/LB, and F3/B3 primers, *Bst* 3.0 polymerase, and 1 μl of fungal DNA. Five parameters (i.e., the concentrations of magnesium [Mg^{2+}], dNTPs, *Bst*, and the primers) were tested and optimized at different temperatures. Thus, Mg^{2+} was tested at 4, 6, and 8 mM; dNTPs were tested at 0.4, 0.6, 0.8, 1.0, and 1.4 mM; and *Bst* DNA polymerase was tested at 3, 6, 8, 10, and 14 U per reaction volume. Additionally, four concentration ratios of the inner to outer primers (2:1, 4:1, 6:1, and 8:1) and the concentrations of each primer (FIP/BIP 1 to 3.6 μM , LF/LB 0.5 to 1 μM , F3/B3 0.2 to 0.5 μM) were tested. All the combinations listed above were tested at 61, 63, and 65°C for 30, 45, and 60 min. Reactions were run in a thermocycler (Veriti, Applied Biosystems, Foster City, CA) and were terminated with a cycle of 5 min at 4°C following the manufacturer's recommendation. LAMP products were analyzed by gel electrophoresis in 1% agarose gel, and amplicons were visualized and photographed using a Gel Doc-It-UVP imaging system (Invitrogen, Carlsbad, CA).

Optimization of a portable LAMP device. After the optimal LAMP conditions had been determined using a traditional

thermocycler, the following step consisted of optimizing the assay for use with a portable instrument for laboratory and laboratory-independent conditions using the Genie II instrument (OptiGene, Horsham, U.K.). Genie II was tested to run reactions for 10, 15, 20, 30, and 45 min to optimize and select a reaction time for *N. perennans* detection. The LAMP reactions in the Genie II were conducted at 65°C in a 15-µl volume including nuclease-free H₂O, 1× isothermal buffer, 6 mM MgSO₄, 0.8 M betaine, 1.4 mM dNTPs, 0.5 µM LB, 1.6 µM each of FIP/BIP, and 1.6 µM each of F3/B3 primers, 8 U of *Bst* 3.0 polymerase, 0.6 µl of green fluorescent dye (Lucigen, Middleton, WI), and 1 µl of fungal DNA at 1 or 100 ng/µl. Following the preliminary optimization steps aimed to define optimal LAMP parameters, further optimizations consisting of removal of the lop primer LF were conducted to investigate the effect on reduction of off-target amplification as described previously (Li et al. 2019; Vielba-Fernández et al. 2019).

Specificity and sensitivity of LAMP primers to detect *N. perennans*. The specificity was tested to assess the ability of the selected primer set to correctly amplify and detect *N. perennans* only. The *N. perennans*-specific primer set 22 (Table 2) was tested against *N. alba*, *N. malicorticis*, *N. kienholzii*, and four of the other most common pome fruit pathogens (i.e., *A. alternata*, *N. galligena*, *B. cinerea*, and *P. washingtonensis*) (Table 1). The sensitivity of LAMP and its ability to detect different concentrations of *N. perennans* purified DNA was conducted by making 10-fold serial dilutions of the extracted DNA from *N. perennans* isolate Np-4122. DNA was diluted in sterile distilled water to final concentrations of 0.0001, 0.001, 0.01, 0.1, 1, 5, 10, 25, 50, and 100 ng/µl. LAMP specificity and sensitivity reactions were run at 65°C in a traditional thermocycler (Veriti, Applied Biosystems) and in the Genie II in a 15-µl final volume using the reaction parameters described above for Genie II optimization.

Detection threshold on apples inoculated with different concentrations of *N. perennans*. To evaluate the ability of LAMP to detect *N. perennans* on apples, trials were conducted at the WSU-Sunrise experimental orchard in East Wenatchee, Washington, during the summer of 2018. Apple trees of Golden Delicious, a highly susceptible cultivar to *Neofabraea* spp., were inoculated with spore suspensions of *N. perennans* (isolate Np-4122) at final concentrations of 0, 10², 10³, and 10⁴ spores/ml. Three replicate trees, set in a completely randomized block design, were inoculated for each concentration. Spore suspensions were prepared by scraping 7-day-old tomato agar plates with 10 ml of sterile distilled water and filtering through cheesecloth. The concentration was determined using a hemacytometer and then adjusted in sterile water to obtain the desired final concentrations. Apples on trees were sprayed to run-off with a hand sprayer on August 27, 2018. Control fruit were sprayed with

sterile distilled water. Fruit were sampled from each replicate tree at 0 days postinoculation (DPI), at 14 DPI, and at harvest (21 DPI) on September 18, 2018. At each sampling time, three fruit were sampled from each replicate tree for a total of nine fruit per treatment (concentration), placed in bags, and stored at –20°C until used for LAMP detection. In order to detect *N. perennans* on inoculated fruit in cold storage, 10 apples were harvested from each replicate tree for a total of 40 fruit per treatment and stored in boxes at 1°C in regular atmosphere. Fruit were sampled at 30, 60, and 90 days postharvest as described above and used immediately or stored at –20°C for LAMP detection.

