Project Title: **Mechanical methods for improving LFD DNA extraction in support of the diagnosis of regulated *Phytophthora* species**

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Summary

A recently developed method for the extraction of DNA using Lateral Flow Devices (LFDs) allows molecular tests such as species-specific real-time PCR to be carried out on positive *Phytophthora* spp. LFDs, without the need for duplicate samples to be submitted to the laboratory. Sample preparation for LFD testing consists of shaking sample tissue in LFD extraction buffer in a bottle containing ball bearings. In order to obtain a positive LFD result when testing *P. ramorum* / *P. kernoviae* hosts such as rhododendron, it is necessary to shake the sample for only a short period of time; however, the amount of tissue disruption required for optimal extraction of nucleic acids is likely to differ from that required for optimal performance of the immunoassay. Excessive disruption of plant tissue has been found to release inhibitors which prevent the amplification of DNA by PCR, so the optimal amount of tissue disruption for LFD extraction of DNA will be enough to release DNA without causing the release of excessive amounts of PCR inhibitors. The objectives of this work were to establish the required amount of tissue disruption by shaking for optimal extraction of DNA from rhododendron, camellia and viburnum, and to investigate modifications to the extraction method that could improve the efficiency of extraction or reduce the duration of shaking required for optimal results.

The optimal amount of shaking was determined to be 1 – 2 minutes for rhododendron and camellia and 30 – 45s for viburnum. In particular, excessive disruption of viburnum causes release of inhibitors, so samples should not be shaken for more than 45s. Efficiency of extraction was improved by using smaller pieces of tissue, faster shaking and larger samples. The use of polystyrene bottles expedited disruption of tissue but also the release of inhibitors.

Materials and methods

Samples. Extractions were carried out using the LFD extraction methods from samples of *Rhododendron catawbiense*, *Camellia japonica* and *Viburnum tinus* leaf material, and from *P. ramorum*-inoculated *R. catawbiense* leaves.

LFD extraction. Two-minute lateral flow devices (produced without antibodies and in a dipstick format) were used for extraction of DNA from non-inoculated material, and *Phytophthora* spp. LFDs were used for extraction of DNA from *P. ramorum*-inoculated material. Samples were placed in 5 ml LFD Buffer C in a plastic bottle containing ball bearings (as supplied by Forsite Diagnostics for use with *Phytophthora* spp. LFDs) and shaken to disrupt the sample, except where otherwise described. Samples typically consisted of 300 mg of leaf material cut into smaller pieces (approx 15 pieces per sample). A 70 µl sub-sample of the disrupted material in Buffer C was applied to a device and allowed to run across the membrane, and devices were stored at room temperature before testing. In general, sub-samples were run on LFDs over the course of a period of shaking (typically 2-3 minutes) in order to monitor the progress of tissue

disruption and DNA release over time. For testing by TaqMan real-time PCR, a 2 mm strip was cut from the membrane using a scalpel. Each strip was then cut into 2 pieces and each piece added to an aliquot of real-time PCR reaction mix in a 96-well plate.
Assessment of the extent of tissue disruption. The amount of tissue disruption achieved using different shaking protocols was assessed using an Ultrospec 3300 Pro to measure approximate absorbance at 550nm, using LFD Buffer C as a reference.

TaqMan real-time PCR. LFDs were tested by TaqMan real-time PCR for plant DNA (cytochrome oxidase, COX) or *Phytophthora ramorum* (inoculated samples only). TaqMan real-time PCR reactions were set up in 96-well reaction plates using TaqMan Core Reagents (Applied Biosystems) consisting of 1 x Buffer A (50 mM KCl, 10 mM Tris-HCl pH 8.3, ROX passive reference dye) and 0.025 U/µl AmpliTaq Gold, plus 0.2 mM each dNTP 5.5 mM $MgCl_2$, 300 nM each primer (forward and reverse), and 100 nM TaqMan probe. Individual pieces of membrane were placed directly into the wells containing master mix before thermal cycling. Negative controls containing no membrane were included in every run. Real-time PCR was carried out on an ABI Prism 7900HT (Applied Biosystems) using generic cycling conditions (95°C for 10 minutes, followed by 40 2-step cycles of 95°C for 15 sec and 60°C for 1 min). Results were analyzed in terms of Ct values (the PCR cycle at which background-corrected fluorescence exceeds the threshold value); a negative result is represented as a Ct of 40. A decrease in Ct of 1 cycle indicates a 2-fold increase in target DNA concentration. All reactions were carried out in duplicate and mean Ct values and standard deviations were calculated.

Results and discussion

Manual vs automated shaking. It was initially intended to use mechanical grinding equipment (Kleco 96) to standardize tissue disruption; however, it was found that, while this equipment generates a faster and more uniform rate of shaking, disruption was less efficient than was achieved by manual shaking, as indicated by higher Ct values in real-time PCR (**Figure 1**). For this reason, manual shaking was used for the remaining experiments.

