Needle disease strategy Research Programme

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Technical Note

High-throughput automated qPCR for increased research outputs from forest pathology trials

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Summary: Needle diseases of *Pinus radiata* caused by species of *Phytophthora* are being increasingly recognised, creating a need for rapid diagnostic screening of numerous samples. Automated and high-throughput capable DNA extraction and qPCR provides the opportunity to expand the capacity of research trial analysis and a potential alternative to laborious isolation and plating. The use of a high-throughput format for qPCR assays targeting *Phytophthora pluvialis* and *Phytophthora kernoviae* was validated on a robotic platform, proving to be consistently more sensitive than isolation. Automated qPCR can therefore be used for detection and quantification of these pathogens with confidence in future research trials.

Introduction

Two species of *Phytophthora*, *P. pluvialis* and *P. kernoviae,* cause needle diseases in radiata pine in New Zealand. Detection, identification and quantification of these species is important in several areas of research and in the development of control strategies. However, the use of isolation for detection and identification of these pathogens is time consuming, resource demanding and often lacks sensitivity. Although collection of isolates is important for ongoing research and diversity studies, more efficient quantitative detection is needed to inform epidemiological studies and ongoing disease monitoring.

Quantitative PCR (qPCR) offers a potential solution to these problems and an opportunity to develop a high-throughput protocol for the detection and quantification of these pathogens. qPCR assays for *P. pluvialis* and *P. kernoviae*, alongside a host reference assay for *P. radiata*, are already established (McDougal et al, unpublished data; Schena et al, 2006; and Chettri et al, 2012).

In this Technical Note we report collaborative work between Scion and Slipstream Automation (Palmerston North) to develop and validate a high-throughput detection protocol for both pathogens. The aims of this study were to (i) establish and validate qPCR analysis for detection and quantification on Slipstream's automated platform (Fig. 1) (ii) establish qPCR methods that are scalable to field studies (iii) recognise the true benefits of high throughput DNA based detection.

Figure 1. Robotic sample handling at Slipstream Automation's facility in Palmerston North.

Method

A concentration series of inoculated pine needle material was used to investigate the sensitivity of established qPCR assays for both *P. pluvialis* and *P.*

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kernoviae on Slipstream's automated system. Pine needles were inoculated with zoospore suspensions of either pathogen and incubated under optimal conditions for symptom development. Resulting symptomatic needles were used to create a concentration series (100%, 75%, 50%, 25%, 10%, 1% and 0% symptomatic material) mixed with the applicable percentage of healthy (un-inoculated) needle fragments. Six biological replicates of each concentration was sent to Slipstream for analysis.

The sensitivity of automated qPCR compared to traditional isolation methods was also investigated. For *P. pluvialis*, 100 inoculated needle fragments and 100 un-inoculated (healthy) needle fragments were plated and incubated under optimal conditions for culture growth. Field collected material was used to compare the sensitivity of both isolation and qPCR detection methods for both pathogens.

Processing of samples for several studies allowed the development of a high-throughput sample preparation protocol. This protocol greatly increases the efficiency of sample processing before downstream automated DNA extraction and qPCR at Slipstream (Fig. 2).

Figure 2. Sample processing for automated DNA extraction: fascicles collected from target trees (1), needles chopped into 1-3mm fragments (2), sub-sample loaded into the plate via funnel (3) and an example extraction plate with partially loaded wells (4).

Results and further work

Analysis of the inoculated needle series on the automated qPCR system achieved consistent detection across biological and technical replicates for *P. pluvialis* and *P. kernoviae* (Figure *3* and Figure *4*). The inoculation protocol for *P.* kernoviae achieved more uniform symptom development than *P. pluvialis* where symptoms were not as homogenous across the inoculated needles. Consistent detection by the automated system was achieved down to 1% diluted inoculated material for *P. kernoviae* and 10% for *P. pluvialis* (Figure *3*). The difference in detection sensitivity between *P. kernoviae* and *P. pluvialis* could be due to differences in infection homogeneity between the zoospore inoculations, higher levels of symptomatic lesions that resulted on the *P. kernoviae* inoculated needles, and/or better sensitivity of the *P. kernoviae* qPCR assay.

qPCR detection rates for *P. pluvialis* were significantly higher than those obtained from plating isolation (Figure *5*), with 60% of inoculated symptomatic material being detected by isolation but 100% detected by qPCR. Importantly, detection rates within samples of naturally infected field material were greater with automated qPCR (Figure *5*), even though a greater amount of needle tissue was used in isolations, and lesions were specifically targeted with this method.