Detection on commercial apples pre- and postharvest. To detect *N. perennans* on fruit from commercial orchards, apples were sampled from one conventional and one organic Piñata orchard located in Quincy, Washington. The cultivar Piñata is highly susceptible to BER, and the two orchards were selected for their BER history (Amiri and Ali 2016). Each orchard was divided into five subblocks (one at each corner and one in the center) including two replicate trees each for a total of 10 trees per orchard. Trees were scouted for cankers and were tagged with tape in July 2018. Eight apples were sampled from each tree 90, 60, and 0 days preharvest for a total of 80 fruit per orchard. Four fruit were harvested from each tree side in a way to include two fruit from the lower canopy and two from the upper canopy. Fruit were placed in separate bags and stored at –20°C until used for LAMP detection. At fruit commercial maturity on September 20, 2018, 24 additional fruit were harvested from each tree and stored in boxes at 1°C in regular atmosphere. Eight apples were sampled randomly from the boxes at 30 and 90 days postharvest to mimic commercial conditions and were used for LAMP detection.

Fruit processing for LAMP detection in artificially and naturally infected apples. Fresh apples were processed in a way to avoid DNA extraction for LAMP detection. Fruit that had been frozen at –20°C were first thawed for a few hours or overnight at room temperature. The artificially inoculated fruit collected from the experimental orchard were analyzed individually. For the apples collected from the two commercial orchards in Quincy, two apples from each tree (two apples from each tree side) were blended together to make a single sample for a total of four samples for each sampling time and each subblock and an overall total of 36 samples for each orchard. For fruit stored in cold rooms, the same number of fruit per subblock and orchard were randomly taken from the storage boxes and were blended as explained above. Cores from 1-inch depth around the calyx and the stem ends along with a 1-cm-deep peel of each individual apple were taken and blended together. A sample of the blend was transferred to 15-ml tubes in a way to have an equal volume for each sample. For detection, 200 mg of the homogenized blend was spun down in a 2-ml microcentrifuge tube, and 1 µl of supernatant was serially

Table 1. Characteristics of the fungal isolates used for LAMP optimization and sensitivity in the laboratory

Species	Isolate	Year of isolation	Host	Origin		Isolate used for LAMP ^a		LAMP sensitivity
				State	County	Specificity	Amplification ^b	
<i>Neofabraea perennans</i>	Np-4122	2005	Apple	Washington	Chelan	+	P	+
<i>N. perennans</i>	Np-4345	2005	Apple	Washington	Chelan	+	P	+
<i>N. perennans</i>	Np-4132	2005	Apple	Washington	Chelan	+	P	–
<i>N. perennans</i>	Np-4463	2005	Apple	Washington	Okanogan	+	P	–
<i>N. kienholzii</i>	Nk-4144	2005	Apple	Washington	Grant	+	N	–
<i>N. kienholzii</i>	Nk-4476	2005	Apple	Washington	Okanogan	+	N	–
<i>N. kienholzii</i>	Nk-4480	2005	Apple	Washington	Chelan	+	P	+
<i>N. kienholzii</i>	Nk-4302	2005	Apple	Washington	Okanogan	+	N	–
<i>N. kienholzii</i>	Nk-4477	2005	Apple	Washington	Okanogan	+	N	–
<i>N. malicorticis</i>	Nm-7056	2008	Apple	Oregon	Hood River	+	N	+
<i>N. alba</i>	Nb-7055	2008	Apple	Oregon	Hood River	+	N	+
<i>Alternaria alternata</i>	Aa-6226	2007	Pear	Washington	Leavenworth	+	N	–
<i>Nectria galligena</i>	Ng-18-684	2018	Pear	Oregon	Hood River	+	N	–
<i>Botrytis cinerea</i>	Bc-290	2008	Apple	Washington	Yakima	+	N	–
<i>Phacidiopycnis washingtonensis</i>	Pw-4169	2012	Apple	Washington	Quincy	+	N	–

^a + and – indicate an isolate used or not, respectively, for LAMP specificity or sensitivity.

^b P and N indicate positive and negative reaction (amplification), respectively, with LAMP detection.

diluted to $\times 1,000$ and used for LAMP reactions following the parameters described above for Genie II optimization. Preliminary tests conducted using 1 μl of the supernatant without prior dilution resulted in negative LAMP detections. The number of samples that amplified positively with the primer set 22 in experimental and commercial orchards was computed and expressed as the frequency (%) of samples positive to *N. perennans* at each sampling time.

