Minimum zoospore concentration for red needle cast infection

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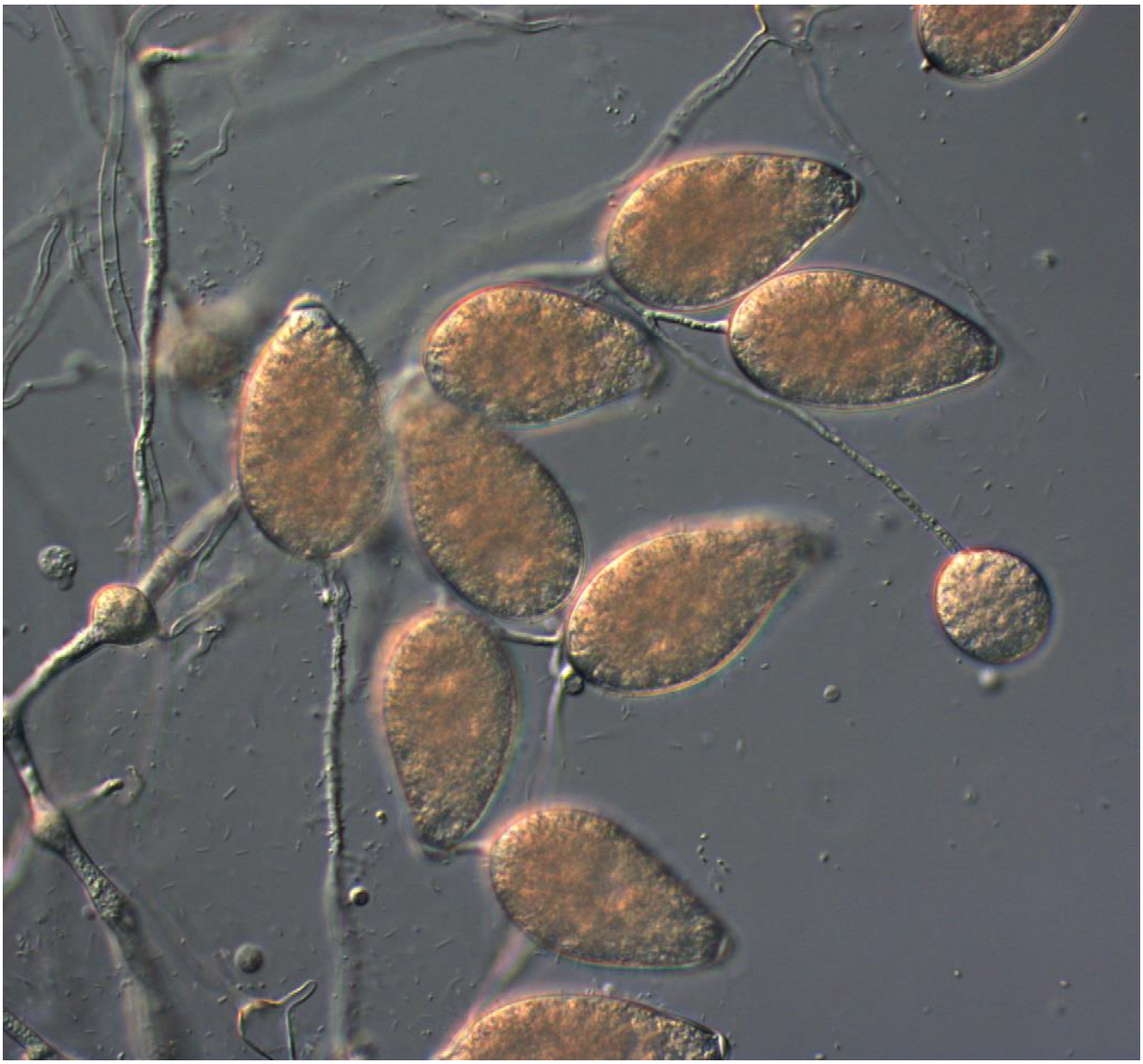

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EXECUTIVE SUMMARY

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The problem

Artificial inoculation methods for infecting *Pinus radiata* needles with *Phytophthora pluvialis* have been developed. The minimum zoospore concentration needed to get reliable infection using these artificial inoculation methods is not known. Knowing this lower limit will allow researchers to substantiate findings from artificial inoculation assays, contribute to our understanding the minimum zoospore levels required for field infection to occur and inform decision making about the biology of the organism.

