Pathogen Detection Assays for Fire Blight Management in Apple Orchards

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The fire blight susceptibility of top commercial scion and rootstock cultivars is a great risk to the profitability of apple orchards. Chemical use can prevent fire blight infection, but not if the pathogen is already in plant tissue. Moreover, chemical sprays add to the production costs of orchards and could lead to antibiotic resistant strains (Norelli et al. 2003). Pruning out fire blight strikes during the growing season can limit its spread. However, if one pruning cut does not remove bacteria, it can move into the main stem and rootstock that can potentially kill high value trees. The infected trees become a source of inoculum that can lead to epidemics and leaving an orchard without production for at least 5 years. Most new cultivars with high economic value in New York State are planted in high-density orchards, which are prone to the spread of fire blight because of the smaller distance between trees, narrow canopy, and small branching system. In high density orchards, fruit spurs are close to main stem so if infection is not completely removed, it can easily move into the stem.

> **"We have studied two lateral-flow immuno kits and a LAMP assay for rapid fire blight detection in the orchard. These kits can help give timely diagnosis and identification of sources of inoculum to help deploy appropriate fire blight control measures and contain pathogen spread to other tissues, thereby limiting significant damage."**

Rapid pathogen detection in the orchard can help timely diagnosis and identification of sources of inoculum to help deploy appropriate fire blight control measures and contain pathogen spread to other tissues, thereby limiting significant damage. Fire blight symptoms in shoots can be confused with nectria twig blight or spray damage, while infected tree suckers and watersprouts can be misdiagnosed with spray damage, leading to rootstock blight. Lateral flow Immuno-assay kits can be used for testing symptomatic tissue to confirm presence of *E. amylovora* when there are uncertainties between spray damage, nectria, and fire blight infection. The Loop Mediated Isothermal Amplification (LAMP) based pathogen detection is a simple and cost-effective method that relies on specific set of DNA primers to amplify and detect target pathogen genes in 15-60 minutes (Buhlmann et al. 2013; Kubota et al. 2015). This assay can also be used to monitor asymptomatic progress of fire blight infection, but needs to be optimized for precision, sensitivity, speed, and cost (Notomi et al. 2015; Tomlinson, 2013). It is in use for citrus, tomato, potato, and grapes by growers and USDA-ARS for on-site detection of pathogens.

We have optimized the use of two commercially available immuno-kits and have tested a commercial battery-operated device for LAMP-based detection of fire blight. These detection methods can distinguish fire blight symptoms from other similar disorders and save growers the time it would take to send symptomatic tissue for diagnosis.

Experimental Methods

Plant material for optimizing the kits: The dormant budwood of fire blight susceptible scion cultivars was grafted on M.9 rootstocks obtained from Willamette Nursery, Canby, OR. These grafted scion cultivars were grown in the greenhouse at Plant Pathology in Geneva in deep pots at 18-22 °C and regularly watered. Young plants were inoculated with two different strains of *Erwinia amylovora,* once shoot length of a majority (90%) of the plants was above 25 cm. Inoculum was prepared with a concentration of 10^{-8}, as described in Khan et al. (2006). Scissors were dipped in the inoculum and used to cut young leaf at the shoot tip for inoculation. The entire infected branch (infected and healthy tissue) of each plant was harvested and brought to the lab for bacterial detection in the tissue using the different kits. In addition, fire blight infected shoots with branches were sent to our lab by growers during the growing season. Infected branches were cut using sterilized pruners and also used for detection tests.

In vitro-cultured fire blight bacteria: Fire blight infected branches from the greenhouse and growers were also used to isolate and culture bacteria on selective media plates at a surface sterilized bench in the lab. Media plates were incubated at 24-26 °C for 2 days and then bacterial culture was directly used to test immuno-kits or DNA was extracted to use with LAMP assay.

Optimization of fire blight bacterial with Lateral flow Immuno Kits: Commercial kits, AgriStrip kit (Bioreba, Reinach, Switzerland) and Pocket Diagnostics kits (Abingdon Health, York, UK), were optimized and tested for reliability, detection threshold and ease of use. The AgriStrip Kit is provided with a large bottle of extraction buffer and immuno-strips. Plastic bags, cuvettes and pipettes can also be purchased (Figure 1). The Pocket Diagnostic kit comes with extraction buffer, a dropper, metal beads and immuno-strips (Figure 2). Both kits were tested with fresh infected material and old infected branches. The test branches were placed in a plastic bag and refrigerated overnight before testing. The tissue (0.5 g) was ground in 4 ml of extraction buffer solution in the test bag and macerated using a hammer for the extraction of pathogen proteins. Total 150 µl resulting liquid was pipetted into a cuvette and the strip was immersed in it for 10-15 min-