Results

Characterization of fungal isolates. A previously developed multiplex-PCR assay was used to confirm the identity of the four *Neofabraea* species. The specific primers amplified approximately 178, 300, 360, and 190 bp of the β -tubulin gene of *N. malicorticis*, *N. perennans*, *N. alba*, and *N. kienholzii*, respectively, corresponding to the approximate expected fragment sizes reported previously (Michalecka et al. 2016). The identity of the other fungal species (Table 1) was confirmed using ITS1 and ITS4 primers, and BLAST search on NCBI revealed >90% homology and 100% coverage with *A. alternata*, *N. galligena*, *B. cinerea*, and *P. washingtonensis* (data not shown).

Specificity of LAMP primers for *N. perennans*. The eight LAMP primer sets targeting the β -tubulin gene were tested for their specificity to amplify the target DNA of *N. perennans* only. Primer set 22 was selected because it produced the most consistent amplification and strong banding patterns when checked with gel electrophoresis. The ΔG of the 3' ends of the F3, B3, F2, and B2 primers were -6.02 , -4.91 , -4.21 , and -4.41 , respectively, whereas the 5' ends of the F1c and B1c primers had respective ΔG values of -4.6 and -5.75 .

The specificity of the primer set 22 was then tested against the three other *Neofabraea* spp. and four other common pome fruit pathogens. Initial reactions using template DNAs at 100 ng/ μl provided negative reactions for all fungal species tested except for all *N. perennans* isolates and one *N. kienholzii* isolate (Nk-4480) (Table 1). Additional optimization steps continued to result in nonspecific amplification of Nk-4480 at the same time as *N. perennans* (data not shown). The reduction of the template DNA concentration from 100 to 1 ng/ μl of DNA and the removal of the LF (Li et al. 2019; Vielba-Fernández et al. 2019) primer from the reaction, after preliminary tests (data not shown), significantly increased the specificity of the primer set 22 to *N. perennans* and reduced off-target amplifications during the 35-min reaction time. Although primer set 22 continued to amplify Nk-4480 in further reactions in the Genie II with reactions containing 1 ng/ μl of DNA, the amplification of Nk-4480, expressed as fluorescence units, was six times lower and

significantly delayed (15 min) after that of *N. perennans* (Fig. 1) and even beyond the desired testing time of 35 min (data not shown).

LAMP sensitivity in vitro. The sensitivity of the LAMP assay was tested using different concentrations of purified DNA of *N. perennans* at 100, 50, 25, 10, 1, 0.1, 0.01, 0.001, and 0.0001 ng/ μl . A 30-min isothermal reaction in a traditional thermocycler followed by gel electrophoresis analysis showed that DNA concentration as low as 0.1 ng/ μl of DNA could be detected (Fig. 2A). LAMP reactions conducted in the portable Genie II instrument were able to detect *N. perennans* DNA concentrations as low as 0.01 ng/ μl within 17 min and 0.001 ng/ μl after 45 min (Fig. 2B). When the DNA concentrations were higher than 0.1 ng/ μl , amplifications occurred between 9 and 15 min after the reaction start.

Detection threshold of *N. perennans* on preinoculated apples. Golden Delicious apples, highly susceptible to *Neofabraea* spp., were inoculated with spore suspensions at 0 to 10^4 spores/ml 3 weeks prior to harvest and sampled for LAMP detection in the Genie II without prior DNA extraction. The fluorescence was visible after 16 to 25 min for 1 ng/ μl of pure DNA of *N. perennans*, whereas the minimal time required for inoculated fruit samples was 30 to 35 min, as shown in Figure 3A. There was a positive correlation ($R^2 = 0.619$) between the spore concentration (spores/ml) at inoculation and the fluorescence units detected in the Genie II at all sampling times (Fig. 3B). The correlation was stronger ($R^2 = 0.823$) with spore concentrations of 0, 10^2 , and 10^3 spores/ml (10^4 removed) at inoculation (data not shown).

The LAMP assay was used to detect *N. perennans* in all the pre- and postharvest samples to determine the frequency of samples positive to *N. perennans* based on a minimum fluorescence threshold of 30,000 units. This threshold was set after several LAMP reactions (i.e., purified DNA) were conducted. Only 5 to 20% of samples were positive to *N. perennans* in noninoculated fruit or those inoculated with a spore suspension at 10^2 spores/ml regardless of the inoculation or sampling time (Fig. 3C). On apples inoculated at 10^3 spores/ml, LAMP detected *N. perennans* in samples collected 0 DPI as well as those sampled during storage, whereas *N. perennans* was detected at all sampling times on almost all fruit inoculated with a spore suspension at 10^4 spores/ml (Fig. 3C).