This project

The objective of this study was to determine the minimum *P. pluvialis* zoospore concentration required to get infection in a detached needle assay. Needles from three different *Pinus radiata* clones were inoculated with four different zoospore concentrations plus a control, using 10 ramets per clone per treatment. Needles were scored for lesions after 11 days incubation. A generalised least-squares model (GLS) was used to analyse the average total lesion length per fascicle, and a bias-reduced generalised linear model (GLM) was used to analyse the percentage of needles and fascicles infected.

Key Results

High *P. pluvialis* zoospore concentration is required to achieve high levels of infection., The degree of infection at low inoculum concentration is strongly influenced by the clone. The use of average lesion length provided slightly different results from the percentage of infected fascicles or needles and could potentially be assessing different aspects of the epidemiology of *P. pluvialis*. There was no significant difference in lesion development between ramets of the same clone but there was a significant difference between clones.

There was a significant decrease in both lesion length and the percentage of lesioned fascicles or needles between 200 and 5000 zoospores per ml concentrations for clones A and C, but no significant difference between concentrations of 0, 50, or 200 zoospores per ml. For clone B there was no significant difference in the percentage of lesioned fascicles or needles between zoospore concentrations of 5000, 200 and 50 zoospores per ml, and there was no significant difference in lesion length between any of the zoospore concentrations.

Based on these results it would not be recommended to undertake artificial inoculations with concentrations using 200 or fewer zoospores per ml. From a risk assessment perspective, these results show that while infection could occur at all concentrations, there was no significant difference in infection up to concentration of 200 zoospores per ml.

Implications of Results for Client

Research into the control of RNC depends on artificial inoculation to test treatment efficacy because of the risk of relying on the vagaries of weather and natural inoculum for disease development. For artificial inoculations, knowing what zoospore concentrations can produce reliable results allows researchers to maximise operational screening. This research is a first step towards determining a zoospore concentration threshold.

The results are important for understanding RNC in the field and how disease severity could be reduced through control methods that target a reduction in inoculum production. As RNC lesions developed at very low zoospore concentrations there is unlikely to be a minimum

number of zoospores required for infection to occur. However, the significant difference in lesion length at concentrations below 200 zoospores per ml suggests inoculum density has an impact on disease severity.

Further Work

These results are from a detached needle assay. It is imperative that the same experiment is completed *in planta* and comparisons between the two methods are used to validate the detached needle assay results as well as the minimum zoospore concentrations required for infection. Testing concentrations between 200 to 5000 zoospores per ml would be recommended.

Further testing and research into the physiological differences between host material of different ages is required to elucidate any influence this has on disease progression and expression. Determining what mechanisms are responsible for the lesion formation changes observed between the different zoospore concentrations tested, and also for concentrations above 5000 zoospores per ml, would also be recommended.

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Introduction

Red needle cast (RNC), caused by *Phytophthora pluvialis,* is an emerging disease of radiata pine in New Zealand (Dick *et al.* 2014). Artificial inoculation methods for *P. pluvialis* have recently been developed which include both *in planta* inoculations and detached needle assays (Dick *et al.* 2014; Williams unpublished data[1]). Grafted radiata pine material, mature trees and cuttings have been successfully inoculated with *P. pluvialis* using these techniques; however, artificial inoculation of seedlings younger than 18 months old has not been successful. These results fit with field observations where RNC symptoms are most commonly seen on older trees. Although younger material (less than three years old) can become infected, infection is rare and appears to occur on sites with very high inoculum loading.

The artificial inoculation methods use active zoospore solutions for inoculum and a standardized method of zoospore production has been developed (Williams unpublished data[1]). Concentrations of between $5x10^3$ - $1x10^4$ zoospores per ml were initially used for inoculations, and since the experimental component of this experiment was conducted, successful inoculations, with comparable lesion lengths, have been completed using concentrations of 1000 zoospores per ml (Rolando unpublished data[2]). However, the lower limit for statistically significant infection using these artificial inoculation methods is not known. Knowing the minimum zoospore concentration where statistically significant infection occurs will allow researchers to substantiate findings from artificial inoculation assays, contribute to our understanding the minimum zoospore levels required for field infection to occur and inform decision making about the biology of the organism. Furthermore, this information can be used for risk assessments to determine the minimum zoospore levels required for infection to occur.

The objective of this study was to determine the minimum *P. pluvialis* zoospore concentration required to get infection in a detached needle assay.

