Needle disease strategy Research Programme

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Understanding red needle cast inoculum dynamics in the field

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EXECUTIVE SUMMARY

1.1 The Problem

Three winters of baiting to detect the presence of *Phytophthora pluvialis* and *P. kernoviae* from red needle cast (RNC) affected forests have provided qualitative data showing that *P. pluvialis* inoculum is produced across the year with a loose association to rainfall. However, the qualitative nature of baiting provides little data on the environmental thresholds that trigger inoculum production and increased disease pressure. Such analysis requires a means of quantifying inoculum densities.

The aim of this project was to establish qPCR protocols for detection and quantification of *P. pluvialis* in environmental samples using filtration, similar to those designed for other forest pathogens (Scibetta, et al., 2012) and (Schweigkofler, et al., 2004).

The objectives of this work were to establish a quantitative qPCR-based assay for assessing inoculum loads from canopy drip or water filtrate from forest canopies. This involved three stages:

- Develop and demonstrate a hydrolysis probe or SYBR qPCR assay for quantifying *P. pluvialis* DNA.
- Demonstrate the application of the assay to filter membranes and assess the sensitivity limits of detection from membranes from zoospore suspensions.
- Demonstrate the application and relative sensitivity of the assay in detecting inoculum from the surface of infected needles.

1.2 This Project

Previously, species-specific PCR primers were designed for *P. pluvialis*. However, upon testing with further clade 3 *Phytophthora* species, which are not present in New Zealand, the assay conditions required a much higher PCR annealing temperature to increase the stringency of the PCR and therefore exclude amplification from those clade 3 *Phytophthora* species. Increasing the annealing temperature also had the effect of decreasing the sensitivity of the PCR assay, meaning that detection of *P. pluvialis* required a much higher level of DNA to be present in the sample. In an attempt to overcome this, a hydrolysis probe was designed, which should increase the sensitivity of the assay (R. McDougal, unpublished data).

The specificity of the new hydrolysis probe was determined with DNA from all clade 3 *Phytophthora* species, a range of other *Phytophthora* and *Pythium* species as well as some fungal species associated with pine. Reaction conditions were optimised to ensure no amplification from any of those species tested (data not shown).

To determine the best method for quantifying zoospores from membranes and environmental samples hydrolysis probe and SYBR qPCR assays were compared. Standard curves were established using genomic DNA extracted from pure cultures of *P. pluvialis*. Real-time PCR reactions were performed using PerfeCTa™ SYBR® Green FastMix™ (Quanta Biosciences, Gaithersburg, MD, USA) on an Eco™ Real-Time PCR System (Illumina Inc., CA, USA) and hydrolysis probe assays were performed using (TaqMan® Environmental Master Mix 2.0, Life Technologies).

To test the sensitivity of the hydrolysis probe qPCR assay in detecting zoospores deposited on membranes, zoospores were mounted on 1 µm membranes (Millipore) at concentrations from 0, to 1.6 x 10^5 zoospores per cm^2. DNA was extracted from the membranes and the hydrolysis probe qPCR assay was used to quantify the number of spores present.

To test the sensitivity of the assay in detecting inoculum from infected needle surfaces, susceptible *Pinus radiata* clones were inoculated with *P. pluvialis in planta.* Twelve plants were inoculated in total and for each plant, one branch per tree was inoculated with 5000 zoospores per ml, using the *in planta* inoculation protocol as described in Ganley et al 2015[1]. Eleven days after inoculation sporangia and mycelium were observed on the needles, and the needles were washed and shaken to remove surface inoculum. The suspensions prepared from the needles, one suspension per inoculated branch, were filtered through 1 µm membranes. DNA was extracted from four of these samples and the resulting DNA was tested using the hydrolysis probe qPCR assay. The hydrolysis probe qPCR assay was completed using undiluted and diluted (1/10) DNA.

1.3 Key Results

Comparison of the hydrolysis probe and SYBR qPCR assays showed that the hydrolysis probe assay was more sensitive and was determined to be the best method for quantification of zoospores, as the SYBR assay could typically detect 2 pg of DNA (data not shown) whereas the hydrolysis probe assay could detect 0.2 pg of DNA. Standard curves produced for the hydrolysis probe assay showed a high correlation between DNA concentration, using genomic DNA, and Cq values (Figure 1).