LAMP detection of *N. perennans* in apples from commercial orchards. The LAMP assay designed and optimized using pure fungal DNA and artificially inoculated fruit was used to detect *N. perennans* in asymptomatic Piñata apples from commercial

Table 2. Primers used in this study

Target species	Primer name	Primer sequence	Target gene	Reference
<i>Neofabraea alba</i>	Neo_alba-loTub-439	AGCAGAGCAGGTCAAGTAA	β -Tubulin	Michalecka et al. (2016)
<i>N. kienholzii</i>	Neo_per-loTub-382	GGGTGCAACATCTGTTGT		
<i>N. malicorticis</i>	Neo_spnov-loTub-319	TGGTGAGAGGAGCGAAC		
<i>N. perennans</i>	Neo_mal-loTub-262	GACAGCCAACCTTGCGG		
	Neofab-alba-up	TGATGAGACCTTCTGTATCG		
<i>Alternaria alternata</i>	ITS1	TCCGTAGGTGAACCTGCGG	ITS	White et al. (1990)
<i>Nectria galligena</i>				
<i>Botrytis cinerea</i>				
<i>Phacidiopycnis washingtonensis</i>	ITS4	TCCTCCGCTTATTGATATGC		
<i>N. perennans</i>				
	FIP	TGAGAGGAGCGAATCC	β -Tubulin	This study
		AACCATGATTTTCTTC		
		GCAAGTTGGCTGTCAA		
		CAT		
	F3	CCAGGTCAACTCAACTCCG		
	LF	GAAATGAAGACGAGG		
		GAAAGGAACC		
	BIP	GTGCTCACTCTTTCCGT		
		GCTGTCTTTTGTCTT		
		TGGGTGCAACATCTGTT		
	B3	GAAGTCAGAAGCAGCC		
		ATCA		
	LB	ACCGTCCCAGATTGACAC		

orchards. Fluorescence values fluctuated between commercial samples (data not shown), but positive detection of *N. perennans* as shown in Figure 4A demonstrates that LAMP detection in such samples can be efficient with a fluorescence significantly higher than that of a sample containing 1 ng/ μ l of pure fungal DNA. Natural infections of *N. perennans* were detected in samples collected 90 days preharvest in both organic and conventional orchards, and the frequency of fruit carrying *N. perennans* increased during the growing season and reached the maximum values of 66 and 70% in conventional and organic orchards, respectively, 30 days postharvest and remained steady for up to 90 days postharvest (Fig. 4B).

Discussion

The detection of infections caused by latent and slow-growing pathogens such as *Neofabraea* spp. is very challenging based on traditional morphological traits and often requires a waiting period until symptoms become visible. Such limitations can hinder the implementation of appropriate and timely disease management decisions. The use of PCR-based techniques has improved the accuracy and reliability of detection (Gariépy et al. 2003; Michalecka et al. 2016). However, the requirement of specialized equipment and highly trained personnel and the need for extracted DNA to run the reactions have restricted the use of PCR-based techniques to research and diagnostic laboratories only. We developed a LAMP assay targeted to the β -tubulin gene of *N. perennans* and evaluated the sensitivity and ability of this assay to detect the pathogen in artificially inoculated and naturally infected apples collected at different points during the growing season from research and commercial orchards and after several months in cold storage. The main rationale behind developing this LAMP assay was to be able to efficiently detect *N. perennans*, and eventually other *Neofabraea* species, in local orchards well before symptoms are expressed to design appropriate spray programs and reduce losses due to BER in packinghouses.

The initial steps of primer design and screening were the most critical and challenging. We initially designed eight LAMP primer sets, of six primers each, and primer set 22 was selected and used to optimize the LAMP assay in the laboratory. Because of its high sensitivity, the most challenging aspect in the early stages of any LAMP assay development consists of potential carryover contaminations, which often can result in false positives (Hsieh et al. 2014). However, after extreme sanitation measures in laboratory spaces used for the assay and spatiotemporal separation of target DNA from the master mix, nonspecific amplifications were reduced significantly. Afterward, the LAMP assay has proven to be specific when used to detect

N. perennans among several other apple pathogens (i.e., other *Neofabraea* species) in less than 30 min. However, some mis-priming of primer set 22 was still observed for pure DNA samples of one *N.*

Fig. 2. A, Image of 1% agarose gel demonstrating the sensitivity of the LAMP assay using primer set 22 at 65°C to detect different DNA concentrations of *Neofabraea perennans*. Lanes 2 to 7: DNA at 100, 50, 25, 10, 1, and 0.1 ng/ μ l, respectively. Lanes 8 to 10: DNA concentrations of 0.01, 0.001, and 0.0001 ng/ μ l, respectively. Lanes 1 and 11 are molecular-grade H₂O, and lanes 12 and 13 are master mix only without water. **B,** LAMP sensitivity in a Genie II portable instrument at 65°C for detection of *N. perennans* at different DNA concentrations between 100 and 0.001 ng/ μ l. Negative control reactions are not shown because they were included with reactions with DNA concentrations from 1.0 to 0.0001 ng/ μ l.