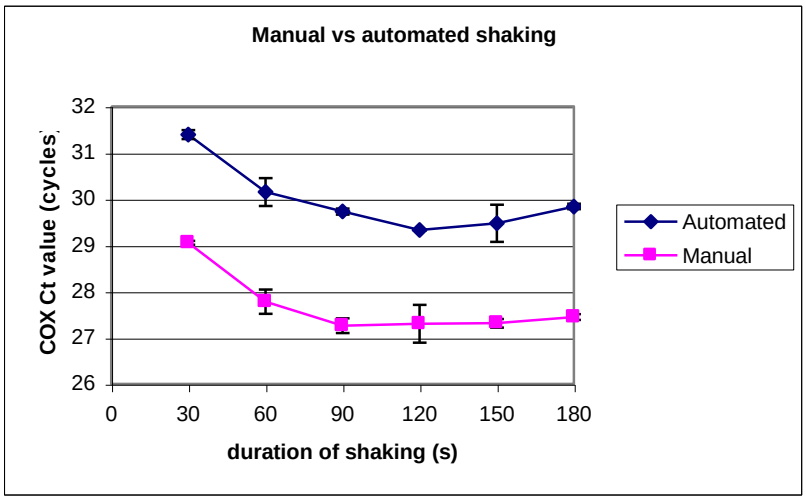
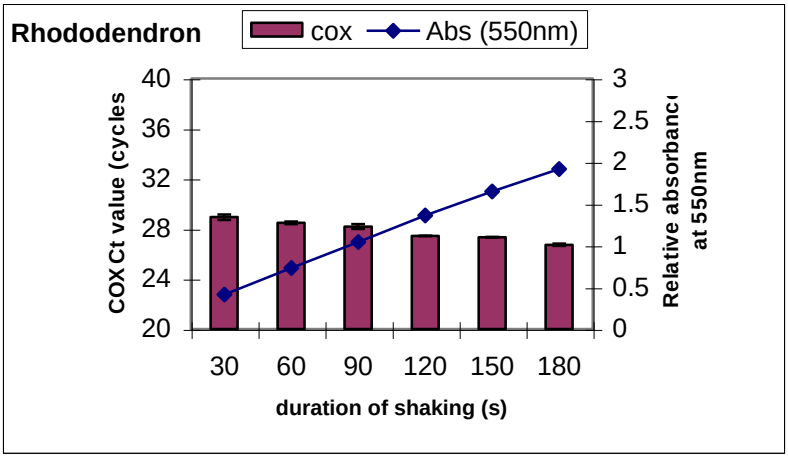
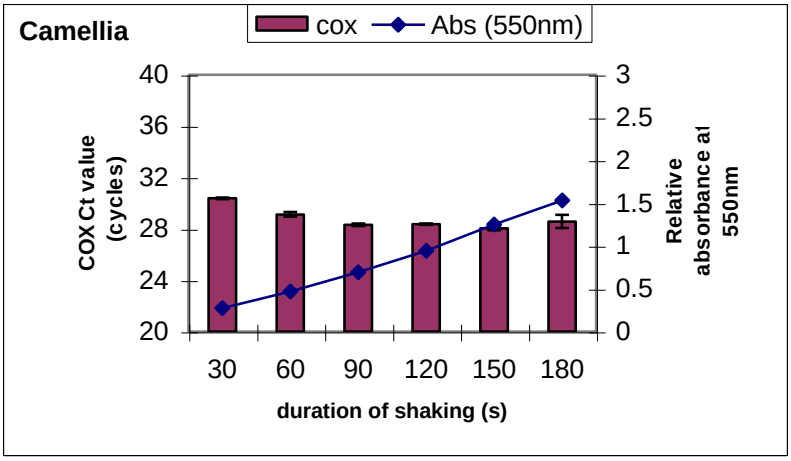


Figure 1. Real-time PCR results (COX Ct values) for rhododendron leaf shaken manually or using the Kleco 96 for 30 – 180 s.

Amount of tissue disruption and DNA amplification. Excessive tissue disruption has been found to release substances from plant material that are inhibitory to subsequent DNA amplification by real-time PCR. The extent of disruption with different amounts of shaking, and the real-time PCR results for these samples run on LFDs, are shown in **Figure 2**. Disruption of viburnum leaf tissue required much less shaking than rhododendron or camellia, and the rapid disruption corresponded to a rapid release of inhibitors, resulting in negative real-time PCR results with more than 60 seconds of shaking. In contrast, 60 seconds of shaking caused much less disruption of rhododendron and camellia leaf tissue, and positive real-time PCR results were obtained for these hosts. **Figure 3** shows the real-time PCR results obtained with different amount of shaking for replicate samples of rhododendron, camellia and viburnum.





