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qPCR methods for quantification of RNC inoculum in the field

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EXECUTIVE SUMMARY

The Problem

Previous work has successfully developed a reliable qPCR protocol for the detection and quantification of *Phytophthora pluvialis* (McDougal, et al., 2015). This used a hydrolysis probe designed to increase PCR sensitivity at the higher annealing temperatures required to eliminate the amplification of other clade 3 *Phytophthora* species not present in New Zealand. This probe is routinely used in Scion's diagnostic laboratory to confirm the presence of *P. pluvialis* from needle samples. The probe assay is able to detect *P. pluvialis* DNA at levels as little as 0.2 pg, which is more sensitive than using the SYBR-green qPCR that had a 2 pg DNA limit of detection. Therefore it was deemed promising for quantification of inoculum which may be present at low concentrations in the field.

The application of filter membranes to capture zoospores was also explored in the previous study, however it was concluded that further investigation was needed to develop methods that maximise zoospore concentration on the surface of these filter membranes and increase the effectiveness of subsequent DNA extraction. Hence, the objectives of this project were to:

- Investigate the effectiveness of different filter membranes (nylon & PET [Polyethylenterephthalat]) for zoospore capture and concentration.
- Develop effective methods for DNA extraction from these filter membranes.
- Investigate the viability of these techniques when used in conjunction with the *P. pluvialis* probe assay with emphasis on application for accurate and reliable detection and quantification of inoculum in the field.

This Project

Preparation of membranes:

Four different concentrations of zoospores suspended in sterile deionised water (SDW) were filtered through nylon filter membranes (pore size 1 µm) (Membrane Solutions: MS® Nylon Membrane Filter) to give membranes with 10^2, 10^3, 10^4 and 10^5 zoospores/membrane. A similar method was used on the PET filter membranes (pluriSelect Lifescience, pluriStrainer) to prepare surface concentrations of 3.16 x 10^2 zoospores/ membrane (mesh size 1 µm). For both membrane types, negative and positive controls were created by pipetting SDW and *P. pluvialis* genomic DNA (extracted from a pure culture) respectively onto the surfaces of membranes. Each filter membrane was divided into four segments using a sterile scalpel and tweezers, with one segment extracted using each of the three DNA extraction methods below.

DNA Extraction and Purification:

Three extraction methods were trialled on the PET and nylon membrane segments mentioned above: (i) *microwaving* (heat-based disruption), (ii) *sonication* (sound-based disruption) and (iii) *vortexing* (physical-disruption). *Microwaving* involved placing each membrane segment in a sterile 25 ml beaker with 2 ml SDW and microwaving for 30 seconds. For the *sonication* method, membrane segments were placed in 50 ml tubes and sonicated with zirconia/silica beads (0.5 mm) in 2 ml SDW for 3 minutes at settings: 30% power, 21°C, pulse on 3.0 seconds and pulse off 1.0 second. *Vortexing* involved continuous vortexing of membrane segments in 10 ml tubes with 2 ml SDW and zirconia/silica beads (0.5 mm). After each extraction was completed, 1 ml of supernatant was removed and placed in a new sterile microfuge tube (Axygen Max Recovery tubes – low DNA adherence) and stored at 4°C until further analysis. A Chargeswitch kit (Invitrogen) was used to purify each raw extract produced using the above techniques on nylon membranes (extracts of samples with original concentration [10^5] and all controls) and PET membranes (all extracts). The FastDNA kit (MP Biomedicals) was used on the remaining segment from each PET filter as an additional method for DNA extraction, and unpurified raw extracts were used in subsequent analysis.

PCR:

All PCR reactions were done with an Eco™ Real-Time PCR System (Illumina Inc., CA, USA). Hydrolysis probe assays were performed using TaqMan® Environmental Master Mix 2.0 (Life Technologies), and *P. pluvialis* specific primers (Ypap2F/Ypap2R) alongside the *P. pluvialis* specific probe mentioned above. Real-time PCR reactions were done with PerfeCTa™ SYBR® Green FastMix™ (Quanta Biosciences, Gaithersburg, MD, USA) and primer sets ITS-4/ ITS-6 or Ypap2F/Ypap2R.

- The hydrolysis probe assay was performed with the undiluted raw extracts produced by the three extraction methods on both membrane types.
- The raw extracts (diluted 1/10 and undiluted) produced using the FastDNA kit on the PET membranes were also tested, in this way, along with all undiluted raw extracts purified with the Chargeswitch kit.
- Additionally, the SYBR-green PCR (primers ITS-4/ITS-6) was conducted with the undiluted purified extracts from the PET filters to compare its sensitivity with that of the probe assay.
- The sensitivity of the SYBR green ITS PCR was also compared to that of the SYBR green assay with Ypap primers.