Fig. 1. Specificity assessment of primer set 22 to amplify *Neofabraea perennans* (Np-4122) and *N. kienholzii* (Nk-4480) using the portable LAMP Genie II instrument. All reactions were run at 65°C and a DNA concentration of 1 ng/ μ l for each pathogen without the LF primer. Nontreated control (NTC) consist of master mix only without water added.

kienholzii isolate (Nk-4480), but it was detected 15 min after *N. perennans*. This lapse between the two *Neofabraea* species was helped by the removal of the LF primer from the reaction and by lowering the DNA concentration. This indicates that further primer design is possible to develop more species-specific primers that should resolve such mis-priming. Moreover, it is noteworthy that the primer set 22 did not mis-prime with *N. malicorticis*, which has been suggested to be more closely related to *N. perennans* than *N. kienholzii* in some studies (Chen et al. 2016; de Jong et al. 2001). Another possible explanation could be that isolate Nk-4480 is a variant of *Neofabraea* other than a sensu stricto *N. kienholzii*, which explains its delayed amplification by the *N. perennans*-specific primer set 22. Although this study focused on detection of the most economically important species *N. perennans*, research is ongoing to characterize a larger number of isolates and also to develop primers for detection of the three other *Neofabraea* species.

Since it was developed by Notomi et al. in 2000, LAMP has revolutionized disease diagnostics. However, its initial expansion was hindered because it could only be performed in research or diagnostic laboratories with access to thermocyclers or a water bath. Tremendous progress has been made since then to remove this obstacle by developing portable LAMP instruments that can be used for detection at points of care. In our study, we used the Genie II developed by OptiGene, which is battery powered and has an efficiency of 4 h. This model can run up to 16 samples at a time. Other LAMP-

based portable instruments such as BioRanger (Diagenetix) are also available for detections. The Genie instrument was reliable and sensitive in detecting *N. perennans* as low as 0.01 ng after 15 min compared with 0.1 ng with a traditional thermocycler through gel electrophoresis. The green fluorescent dye used in our assay seems to be efficient, but other more sensitive dyes could be tested in the future to further increase the sensitivity of the assay below 0.01 ng of DNA if needed.

The elimination of the DNA-extraction step and the tremendous reduction of carryover contaminations rendered the portable device very useful for quick and accurate diagnostics. LAMP reaction in the portable device was successfully used to detect *N. perennans* on Golden Delicious apples, and its sensitivity was proven as it detected *N. perennans* in almost all samples inoculated at 10^4 spores/ml and in about 25 to 100% of apples inoculated with spore suspensions at 10^3 spores/ml. The level of detection by LAMP decreased in noninoculated apples or on those inoculated with a spore suspension at 10^2 spores/ml. An appropriate protocol to detect a fungus with LAMP in fruits is critical because apples are rich in compounds that may inhibit or interfere with the reaction. In our assay, we initially used 1 μ l of the supernatant from 200 mg of a homogenized sample, and this yielded either no amplification or nonspecific and unrelated amplifications in all samples. Therefore, we diluted the 1 μ l of the supernatant $\times 1,000$ for further LAMP reactions, which yielded appropriate detection for up to 10^3 spores/ml. However, it is possible that the lower sensitivity of LAMP at spore concentrations below 10^3 spores/ml was reduced by the $\times 1,000$ dilution. Future tests (i.e., intermediate dilution factors) will help fine tune the assay and improve its specificity at lower inoculum loads.

The main objective of our work was to develop a tool to be used in commercial settings. Therefore, the final step was the validation of LAMP for *N. perennans* detection in commercial field apples. LAMP reactions in the Genie II detected *N. perennans* on apples collected in July, and the frequency of samples positive to the fungus increased until harvest and remained steady for up to 90 days of cold storage. Besides the usefulness of early detection by LAMP, our findings are relevant to better understand the epidemiology of *Neofabraea* spp.

Fig. 3. **A**, Examples of LAMP amplifications in a Genie II portable instrument from Golden Delicious apples sampled 3 weeks postinoculation with *Neofabraea perennans* at 10^3 spores/ml; **B**, correlation between spore concentrations of *N. perennans* used at inoculation and mean (36 values/concentration) fluorescence units detected by LAMP in a Genie II; and **C**, frequency of positive Golden Delicious samples detected by LAMP in a Genie II from fruit preinoculated with different *N. perennans* spore concentrations. For each sampling time, frequencies are calculated from nine values from three replicate trees and three sample fruit each. DPI and DPH indicate days postinoculation and days postharvest, respectively.