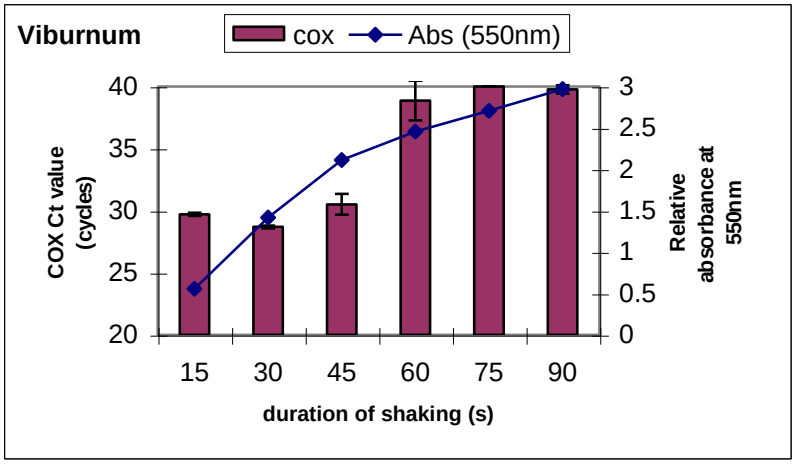
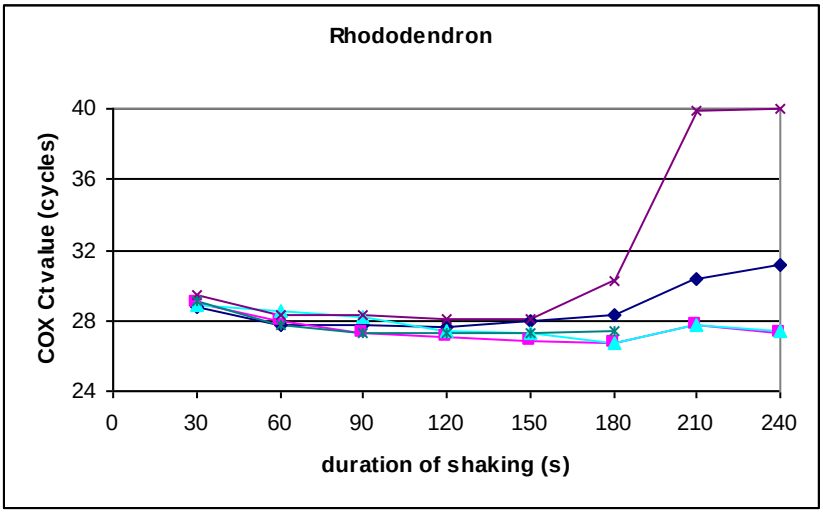
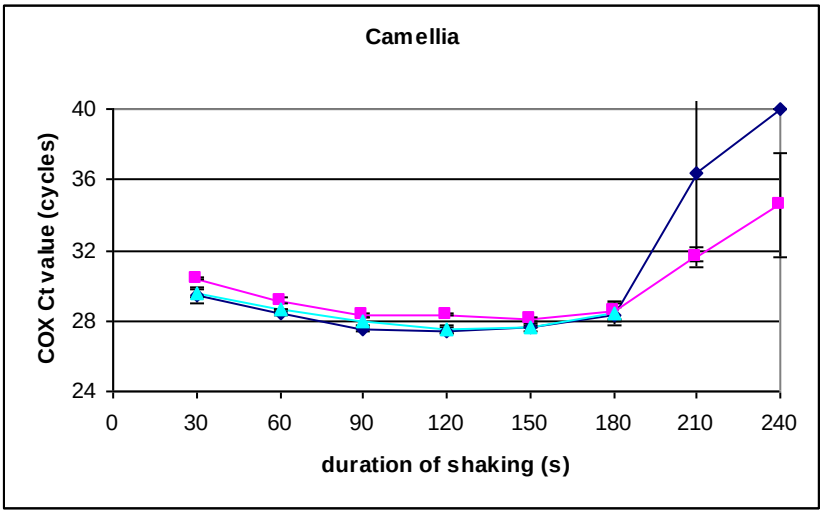


Figure 2. Real-time PCR results (bars) and tissue disruption (lines) as assessed by relative absorbance at 550nm for samples of rhododendron, camellia and viburnum leaf shaken for 30 – 180s.