utes. The samples used for both kits were taken from 1 cm below the obvious infection zone into healthy looking material. For the Pocket Diagnostics kit the buffer solution from the kit (provided in individual bottles) was placed into a plastic test bag and macerated with a hammer as above or the test sample (0.5g) was cut into less than 0.25 cm pieces and placed in the bottle of extraction buffer (4 ml) containing beads for the extraction of pathogen proteins. The bottle was shaken for 30 seconds to 1 minute. Two to three drops of the resulting liquid were put onto the strip, using the dropper provided with the kit. If both a red (AgriStrip) or blue (Pocket Diagnostic) test line and a control line were observed, the sample was declared fire blight positive. If the control line was clearly visible but test line did not appear within 15 minutes, the sample was considered fire blight negative (Figure 3). If no lines appeared, test was considered invalid and was repeated. We have also used old test sample branches to perform the test in the same way as described above.

Figure 1. AgriStrip kit components, a hammer and a test branch sample

Figure 2 Pocket Diagnostic kit components and a test branch sample

Optimization of Loop Mediated Isothermal Amplification (LAMP) detection kit: LAMP reaction was performed in a handheld battery powered portable commercial device called 'BioRanger' (Diagenetix, Inc.) (**Figure 4**). This device is connected through Bluetooth to an Android tablet that has the BioRanger app installed to carry out the reaction for the site-specific amplification of pathogen genome and fluorescent detection Different concentrations of reagents, including Isothermal enzyme, LAMP and *E. amylovora* specific primers and pathogen DNA are combined in a PCR tube using a pipette and then loaded in the BioRanger device. Real-time LAMP reaction can be graphically visualized on the tablet to confirm positive and negative samples. The fire blight bacterial DNA was directly isolated from infected branches and/or from pure culture of bacterial cells by boiling cells for a short time in water followed by quick centrifuging. We have tested different indicators to detect changes in LAMP reaction, different LAMP primers, and optimized reaction conditions to determine minimum amount of DNA, reaction times, and amounts of reagents required for sensitivity, for direct use in the field. We have tested two different reaction mixes from two different commercial providers for LAMP assay *i.e.* Lucigen mix (Middleton, WI) and OptiGene ISO-001 and ISO-004 (OptiGene Ltd, Camberley, UK). In addition, different dyes including Syber Green, Green Dye (Lucigen), Eva Green, and Gel Red were also tested with in-house LAMP reaction mix. The reaction temperature was set to 65°C for 30 min followed by 80°C for 5 min in the BioRanger. We designed our own LAMP primers but the main LAMP reactions were conducted using the DAS primer set (Shin, et.al 2018). LAMP amplification products from the LAMP reactions were also visualized on an agarose gel. The LAMP assay was optimized for better bacterial cell lysis and DNA release. Also, different bacterial DNA concentrations were used to optimize the detection threshold.

Results and Discussion

Optimization of Immuno-kits: Based on our optimization protocols, for both immuno-kits, it is best to use bacterial ooze or young twigs for test sample preparation. Bacterial ooze can be sampled by touching the tip of a toothpick to the ooze and suspending it in the cuvette or a small cup. Tissue from young twigs (0.5g) should ideally be collected from the transition zone. For older bark and active cankers, we recommend skimming bark off using a knife to sample the cambium layer (Figure 5).

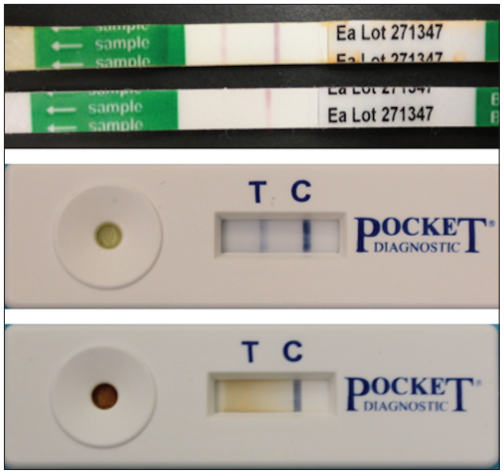

Figure 3. a) AgriStrip (Bioreba, Reinach, Switzerland) showing positive (top) and negative (bottom) results, b) Pocket Diagnostics kit (Abingdon Health, York, UK) showing a positive (top) and negative (bottom) result.

Figure 4. BioRanger device, an android Tablet on top, infected test branch, pipette and PCR tubes below).