Fig. 4. **A**, Example of LAMP amplification for commercial organic and conventional Piñata apples collected in 2018; and **B**, frequency of positive Piñata samples detected by LAMP reactions in a Genie II in fruit from organic and conventional commercial orchards in Quincy, Washington, in 2018. For each sampling time, frequencies are calculated from 40 values from five replicate trees and eight samples each. DPrH and DPsH indicate days preharvest and postharvest, respectively. One microliter of the supernatant, of a 200-mg subsample from each sample, was diluted $\times 1,000$ and used for LAMP reactions.

and to improve recommendation for enhanced BER management. Although additional research is needed to make potential correlations between the levels of detection in the field and BER incidence in storage, our results on commercial Piñata apples support the correlations reported on inoculated Fuji apples, in which about 10 to 20% of fruit inoculated in July developed BER in storage, and the incidence increased to 50% when fruit were inoculated near harvest (Aguilar et al. 2017). Moreover, apples collected from the organic and conventional orchards showed a similar trend in the frequency of positive samples at all sampling points. The two orchards used in this study are only a few miles apart. This could have resulted in some population drift between orchards, although long-distance dispersion of *Neofabraea* spp. has not been reported, because infections occur mainly through water splash. It is unknown if insect movements between orchards can disperse *Neofabraea*. The similarity in the frequency of positive samples between the conventional and organic orchards may not necessarily result in similar BER incidences in storage because inoculum load, not quantified by LAMP, may be different and have a bigger impact. A comparison of spray records at the two orchards used in this study indicates that the conventional orchard received six fungicide applications (i.e., Fungicide Resistance Action Committee [FRAC] groups 3 and 7+11 between April and September). On the other hand, the organic orchard received seven sprays (i.e., four applications of *Bacillus pumilus* strain QST2808 [Sonata] and three applications of *Bacillus subtilis* strain QST 713 [Serenade Opti]). Although FRAC 3 fungicides are not highly effective against *Neofabraea* spp. (Spotts et al. 2009), the efficacy of FRAC 7 and 11 has been shown to be either low or moderate compared with FRAC 1 fungicides (Aguilar et al. 2018; Spotts et al. 2009). Although the efficacy of the two biopesticides applied in the organic orchard against *Neofabraea* spp. is unknown and cannot be compared with that of the conventional fungicides, the latter did not significantly reduce the inoculum load of *N. perennans* in the fruit, as proven by LAMP detection at harvest. The low efficacy of preharvest fungicides observed in our study may indicate that a post-harvest fungicide application will likely be more effective against BER unless FRAC 1 fungicides are applied preharvest in conventional orchards. In organic orchards, however, preharvest sanitations remain critical until more potent organic materials become available.

The ability to determine the infection risks in an orchard earlier in the season would allow timely and effective sprays. Currently, most pome fruit growers apply fungicides, to control BER and other fungi, either 2 weeks prior or at harvest. Portable LAMP detection can be informative in assessing infection risks. However, a robust protocol is needed to ensure accuracy and sample normalization. Although the described sampling protocol of dividing a block into subblocks with a final number of 40 fruit sampled per orchard is appropriate and reasonable for research purposes, it may need some adjustment in settings in which a grower or a fieldman has to run the assay independently. Although variability was seen, and expected, between trees, especially at early sampling stages, it is possible to reduce the number of fruit to four per tree without reducing the accuracy of the assay.

The potential benefit of the LAMP assay once the fruit are harvested can be achieved chiefly at harvest when sampling from representative bins prior to storage can be done easily. Once an infection risk level is determined within an hour, a packer can make an informed decision about the need for a postharvest fungicide application, the type and length of storage, or market decisions such as packing and selling the fruit shortly after harvest to avoid consequent storage losses due to BER. Ultimately, the LAMP assay can be used for fungal detection before fruit shipping at the packinghouse by the packer or by APHIS technicians at ports of export to check for quarantine pathogens.