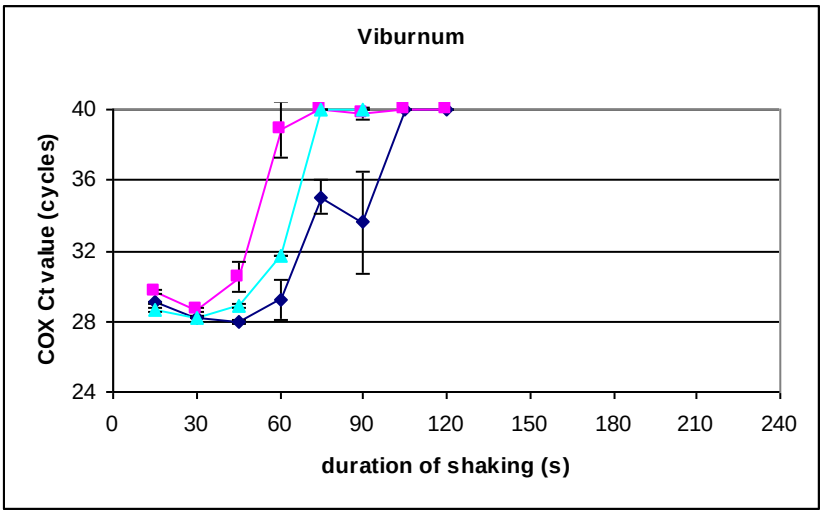


Figure 3. Real-time PCR results for rhododendron (5 samples), camellia (3 samples) and viburnum (3 samples) shaken for 30 – 240s.

The Ct values for rhododendron and camellia decreased by approximately 1 - 2 cycles as the amount of shaking was increased from 30 to 90 seconds, but no consistent improvement in Ct value was observed when the amount of shaking was increased to 3 minutes. With more than 3 minutes of shaking, the results for both rhododendron and camellia were more variable, indicating the release of inhibitors from some samples at this point. As indicated in **Figures 2** and **3**, viburnum required much less shaking, with optimal results obtained with 30 – 45 seconds of shaking, and negative results with shaking for longer than this. Viburnum samples that had been shaken for longer than 60 seconds and subsequently diluted in LFD Buffer C tested positive by real-time PCR (**Figure 4**).

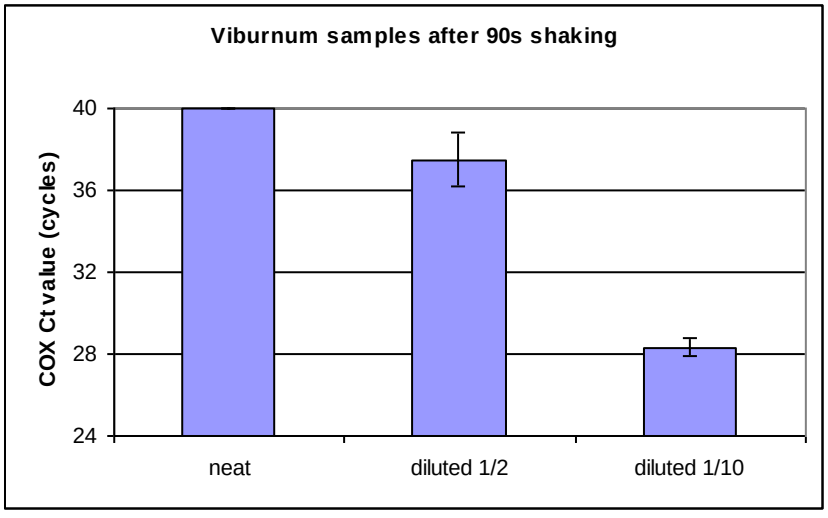


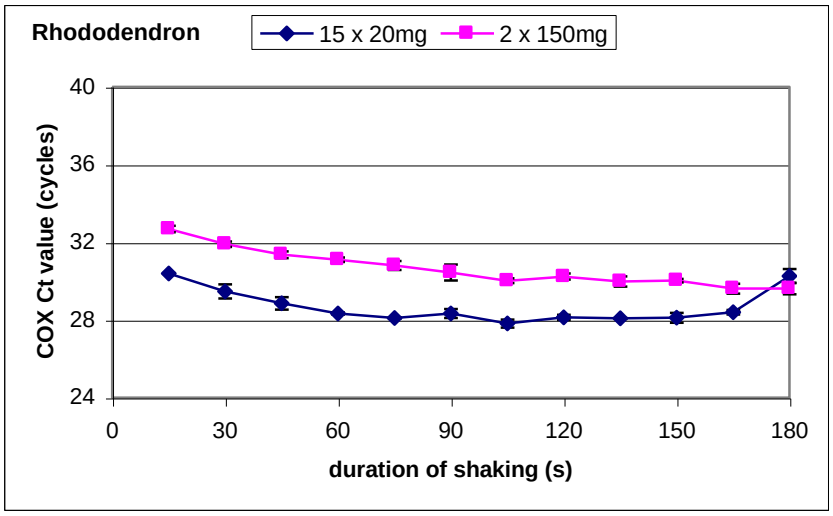
Figure 4. Real-time PCR results for viburnum leaf shaken for 90 seconds and diluted in LFD buffer before running on LFDs.