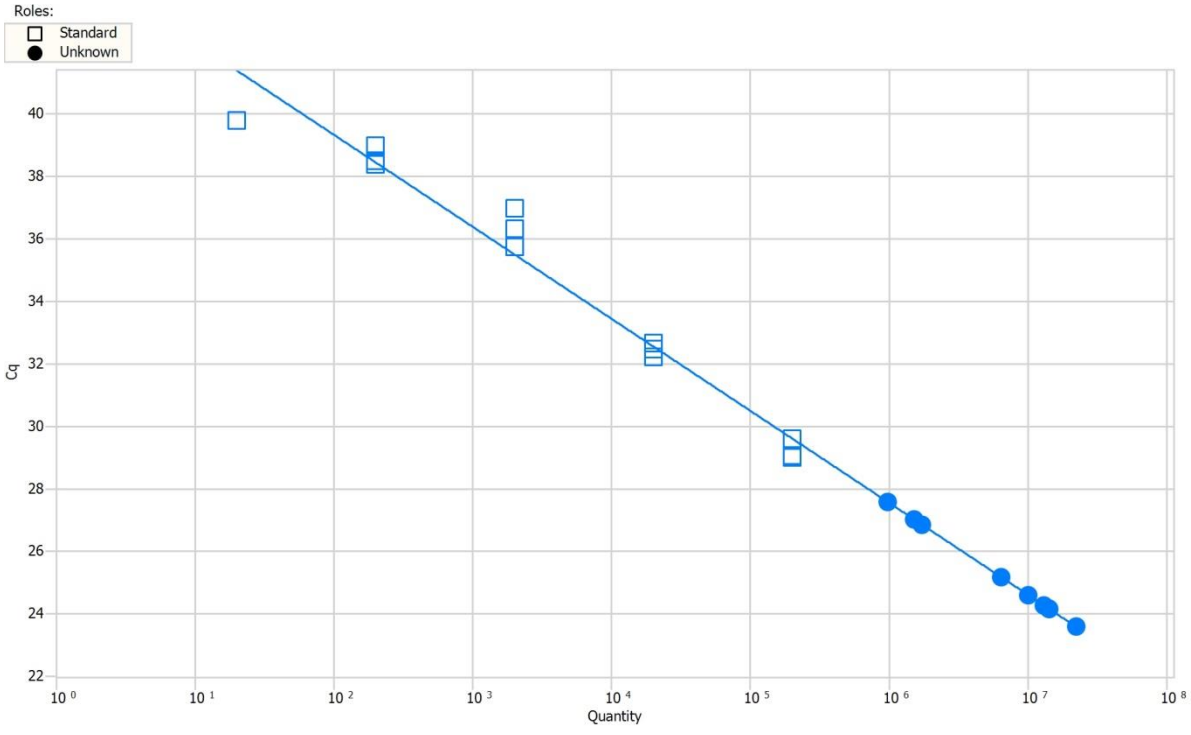

Figure 1. Standard curve of *P. pluvialis* DNA concentration. This standard curve represents one of four produced for the hydrolysis probe assay. Concentrations of DNA at 2x 10^1, 2x 10^2, 2x 10^3, 2x 10^4 fg (x axis) were detected (blue 'standard' boxes). Six positive control DNA samples (blue 'unknown' circles) were also included. Lower Cq values indicate higher concentrations of DNA.

[1] Ganley R, P Scott and M Bader. (2015) Minimum *Phytophthora pluvialis* zoospore concentration for red needle cast infection *in planta*. Scion Sidney Output 56054.

Zoospore concentrations between 1.6 x 10^5 to 3.3 x 10^4 zoospores per cm^2 mounted on 1 µm membranes were able to be amplified using the hydrolysis probe qPCR method, but no DNA was detected from concentrations below 3.3 x 10^4 zoospores per cm^2.

No *P. pluvialis* DNA was detected in any of the needle-wash suspensions mounted on 1 µm membranes.

1.4 Implications of Results

The results of this work show that the hydrolysis probe qPCR assay is functional and sensitive, and able to detect low concentrations of genomic DNA. However, using the current membrane method described in this project, over 33,000 zoospores would be required to get enough genomic DNA that can be detected using the hydrolysis probe assay. Results from the needle-washes suggest the amount of zoospores present is below this level.

1.5 Further Work

A quantitative method to detect *P. pluvialis* zoospores would significantly improve our understanding of inoculum dynamics and could also be used to assess the effectiveness of control products in the field. At this stage the limitation in using the hydrolysis probe qPCR assay for this purpose relates to the number zoospores that are being mounted to the membranes and/or the amount of DNA that is being extracted from them.

For this procedure to be successful further work is required to concentrate zoospores onto the membrane and to optimize the amount of DNA that is extracted from the samples. It is possible that the DNA from zoospores is readily degraded and snap-freeing of samples, storage at -80°C, along with the use of a cryo-grinder for DNA extraction, could improve the yield of DNA obtained.

1.6 Acknowledgments

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1.7 References

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