Literature Cited

- Abeln, E. C. A., De Pagter, M. A., and Verkley, G. J. M. 2000. Phylogeny of *Pezizula*, *Dermea* and *Neofabraea* inferred from partial sequences of the nuclear ribosomal RNA gene cluster. *Mycologia* 92:685-693.
- Aguilar, C. G., Mazzola, M., and Xiao, C. L. 2017. Timing of apple fruit infection by *Neofabraea perennans* and *Neofabraea kienholzii* in relation to bull's-eye rot development in stored apple fruit. *Plant Dis.* 101:800-806.
- Aguilar, C. G., Mazzola, M., and Xiao, C. L. 2018. Control of bull's eye rot of apple caused by *Neofabraea perennans* and *Neofabraea kienholzii* using pre- and postharvest fungicides. *Plant Dis.* 102:905-910.
- Amiri, A., and Ali, E. 2016. Prevalence of storage decays of apple: Lessons from the 2016 statewide survey. Washington State University. <http://treefruit.wsu.edu/article/prevalence-of-storage-decays-of-apple-lessons-from-the-2016-statewide-survey/>
- Amiri, A., and Bompeix, G. 2005. Diversity and population dynamics of *Penicillium* spp. on apples in pre- and postharvest environments: Consequences for decay development. *Plant Pathol.* 54:74-81.
- Best, N., Rodoni, B., Rawlin, G., and Beddoe, T. 2018. The development and deployment of a field-based loop mediated isothermal amplification assay for virulent *Dichelobacter nodosus* detection on Australian sheep. *PLoS One* 13: e0204310.
- Cameldi, I., Neri, F., Ventrucci, D., Ceredi, G., Muzzi, E., and Mari, M. 2016. Influence of harvest date on bull's eye rot of 'Cripps Pink' apple and control chemical strategies. *Plant Dis.* 100:2287-2293.
- Chen, C., Verkley, G. J. M., Sun, G., Groenewald, J. Z., and Crous, P. W. 2016. Redefining common endophytes and plant pathogens in *Neofabraea*, *Pezizula*, and related genera. *Fungal Biol.* 120:1291-1322.
- Cunnington, J. H. 2004. Three *Neofabraea* species from pome fruit in Australia. *Australas. Plant Pathol.* 33:453-454.
- de Jong, S. N., Levesque, C. A., Verkley, G. J. M., Abeln, E. C. A., Rahe, J. E., and Braun, P. G. 2001. Phylogenetic relationships among *Neofabraea* species causing tree cankers and bull's-eye rot of apple based on DNA sequencing of ITS nuclear rDNA, mitochondrial rDNA, and the β -tubulin gene. *Mycol. Res.* 105:658-669.
- Denschlag, C., Vogel, R. F., and Niessen, L. 2013. Hyd5 gene-based analysis of cereals and malt for gushing-inducing *Fusarium* spp. by real-time LAMP using fluorescence and turbidity measurements. *Int. J. Food Microbiol.* 162:245-251.
- Duan, Y., Ge, C., Zhang, X., Wang, J., and Zhou, M. 2014. A rapid detection method for the plant pathogen *Sclerotinia sclerotiorum* based on loop-mediated isothermal amplification (LAMP). *Austr. Plant Pathol.* 43:61-66.
- Feng, W., Hieno, A., Kusunoki, M., Suga, H., and Kageyama, K. 2019. LAMP detection of four plant-pathogenic oomycetes and its application in lettuce fields. *Plant Dis.* 103:298-307.
- Gariépy, T. D., André Vesque, C., De Jong, S. N., and Rahe, J. E. 2003. Species specific identification of the *Neofabraea* pathogen complex associated with pome fruits using PCR and multiplex DNA amplification. *Mycol. Res.* 107: 528-536.
- Goto, K., Uchida, H., Sasa, M., and Ando, M. 2009. Emitting direction control apparatus for LAMP. United States Patent No. 7511440B2.
- Grantina-Ilevina, L. 2015. Fungi causing storage rot of apple fruit in integrated pest management system and their sensitivity to fungicides. *Rural Sustain. Res.* 34: 2-11.
- Hadersdorfer, J., Neumüller, M., Treutter, D., and Fischer, T. 2011. Fast and reliable detection of plum pox virus in woody host plants using the blue LAMP protocol. *Ann. Appl. Biol.* 159:456-466.
- Hsieh, K., Mage, P. L., Csordas, A. T., Eisenstein, M., and Tom Soh, H. 2014. Simultaneous elimination of carryover contamination and detection of DNA with uracil-DNA-glycosylase-supplemented loop-mediated isothermal amplification (UDG-LAMP). *Chem. Commun. (Camb.)* 50:3747-3749.
- Hu, X. R., Dai, D. J., Wang, H. D., and Zhang, C. Q. 2017. Rapid on-site evaluation of the development of resistance to quinone outside inhibitors in *Botrytis cinerea*. *Sci. Rep.* 7:13861.
- Kaczmarek, A. M., King, K. M., West, J. S., Stevens, M., Sparkes, D., and Dickinson, M. J. 2019. A loop-mediated isothermal amplification (LAMP) assay for rapid and specific detection of airborne inoculum of *Uromyces betae* (sugar beet rust). *Plant Dis.* 103:417-421.
- Kingsnorth, J., Perrine, J., Berrie, A., and Saville, R. 2017. First report of *Neofabraea kienholzii* causing bull's eye rot of apple in the UK. *New Dis. Rep.* 36:15.
- Köhl, J., Wencker, M., Groenenboom-de Haas, B. H., Anbergen, R., Goossen-van de Geijn, H. M., Lombaers-van der Plas, C. H., and Kastelein, P. 2018. Dynamics of post-harvest pathogens *Neofabraea* spp. and *Cadophora* spp. in plant residues in Dutch apple and pear orchards. *Plant Pathol.* 67: 1264-1277.
- Li, L., Zhang, S. Y., and Zhang, C. Q. 2019. Establishment of a rapid detection method for rice blast fungus based on one-step loop-mediated isothermal amplification (LAMP). *Plant Dis.* 103:1967-1973.
- Michalecka, M., Bryk, H., Poniatowska, A., and Puławska, J. 2016. Identification of *Neofabraea* species causing bull's eye rot of apple in Poland and their direct detection in apple fruit using multiplex PCR. *Plant Pathol.* 65:643-654.
- Mori, Y., Kanda, H., and Notomi, T. 2013. Loop-mediated isothermal amplification (LAMP): Recent progress in research and development. *J. Infect. Chemother.* 19:404-411.
- Mori, Y., Kitao, M., Tomita, N., and Notomi, T. 2004. Real-time turbidimetry of LAMP reaction for quantifying template DNA. *J. Biochem. Biophys. Methods* 59:145-157.