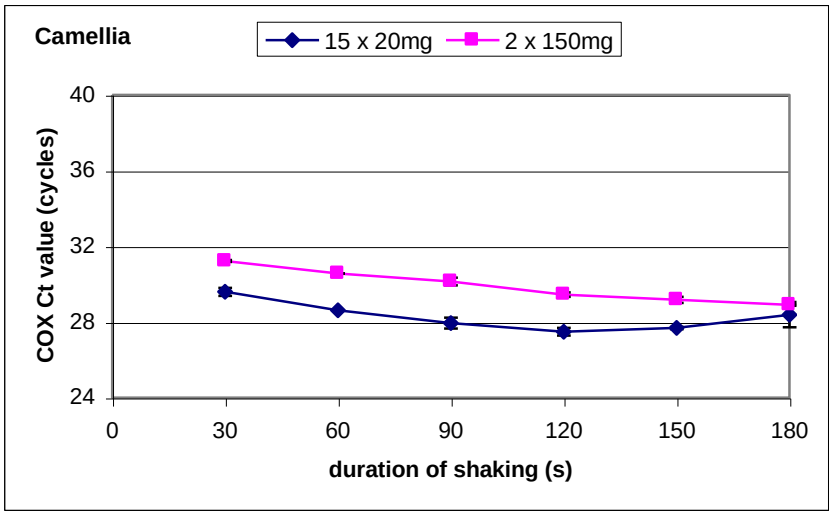
Modifications to improve efficiency of disruption. The results described so far were for samples consisting of 300mg of leaf tissue cut into smaller pieces (approximately 15 pieces per sample) and disrupted in the standard LFD sample bottles supplied by Forsite

Diagnostics containing 5 ml LFD buffer and five small (5mm diameter) ball bearings. The efficiency of tissue disruption is likely to be affected by the size of the pieces of tissue, the size of ball bearings and the type of bottle used.

Figure 5 shows the effect of the size of tissue pieces for rhododendron, camellia and viburnum. For rhododendron and camellia, the use of smaller tissue pieces results in more efficient disruption as indicated by lower Ct values. For viburnum the use of larger tissue pieces results in slightly higher Ct values with 15 – 30 seconds of shaking but also delays the release of inhibitors.

The effect of ball bearing size was investigated for extraction from samples of rhododendron (**Figure 6**). No improvement in Ct value was observed using a larger ball bearing (10mm diameter) after 1 – 2 minutes of shaking, but negative real-time PCR results were obtained after 2 minutes, indicating that although disruption was more efficient, this was not beneficial to DNA extraction.





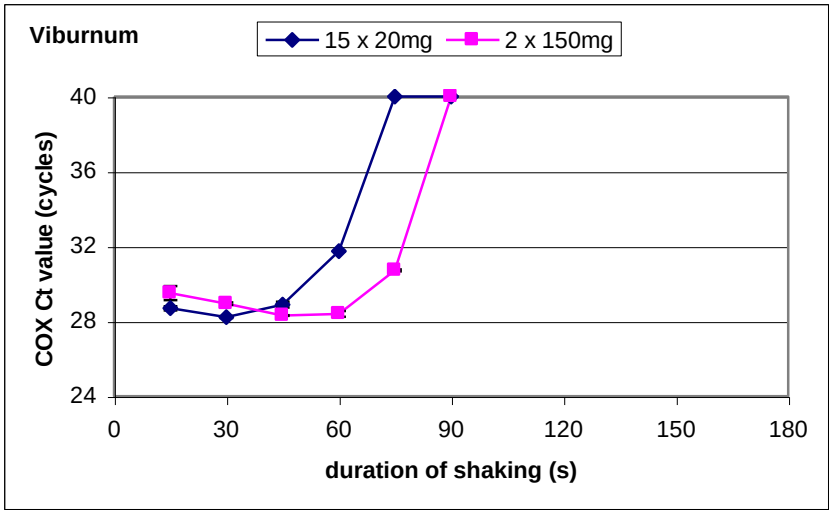
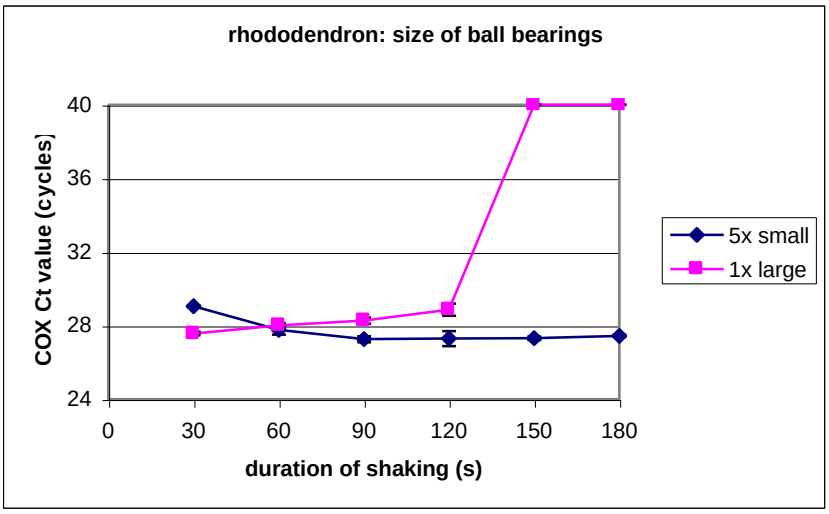