The detection consistency and sensitivity of automated qPCR analysis achieved for *P. pluvialis, P. kernoviae* and *P. radiata* targets in this study provide validation for further research use of this high-throughput diagnostic and quantification tool. Automated qPCR was also found to have higher detection rates than isolation from the same sample material. Full cost-benefit analysis is yet to be undertaken, however automated qPCR methods are scalable to high throughput analysis volumes which would not be practical for isolation plating and therefore provides the opportunity to expand the capacity of research and monitoring programmes as part of integrated disease management systems or biosecurity.

Further work in this area might include;

- Development of a more sensitive qPCR assay for *P. pluvialis*, improving detection at lower abundances
- Development of spore traps that can be combined with the presented assays to monitor and quantify pathogen activity
- Repeated analysis of material, increasing confidence in research applications
- Validation of assays for other target pathogens and assays of other sample types (e.g. spore traps)
- Larger scale operation of field trial sampling
- Pathogen quantification
- Optimisation or automation of sample preparation

References

Chettri, P. et al. (2012). The veA gene of the pine needle pathogen *Dothistroma septosporum* regulates sporulation and secondary metabolism. *Fungal Genetics and Biology* 49: 141-151

Schena, L. et al. (2006). Detection and quantification of *Phytophthora ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina* in symptomatic leaves by multiplex real-time PCR. *Molecular Plant Pathology*, 7: 365-379

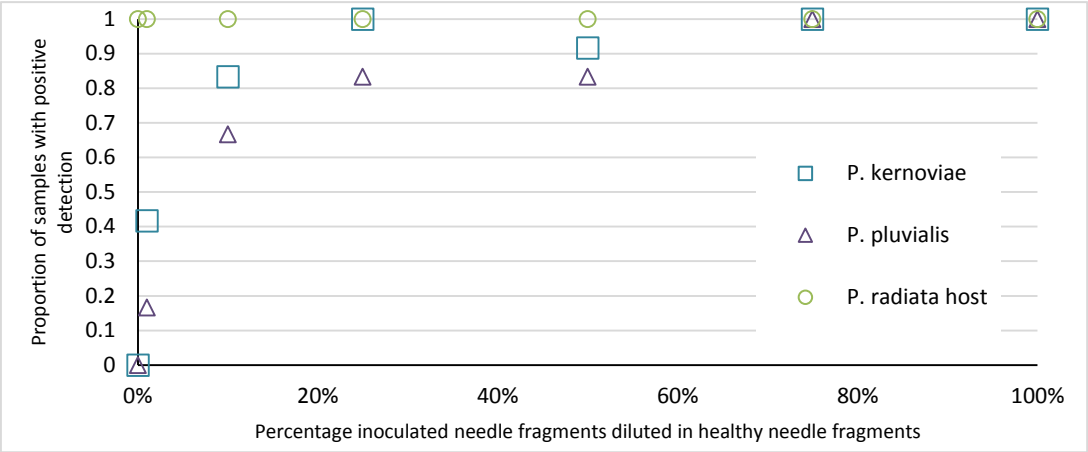

Figure 3. Limits of qPCR detection in *P. pluvialis* and *P. kernoviae* assays. Consistent detection was achieved down to 1% diluted inoculated material for *P. kernoviae* and 10% for *P. pluvialis*.