It is important to clean hands and all tools between samples. A cloudy or opaque solution will interfere with test efficacy. In fact, we found that the detection limit for the AgriStrip kit was 10^7 fire blight bacterial cells per milliliter and for the Pocket Diagnostic Kit, 10^6 cells

per milliliter. For the AgriStrip Kit, it is especially important to not add any sample volume above the green area of the strip, as this will interfere with test accuracy. The Pocket Diagnostic kit well should not be flooded as this will also interfere with test accuracy. For both kits, non-active fire blight cankers, old strikes, or dead tissue did not work.

Optimized protocol and results for LAMP assay: After testing many different variables, we optimized the LAMP reaction in the BioRanger device to use bacterial ooze, young twigs, and/or samples from active cankers. When collecting samples from branches, a drop of sterile water can be added to the exposed cambium area if it looks dry. Both ooze and cambium layer samples should be collected with a clean disposable pipet that is then put into 25 µl of sterile water. Care needs to be taken that the sample remains clear, as a discolored sample will not give good results. A 5 minute lysis at 95°C is needed before running the reaction. The BioRanger has capacity for 8 samples at a time, each reaction should be prepared in PCR tubes, with 15µl Optigene mix ISO-001 (Pro-Lab Diagnostics Inc., Round Rock, TX), 3µl DAS Primers, and 5µl lysed sample and 2µl of sterile water. We found that the ideal reaction times were: isothermal reaction at 65°C for 30 minutes, denaturation at 80°C for 5 minutes, and fluorescence read interval every 30 seconds. The real-time progress of the reaction can be visualized on the tablet connected to BioRanger within 5-10 minutes of the start of the reaction (Figure 6). Sometimes irregular or delayed peaks were seen, which can be ignored by observing and comparing to a negative control. The size of the residual RFU threshold peak will depend on the specific reaction run therefore, it is advised to compare the residual RFU values within a run not between individual runs.

Conclusions

Two lateral-flow immuno kits and a LAMP assay have been optimized for rapid detection of fire blight pathogen. AgriStrip kit (Bioreba, Reinach, Switzerland) and Pocket Diagnostic kit (Abingdon Health, York, UK) are simple to use in the orchards and need less technical expertise. However, these kits are less sensitive compared to LAMP detection assay. LAMP has comparatively high detection sensitivity but needs some level of technical expertise. The LAMP assay requires higher initial investment costs and technical handling and could be used by extension educators or crop consultants as a more sensitive solution for pathogen detection. Before wider adoption, the cost/benefit of the different detection methods needs to be more thoroughly assessed with regard to detection threshold, ease of use, and initial investment. The use of rapid and precise pathogen detection assays in the orchards can improve fire blight management for apple orchards that can minimize the risk of economic loss, particularly in high density orchards with high-value new cultivars. These efforts will ultimately enhance the productivity and profitability of orchard operations, thus indirectly benefiting the entire value chain involved in apple nursery, production, marketing, retail, and product development business in the New York State.

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Figure 5. Sampling procedure for pathogen detection kits and LAMP assay: a) Bacterial ooze, b) Scraped bark to expose cambium layer of fire blight canker, c) Transition zone between healthy and infected tissue.

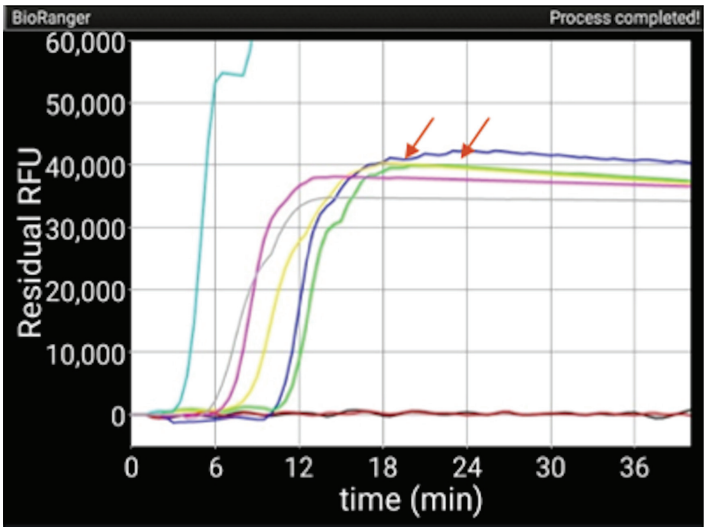

Figure 6. Real time results from LAMP reaction on BioRanger (Diagenetix Inc). Positive reactions can be confirmed in 5-10 minutes as indicated by the peak and subsequent sigmoid curve. Samples shown represent different concentrations of bacterial DNA: Black=1x106, Red= 105, Blue=104, Bright Green= 103, Yellow= unknown sample, Blue=unknown sample, Violet= lysed bacteria, Gray=DNA 2ng/ul.

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