Figure 5. Real-time PCR results for 300mg samples of rhododendron, camellia and viburnum leaf cut into large or small pieces and shaken for 30 - 180s.

Standard LFD bottles supplied by Forsite Diagnostics (low density polyethylene; 15ml volume) were also compared with bottles made out of a harder plastic (polystyrene; 20ml volume), as shown in **Figure 6**. Tissue disruption was more efficient using a polystyrene bottle, although shaking for more than 60s resulted in negative real-time PCR results, indicating the more efficient release of inhibitors with this bottle type.



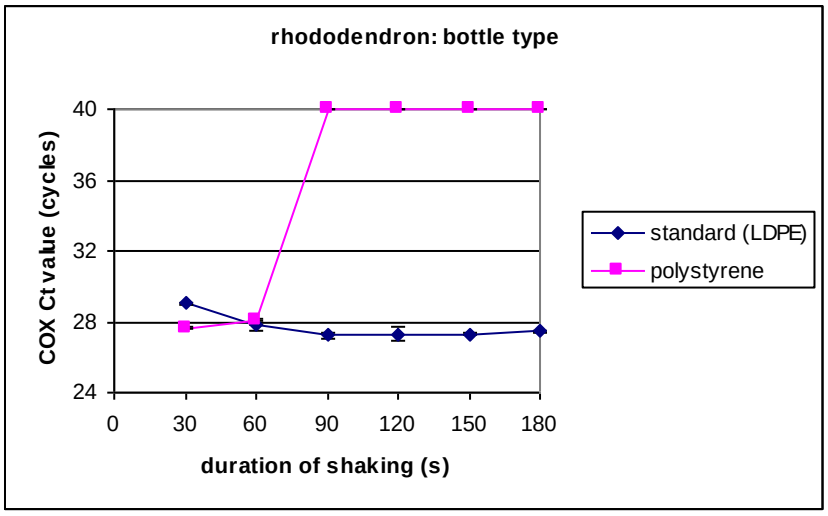


Figure 6. Real-time PCR results for rhododendron samples shaken for 30 – 180s using large (10 mm) or small (5mm) diameter ball bearings (top); or using standard low density polyethylene or polystyrene bottles (bottom).

Other factors affecting efficiency of extraction. There is likely to be an amount of variation in the way devices are run, especially in terms of the speed at which different individuals shake the samples bottles and the amount of sample tested, which is likely to depend on the amount of symptomatic material available. The effect of shaking speed was investigated using samples of rhododendron leaf. **Figure 7** shows the real-time PCR results for samples shaken at relatively low speed (approximately 3 'cycles' - ie returning the bottle to the starting position - per second) or at higher speed (approximately 4 'cycles' per second). The slower rate of shaking is easily achievable for a period of 2 – 3 minutes, while the faster rate is more vigorous and is more easily maintained for periods of 1 minute or less. Similar Ct values were obtained using the faster rate of shaking for 1 minute and the slower rate of shaking for 90s indicating that, while vigorous shaking results in more efficient DNA extraction, a slower rate of shaking can be used and the period of shaking extended by approximately 30 seconds.

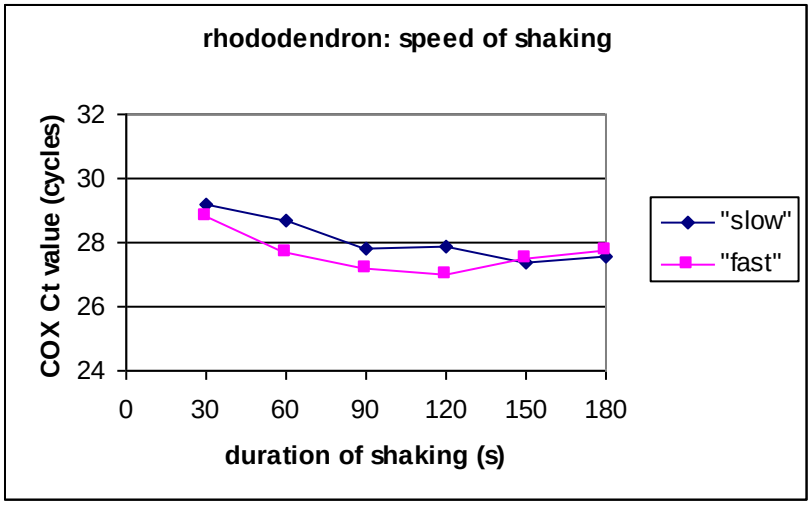


Figure 7. Real-time PCR results for rhododendron samples shaken at different rates for 30 – 180s.