Materials and Methods

Plant material

Three different *Pinus radiata* clones were used in this study, with ten ramets used per clone. Clones A (286.763) and C (889.701) were from grafted material prepared in 2012 from buds taken from mature trees in the field. Clone C has been tested previously against *P. pluvialis* and is known to be susceptible. The susceptibility of clone A against *P. pluvialis* was unknown. Clone B (ortet 6/3 from cross 869.021 x 880.655) were from cuttings prepared in 2012 from an ortet sown in 2011. Clone B is known to be tolerant to *Dothistroma septosporum* (estimated breeding value 30) and in a previous study, siblings (clones 6/1 and 6/2) of clone 6/3 were found to be susceptible to *P. pluvialis* (Ganley and Bader unpublished data[3]). All plant material was propagated and maintained at Scion under standard nursery conditions.

[1] Williams, N. (2013*). Red needle cast SOP*. Scion internal report (SIDNEY output 51194).

[2] Rolando, C, Williams, N., Bader, M. (2014*). Efficacy and persistence of phosphite for control of RNC in* Pinus radiata. Scion internal report.

[3] Ganley, RJ and M Bader. 2013 Milestone 3 (c), Tasks 3 & 4: Test best treatments in young plants against the foliar disease red needle cast. Scion report output 51311.

Detached needle inoculations

Zoospore suspensions of *P. pluvialis* were prepared using the red needle cast Standard Operating Procedure (SOP) (Williams unpublished data[1]). Suspensions were cultured and prepared in Scion's Forest Protection laboratory using *P. pluvialis* isolates 242A, 62-1, 4202 and 3626. Zoospore suspensions produced from each isolate were combined and diluted to form 20, 50, 200, and 5000 zoospores per ml. Autoclaved pond water was used for the control.

Fifty healthy fascicles were harvested from each plant with the bases intact. Ten fascicles were then placed into labelled 50 ml falcon tubes with the fascicle ends down. Inoculum was added to each falcon tube, comprising either 10 ml of *P. pluvialis* at the four different zoospore concentrations or pond water for the control. Needles were left in the inoculum for 20 hours at room temperature.

The following day, the fascicles were carefully removed from the inoculum and laid out using forceps in damp chamber trays, with the fascicles approximately 1 cm apart. Trays were sealed with cling-film and incubated in a growth room for 11 days at 17°C, with a 14 hr day and 10 hr night lighting regime.

Phytophthora pluvialis lesion assessments

The ten fascicles per treatment were assessed after 11 days incubation in the chamber trays. The number of needles present, the length of each lesion on each individual needle, and the total number of lesions per needle were recorded.

A selection of needles displaying RNC-like symptoms from controls and *P. pluvialis*-inoculated needles were tested for the presence of *P. pluvialis*. Lesions were sectioned from the needles, surface sterilised with 70% ethanol for 30 sec, and rinsed twice with sterile distilled water. After blotting dry, the needle sections were plated onto PARP agar (Williams unpublished data[1]) and incubated at 17°C for 5-7 days before assessment.

Data preparation

First the data was transposed into long format and then the cumulative lesion length of lesions per fascicle was derived, followed by the computation of the average per 10 fascicles per ramet and finally the average per ramet.

In order to analyse the data as a percentage of lesioned needles, a binary response variable had to be created first by calculating the number of needles per fascicle and then the number of lesioned needles per fascicle. This information was used to construct a two-column matrix holding the number of 'successes' (lesioned needles) and the number of 'failures' (intact needles) as response variable for a binomial regression model. For an analysis of the percentage of affected fascicles, any fascicle with at least one lesioned needle was considered infected.

Data analysis

A generalised least-squares model (GLS) fitted by restricted maximum likelihood was applied to analyse the average cumulative lesion length per fascicle using R version 3.0.2 (R Development Core Team 2013, R-package nlme). The model contained zoospore concentration, host clone and their interaction as fixed effects. The significance of a ramet random effect was tested by comparing the GLS model (model without random term) with a linear mixed-effects model (model including a ramet random term) using a likelihood ratio test (Zuur et al. 2009). Graphical model validation tools were used to check the model assumptions of variance homogeneity and normality (plots of standardised residuals vs. fitted and explanatory variables and quantile-quantile plots). Variance heterogeneity was detected and modelled applying a combination of an

exponential variance structure using the fitted values as variance covariate and clone as grouping variable and a constant variance function using clone as grouping variable (varExp and varIdent functions in R-package nlme).