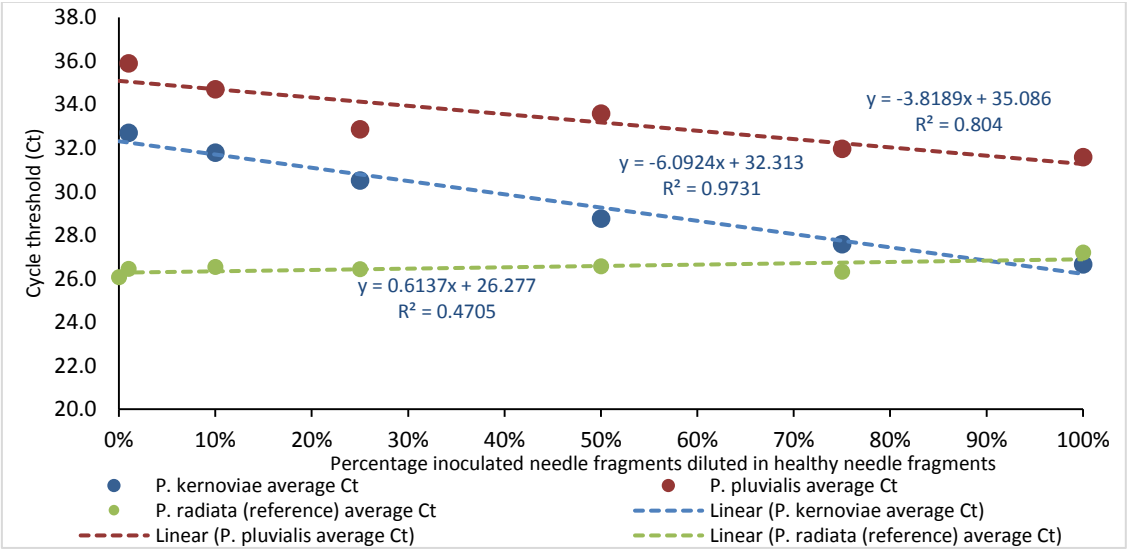

Figure 4. Standard curve for qPCR targeting *P. pluvialis* and *P. kernoviae*. With greater validation, line equations like these may be used to calculate % infection of plant material from the cycle threshold (qPCR result).

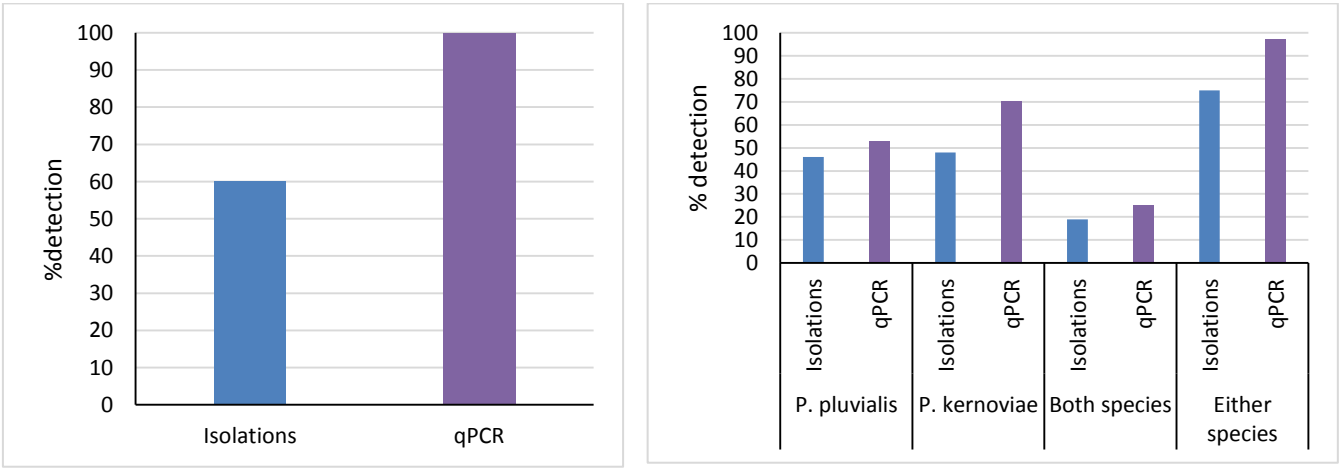

Figure 5. Detection rates of *P. pluvialis* from isolation and qPCR of inoculated needles (1) and both *P. pluvialis* and *P. kernoviae* in field collected needles (2).