DNA was also extracted from samples of rhododendron of different sizes (**Figure 8**). 300mg corresponds to a piece of rhododendron leaf of approximately 7cm^2, or roughly half of a medium sized leaf (or roughly one third of a medium sized camellia leaf, or one medium sized viburnum leaf). Slightly lower Ct values were obtained for larger samples of rhododendron leaf, although after 120s shaking, similar Ct values were obtained for all sample sizes tested, suggesting that although more DNA is released if there is more starting material, the amount of inhibitors released is also increased.

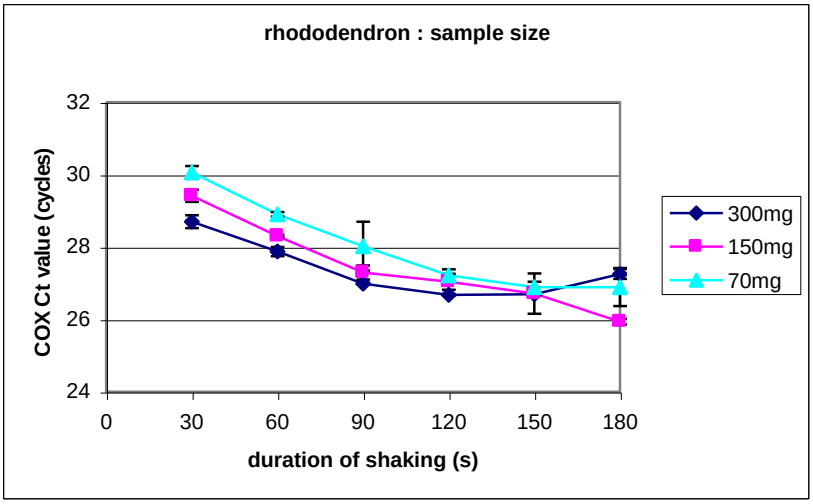


Figure 8. Real-time PCR results for rhododendron samples of different sizes shaken for 30 – 180 seconds.

For camellia, similar Ct values were obtained for all sample sizes tested after 60 – 120s. For viburnum, similar Cts were obtained for all sample sizes after 30 s shaking, although after shaking for 45s only the 70mg sample was positive by real-time PCR, suggesting less inhibition when a small sample size was used.

Testing *P. ramorum*-inoculated material. DNA was extracted from rhododendron leaf inoculated with *P. ramorum* using *Phytophthora* spp. LFDs. Samples consisted of either 300mg necrotic leaf tissue, or 60mg necrotic and 240mg non-inoculated leaf tissue. All LFD results were positive. The LFDs were tested by real-time PCR for both plant DNA (COX) and *P. ramorum* DNA, and the results are shown in **Figure 9**. Both the host and pathogen DNA were amplified by real-time PCR, with a small decrease in Ct value with 15 - 60s of shaking, and an increase in Ct above 135s. The *P. ramorum* Ct values obtained for a sample consisting of 100% necrotic material were lower than for a sample containing only 20% infected material, while the COX Ct values were slightly higher for the 100% necrotic sample.

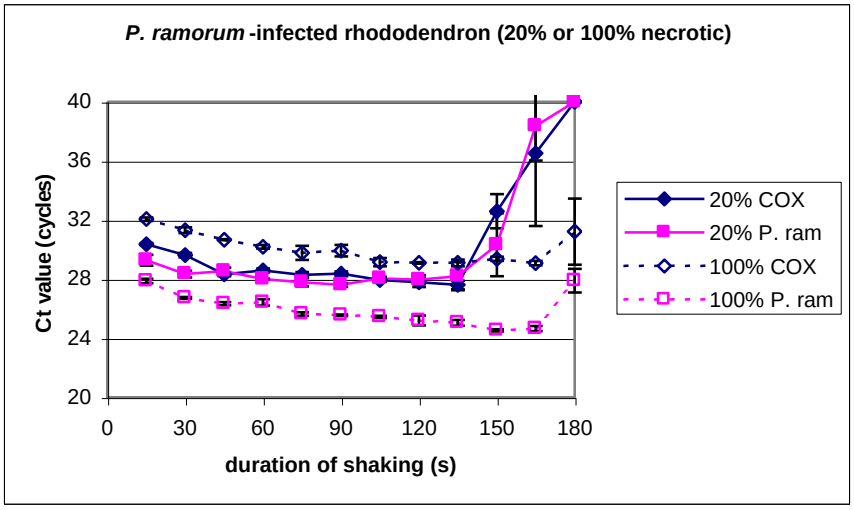


Figure 9. COX and *P. ramorum* real-time PCR results for *P. ramorum*-infected rhododendron samples (100% or 20% necrotic) after shaking for 30 – 180s.