The data set was also analysed as percentage data using a bias-reduced generalised linear model (GLM) with binomial errors and logit link (R-package brglm). The occurrence of quasi-complete separation (statistical phenomenon where the response variable separates one or more predictors to a large degree, here because of the nearly perfect control treatment), required the use of the bias-reduction method developed by Firth (1993).

For all models, the significance of the fixed term was assessed using a backwards selection procedure based on likelihood ratio testing (Zuur et al. 2009). A significant interaction term was followed up with a multiple comparison procedure using Tukey contrasts and the Benjamini & Yekutieli (2001) method to adjust P-values for multiple testing (R-package multcomp).

Results

All three clones were able to be successfully infected with *P. pluvialis* (Figures 1-3) using the detached needle assay. Re-isolation of *P. pluvialis* from inoculated needle lesions was successful. *Phytophthora pluvialis* was not isolated from any of the control needles tested.

Lesion length

The likelihood ratio comparison between models with and without a random term showed that ramet did not have a significant random effect (L = 1.02 × 10-7, df = 1, P = 0.499). There was a significant zoospore × host clone interaction (L = 31.74, df = 8, P < 0.001) indicating that *P. pluvialis* zoospore concentrations had a significant effect on lesion formation that varied among *P. radiata* host clones (Fig. 1). Regardless of host clone identity, cumulative lesion length per fascicle in the control groups and in the plant material treated with 20, 50 and 200 zoospores per ml concentrations were not significantly different from zero (Fig. 1). In clones A and C, the highest zoospore concentration caused significant lesion formation with an average cumulative length per fascicle of 2.7 and 5.3 mm, respectively. However, in clone B not even the highest zoospore concentration produced large enough lesions to yield a significant effect.

Percentage of lesioned needles

When comparing the percentage of lesioned needles per treatment, a significant zoospore concentration × host clone interaction was found (χ^2 = 52.72, *df* = 8, *P* < 0.001). For clones A and C, needles treated with concentrations of 20 – 200 zoospores per ml did not differ significantly from the control and developed fewer lesions compared with the highest concentration. However, for clone B, treatment with 50 zoospores per ml resulted in a significant increase in the percentage of lesioned needles compared to the control (Fig. 2). The highest percentage of lesioned needles was observed when ramets were exposed to 5000 zoospores per ml, this effect was most pronounced in clone C which showed 36 % lesioned needles (Fig. 2).

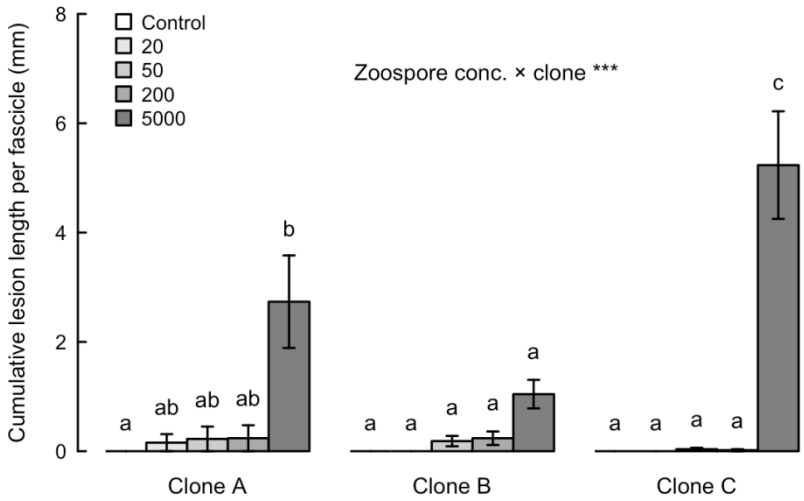

Fig. 1 Cumulative lesion length per fascicle in ramets from three *Pinus radiata* clones that were either inoculated with different zoospore concentrations of *Phytophthora pluvialis* (zoospores per ml) or non-inoculated (control). Different lower-case letters indicate significant differences between plant materials at α = 0.05 (multiple comparison test using Tukey contrasts). Means ± SE, n = 10 ramets, *** P < 0.001.

Percentage of lesioned fascicles

A significant zoospore concentration × host clone interaction was also detected when the proportion of total lesioned fascicles per treatment was used as a response variable (χ^2 = 47.44, *df* = 8, *P* < 0.001).