Key Results

There was no amplification in the real-time qPCR with *P. pluvialis* probe for any of the undiluted raw extracts from nylon filter membranes produced using *sonication, vortexing* or *microwaving* as extraction methods. No amplification was observed when the PCR was repeated using purified 10^5 samples.

Diluted (1/10) and undiluted DNA extracted from PET filters using the FastDNA kit did not amplify in the real-time PCR with *P. pluvialis* probe, therefore this method was not considered for further tests. Undiluted samples of raw and purified DNA extracted from the PET filters using identical methods to the nylon filters showed amplification at high Cq values (very late in PCR cycling) in the real-time qPCR with *P. pluvialis* probe, which means very little *P. pluvialis* DNA was detected. Further testing revealed consistently high Cq values with little consensus amongst replicates of samples, indicating this method has poor reliability and repeatability. Subsequently, SYBR-green with ITS primers was chosen as a potential alternative assay for comparing sensitivity.

Successful amplification and more consistent Cq values were observed in real-time qPCRs using SYBR-green with the primer set ITS-4/ITS-6. These results were then compared to SYBR-green PCRs using Ypap2F/Ypap2R. Figure 1 demonstrates an overall increase in sensitivity when using ITS primers (lower Cq values) in comparison to Ypap primers. *Sonication* appears to be the best DNA extraction method when paired with ITS primers for amplification, with relatively consistent Cq values and lower Cq means (Fig. 1).

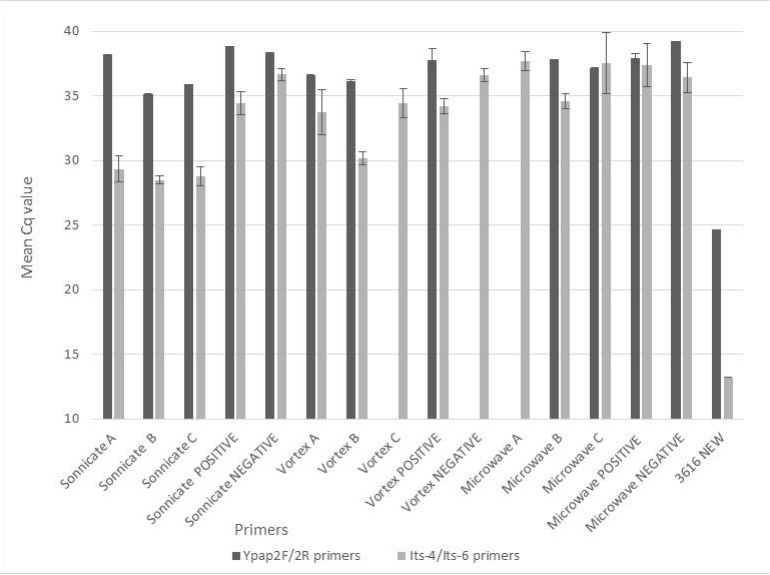

Figure 1: Mean Cq values for SYBR green PCRs using Ypap2F/2R and ITS-4/ITS-6 primers. Error bars are equivalent to one standard deviation. Replicate membranes are identified as A, B or C and POSITIVE and NEGATIVE controls are labelled accordingly. 3616 NEW is a positive *Phytophthora pluvialis* control.

Final Conclusions and Implications of Results

Despite the promising results observed with the ITS primers, the ability of this method to reliably detect inoculum in the field is questionable since these laboratory experiments have used high original concentrations of zoospores on the filters (10^5) and yielded high Cq values (high Cq values indicate low DNA concentrations). It is likely the Ypap and ITS primers were right at the limit of detection, and even then our confidence in detecting *P. pluvialis* inoculum using these primer sets is low. It is not predicted that high zoospores concentrations, such as those use in this study, will be found in samples from the field, therefore these assays are not likely to be sensitive enough to quantify inoculum from field samples.

Further Work

A reliable method for the detection and accurate quantification of *P. pluvialis* inoculum in the field would be an invaluable asset and progress our understanding of zoospore presence and prevalence in the field in response to climatic changes as well as the effectiveness of control methods to curb disease. It would also lead to rapid diagnostic results, with potential application for use alongside portable qPCR machines.

It is recommended that the level of inoculum in the field is quantified to determine whether the proposed methodology is likely to succeed. This could involve, for example, capturing *Phytophthora* sporangia and/or zoospores using a spore trap (West, et al., 2015) and quantifying as described in (Fall, Tremblay, et al., 2015; Fall, Van der Heyden, et al., 2015). The quantification could be done using the current PCR assays described above, or a genus-based assay already designed and validated in recent literature.

If this was successful then a new probe-based assay for field detection of *P. pluvialis* inoculum could be later developed using the ITS genetic region, and to combat the issues of sensitivity highlighted in this project.

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