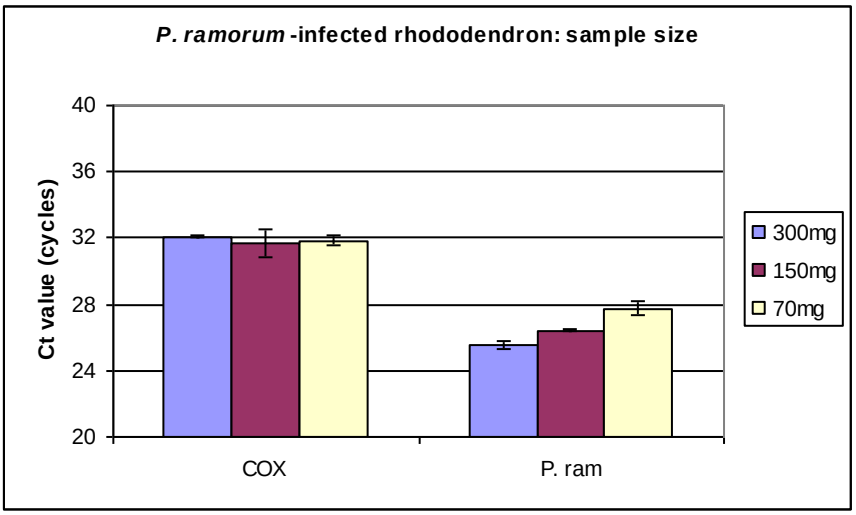


Figure 10. COX and *P. ramorum* real-time PCR results for *P. ramorum*-infected rhododendron samples after 120s shaking.

In addition, samples consisting of different amounts of infected material (100% necrotic) were tested with shaking for 120s (**Figure 10**). The *P. ramorum* Ct values decreased as the sample size was increased, while the COX Ct values were similar for all three sample sizes tested.

Conclusions

The optimal amount of shaking is different for rhododendron and camellia, which have relatively tough leaves, and viburnum, which has much softer leaves which are more easily disrupted. Routine testing of other hosts is likely to require initial experiments to determine the optimal amount of tissue disruption for extraction of DNA using this method. Adequate disruption of viburnum leaves for optimal DNA extraction can be achieved by shaking for 30s, and shaking for longer than this results in the release of inhibitors which are likely to prevent PCR amplification. A sample which has been over-disrupted can be diluted before running the LFD to mitigate the effect of

inhibitors that have been released. The optimal amount of shaking for samples of rhododendron and camellia is approximately 1 – 2 minutes for most samples; shaking for longer than this did not generally improve the real-time PCR results, and shaking for 3 minutes or longer resulted in failure of PCR amplification in some samples.

More efficient DNA extraction from rhododendron and camellia was achieved if smaller pieces of tissue were used (approximately 0.5cm^2). The use of larger ball bearings did not expedite disruption of the tissue. Using bottles made of polystyrene instead of polyethylene resulted in more efficient disruption of rhododendron leaf, but also faster release of inhibitors; the use of this type of bottle could be considered for testing tissue that is more difficult to disrupt than rhododendron or camellia leaf, such as woody stems.

It is likely that sample size and shaking speed will vary substantially between samples. Sample size was not found to be critical and small samples (70mg, corresponding to approximately 1.5cm^2 of rhododendron leaf) gave acceptable results for real-time PCR detection of both pathogen and host DNA, although in general lower Ct values were obtained using larger samples. DNA extraction was slightly more efficient with vigorous shaking, but optimal real-time PCR results could still be obtained using a slower shaking speed if the period of shaking was extended by around 30s.

Recommended conditions for LFD extraction of DNA from rhododendron, camellia and viburnum:

	Minimum shaking	Optimal shaking	Maximum shaking
Rhododendron	>30s	60-150s	Do not exceed 180s
Camellia	>30s	60-150s	Do not exceed 180s
Viburnum	>15s	30-45s	Do not exceed 60s

- Shake vigorously for the shorter amount of time, or increase the amount of time if shaking less vigorously.
- Small (approximately 0.5cm x 0.5cm) pieces of leaf tissue are more efficiently disrupted.
- Use up to 1/2 leaf rhododendron or 1/3 leaf camellia if sufficient symptomatic material is available.