Similar to the needle-level analysis, zoospore concentrations up to 200 zoospores per ml caused only an insignificant percentage of lesioned fascicles, apart from clone B which showed 24 % lesioned fascicles at 50 zoospore per ml concentrations (Fig. 3). The highest percentage of lesioned fascicles (32-48 %) coincided with the highest zoospore concentration and did not differ significantly across *Pinus radiata* clones (Fig. 3).

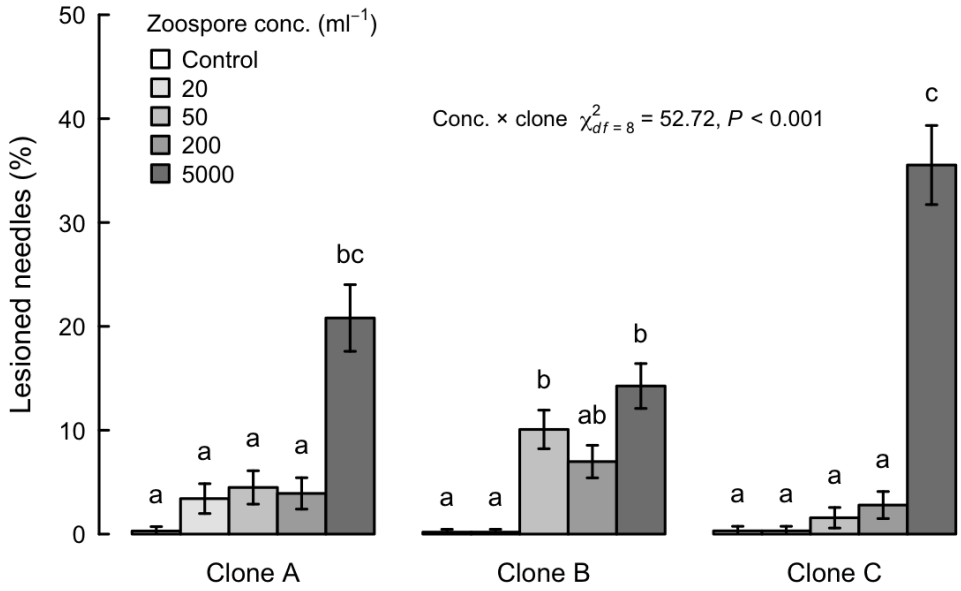

Fig. 2 Percentage of lesioned needles, for three *Pinus radiata* clones, that were either inoculated with different zoospore concentrations of *Phytophthora pluvialis* (zoospores per ml) or non-inoculated (control).Different lower-case letters indicate significant differences between plant materials at α = 0.05 (multiple comparison test using Tukey contrasts). Means ± SE, *n* = 10 ramets, *** P < 0.001.

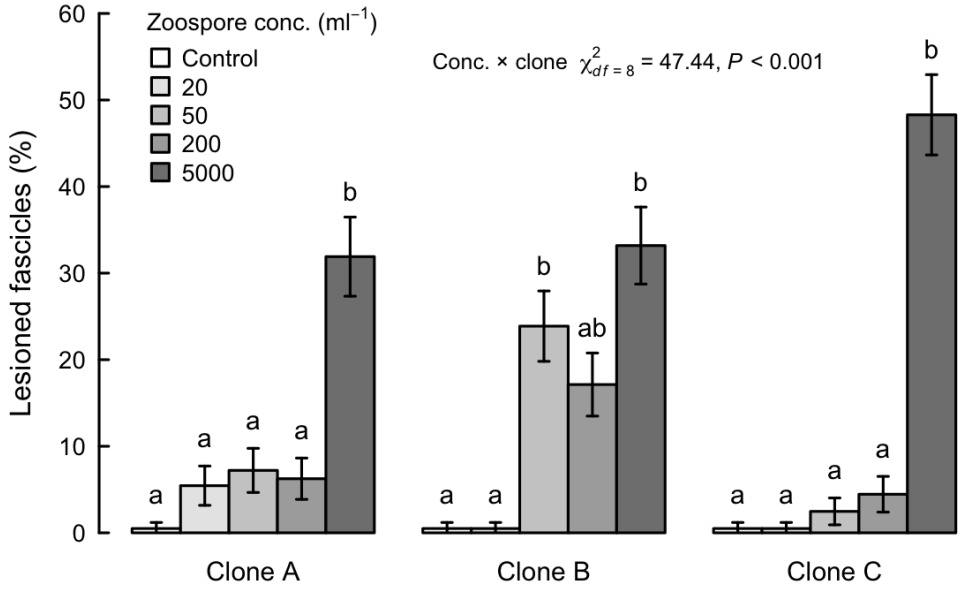

Fig. 3 Percentage of lesioned fascicles, for three *Pinus radiata* clones, that were either inoculated with different zoospore concentrations of *Phytophthora pluvialis* (zoospores per ml) or non-inoculated (control). Different lower-case letters indicate significant differences between plant materials at α = 0.05 (multiple comparison test using Tukey contrasts). Means ± SE, n = 10 ramets, *** P < 0.001.