- Mori, Y., Nagamine, K., Tomita, N., and Notomi, T. 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochem. Biophys. Res. Commun.* 289:150-154.
- Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., and Hase, T. 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.* 28:e63.
- Pešicová, K., Kolařík, M., Hortová, B., and Novotný, D. 2017. Diversity and identification of *Neofabraea* species causing bull's eye rot in the Czech Republic. *Eur. J. Plant Pathol.* 147:683-693.
- Rosenberger, D. A., and Xiao, C. L. 2014. Postharvest Diseases. Pages 75-86 in: *Compendium of Apple and Pear Diseases and Pests*, 2nd Ed. T. B. Sutton, H. S. Aldwinckle, A. M. Agnello, and J. F. Walgenbach, eds. APS Press, St. Paul, MN.
- Sholberg, P. L., and Haag, P. D. 1996. Incidence of postharvest pathogens of stored apples in British Columbia. *Can. J. Plant Pathol.* 18:81-85.
- Soli, K. W., Kas, M., Maure, T., Umezaki, M., Morita, A., Siba, P. M., Greenhill, A. R., and Horwood, P. F. 2013. Evaluation of colorimetric detection methods for *Shigella*, *Salmonella*, and *Vibrio cholerae* by loop-mediated isothermal amplification. *Diagn. Microbiol. Infect. Dis.* 77:321-323.
- Soto-Alvear, S., Lolas, M., Rosales, I. M., Chavez, E. R., and Latorre, B. A. 2013. Characterization of the bull's eye rot of apple in Chile. *Plant Dis.* 97:485-490.
- Spotts, R. A., Cervantes, L. A., and Mielke, E. A. 1999. Variability in postharvest decay among apple cultivars. *Plant Dis.* 83:1051-1054.
- Spotts, R. A., Seifert, K. A., Wallis, K. M., Sugar, D., Xiao, C. L., Serdani, M., and Henriquez, J. L. 2009. Description of *Cryptosporiopsis kienholzii* and species profiles of *Neofabraea* in major pome fruit growing districts in the Pacific Northwest USA. *Mycol. Res.* 113:1301-1311.
- Temple, T. N., and Johnson, K. B. 2011. Evaluation of loop-mediated isothermal amplification for rapid detection of *Erwinia amylovora* on pear and apple fruit flowers. *Plant Dis.* 95:423-430.
- Tomlinson, J. A., Boonham, N., and Dickinson, M. 2010. Development and evaluation of a one-hour DNA extraction and loop-mediated isothermal amplification assay for rapid detection of phytoplasmas. *Plant Pathol.* 59:465-471.
- USDA-NAAS. 2017. United States Department of Agriculture, National Agricultural Statistics Service. <https://www.nass.usda.gov/>
- Varga, A., and James, D. 2006. Use of reverse transcription loop-mediated isothermal amplification for the detection of plum pox virus. *J. Virol. Methods* 138:184-190.
- Vielba-Fernández, A., de Vicente, A., Perez-Garcia, A., and Dolores Fernandez-Ortuño, D. 2019. Monitoring methyl benzimidazole carbamate-resistant isolates of the cucurbit powdery mildew pathogen, *Podosphaera xanthii*, using loop-mediated isothermal amplification. *Plant Dis.* 103:1515-1524.
- Ward, L. I., and Harper, S. J. 2012. Loop-mediated isothermal amplification for the detection of plant pathogens. Pages 161-170 in: *Plant DNA Fingerprinting and Barcoding*. N. Sucher, J. Hennell, and M. Carles, eds. Humana Press, New York, NY.
- Warner, G. 2014. Washington apple producers hope to resume exports to China. Good Fruit Growers. <https://www.goodfruit.com/washington-apple-producers-hope-to-resume-exports-to-china/>
- White, T. J., Bruns, T. D., Lee, S. B., and Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: *PCR Protocols: A Guide to Methods and Applications*. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, New York, NY.
- Xiao, C. L., and Kim, Y. K. 2008. Postharvest fruit rots in apples caused by *Botrytis cinerea*, *Phacidiopycnis washingtonensis*, and *Sphaeropsis pyriputrescens*. *Plant Health Prog.* doi:10.1094/PHP-2008-0919-01-DG.
- Zhang, X., Harrington, T. C., Batzer, J. C., Kubota, R., Peres, N. A., and Gleason, M. L. 2016. Detection of *Colletotrichum acutatum* sensu lato on strawberry by loop-mediated isothermal amplification. *Plant Dis.* 100:1804-1812.