Discussion

Overall, the results from this experiment show that high levels of *P. pluvialis* zoospore inoculum are required to achieve high levels of infection but that the degree of needle infection at low inoculum levels is strongly influenced by the host plant. The use of lesion length versus the percentage of infected fascicles or needles can provide different results and potentially assesses different aspects of the epidemiology of *P. pluvialis*. There was very little difference between the percentage of lesioned fascicles or needles, suggesting in most cases there is only one needle per fascicle that becomes infected. This means that infection is occurring at a low frequency across numerous fascicles (i.e. one needle per fascicle) versus infection occurring in multiple needles for only a few fascicles.

In this study there was no significant difference in lesion development between ramets of the same clone but there was a significant difference between clones. This difference between clones reflects the variation in resistance to *P. pluvialis* that has been observed previously (Graham et al., unpublished data[4]). For both lesion length and the percentage of lesioned fascicles and needles, clone C was significantly the most susceptible clone used when inoculated with high concentration inoculum. This fits with previous assessments of this clone which have found it to be highly susceptible (Williams, N. pers. comm.). Based on the results in this experiment, clones A and B may be considered moderately susceptible, however further testing against a larger number of host genotypes would be required to confirm this. Interestingly, there is potentially a difference in how these two clones would have been classified if only one assessment method had been used. When lesion length is compared, clone A appeared significantly more susceptible than clone B, whereas for the percentage of lesioned fascicles or needles this difference is not significant. Regardless, for both methods of assessment the ranking of susceptibility is the same.

For both lesion length and the percentage of lesioned fascicles or needles there is a significant drop in infection between 5000 and 200 zoospores per ml concentrations for clones A and C, and no significant difference in infection levels between concentrations of 200 or less zoospores per ml with the control. Based on these results artificial inoculations should not be done with concentrations of 200 or less zoospores per ml. From a risk assessment perspective, these results show that while infection could occur at all concentrations, there was no significant difference in infection up to concentration of 200 zoospores per ml. However, results using clone B were somewhat different.

There was no significant difference in the percentage of lesioned fascicles or needles for clone B between zoospore concentrations of 5000, 200 and 50 zoospores per ml, and for the lesion length assessment there was no significant difference in infection levels between any of the zoospore concentrations and the control. For this clone it is clear that the needles are being infected but the severity of the infection is much lower than that observed for clones A and C. Clone B material were cuttings that were considerably younger than the clone A and C grafted material and it is possible that the difference in results between these clones could be due to age-related physiological differences. These results also may explain why field observations have suggested that younger material is more resistant to red needle cast, with symptoms rarely observed in younger material unless adjacent to heavily infected older stands.

[4] Graham, N, Li, Y, and B Ganley. 2014. Screening RPBC Elites for RNC susceptibility. Scion report output 53570.

Recommendations and future directions

It is recommended that zoospore concentrations of 200 or less zoospores per ml should not be used for artificial inoculation experiments and it is recommended that both lesion length and the percentage of lesioned fascicles are used for assessments. Further work is required to determine how well lesion length and the percentage of lesioned fascicle of detached needles predicts susceptibility.

This experiment show that needles can be infected at very low zoospore concentrations but for artificial inoculations it is still unknown what concentrations between 200 zoospores per ml and 5000 zoospores per ml would provide the best results for assessments. Furthermore the effect of zoospore concentrations above 5000 zoospores per ml on disease expression is also unknown. Results suggest that for clone A and C, a significant change in the interaction between the host and pathogen has occurred at concentrations between 200 and 5000, resulting in the step up increase in lesion extension and percentage of lesioned needles. Further work is required to determine what mechanisms are responsible for these changes and how concentrations above 5000 zoospores per ml impact lesion formation.

Further testing and research into the physiological differences between of host material of different ages is required to elucidate any influence this has on disease progression and expression.

These results are based on a detached needle assay. It is imperative that the same experiment is completed *in planta* and comparisons between the two methods are used to validate the detached needle assay results as well as the minimum zoospore concentrations required for infection.

Acknowledgements

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