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Validation of observations of *P. radiata* susceptibility/resistance to red needle cast in potted plants

Authors: Nari Williams, Natalie Graham and Martin Bader

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FOREST OWNERS ASSOCIATION

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REPORT INFORMATION SHEET

REPORT TITLE	VALIDATION OF OBSERVATIONS OF *PINUS RADIATA* SUSCEPTIBILITY/RESISTANCE TO RED NEEDLE CAST IN POTTED PLANTS
AUTHORS	NARI WILLIAMS, NATALIE GRAHAM AND MARTIN BADER
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EXECUTIVE SUMMARY

Report Title: Validation of observations of *P. radiata* susceptibility/ resistance to red needle cast in potted plants
Authors: Nari Williams, Natalie Graham and Martin Bader

The problem

Advancement of knowledge of RNC has required the establishment of a rapid assay for assessing plant responses to infection by *P. pluvialis.* A detached needle assay has been developed based on similar approaches applied in screening plant susceptibility in other *Phytophthora* pathosystems. However, to date, the detached needle protocol has yet to be cross validated with on-plant inoculations for genetic screening of trees in the field.

This project

Compared the use of detached needle assays against on-plant inoculations for screening for resistance to *Phytophthroa pluvialis* infection.

Key Results

On-plant inoculations resulted in fewer RNC lesions and lower lesion lengths than the detached needle inoculation assay with greater variation in infection levels in on-plant assays.

Implications of Results for Client

The detached needle assay is a useful tool for high throughput screening the susceptibility of plant tissues to infection by *Phytophthora.* This methods provides a high level of disease pressure compared to on-plant inoculations.

For breeding and screening purposes, the detached needle assay provides a means of identifying genotypes with a higher likelihood of resistance to RNC that can be carried on to further field and progeny testing.

Further Work

It is recommended that where appropriate, this assay is used to narrow down selections for repeated on-plant and in-field inoculations and/or exposure to natural inoculum wherever possible.

Validation of observations of P. radiata susceptibility/ resistance to red needle cast in potted plants

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31 March 2015

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Introduction

Red needle cast (RNC) of *Pinus radiata* is caused by *Phytophthora pluvialis* is a relatively new disease in New Zealand forests causing premature casting of *P. radiata* needles. Among the options for integrated disease management, the selection of resistant cultivars has been identified as a priority for breeding programmes aimed at the long term management of the disease. To date, various experiments have established a basic understanding of the infection of plant material with detached needle assays deployed for primary screening of plant material. While these have shown quantitative variation in the response of different genetic lines to infection by *P. pluvialis,* the results of detached needle assays are yet to be validated against on-plant inoculations and plants exposed to inoculum in the field. The use of detached needle assays as a breeding and assay tool and its impact on the genetic improvement of *Pinus radiata* are discussed.

While field studies have indicated a genetic basis to RNC resistance (Dungey and others, 2014), there are several limitations in relying on field assessments to evaluate both treatment and genetic responses to red needle cast. RNC has been shown to vary considerably across sites and seasons making disease assessments unpredictable, potentially costly and highly variable. Under normal conditions, the disease is most severe from canopy closure when plants are 8 years or older, leading to a significant time lag screening clonal material.

Advancement of knowledge of RNC has required the establishment of a rapid assay for assessing plant responses to infection by *P. pluvialis.* A detached needle assay was developed based on similar approaches applied in screening plant susceptibility in other *Phytophthora* pathosystems (Denman and others, 2005; Hansen and others, 2005; Tooley and Browning, 2009; Tooley and Kyde, 2007). Since 2013, this assay has been applied to identify potential genetic resistance to RNC and assessing responses to chemical treatments for RNC control. Correlation between on-plant inoculations and the detached needle assay have shown the utility of this assay for assessing chemical control treatments (Rolando and others, 2014a; Rolando and others, 2014b; Rolando and others, 2014c). However, to date, the detached needle protocol has yet to be cross validated with on-plant inoculations for genetic screening of trees in the field.

Objective

To compare the use of detached needle assays against on-plant inoculations for screening for resistance to *Phytophthroa pluvialis* infection.

Materials and Methods

Plant material - Pinus radiata genotypes

Twelve genotypess were selected from 12 different families within the RPBC "Elite Clone" population (Table 1). Cuttings planted in stool beds in the Scion nursery 2013 were potted up into 10 cm pots in September 2014 and grown in the shade-house for 10 weeks to overcome transplantation stress. Prior to the experiment, needles showing signs of prior infection with needle pathogens and/or browning due to suppression when grown in the glasshouse were removed from each plant to leave a predominance of healthy fascicles. At the time of inoculation, each plant was approximately 35 cm high with a single dominant shoot.

Table 1: Genotypes from the RPBC Elite Clone population used in the experiment

01_07	23_07	43_24
05_08	25_09	47_16
09_19	29_24	57_24
17_11	38_10	61_21

Inoculum preparation

Zoospore inoculum was prepared in accordance with Scion's standard protocol (Williams, 2014). In brief, zoospore inoculum was prepared by growing isolates on carrot agar at 17 ^{o}C for three days. Plugs of agar and mycelium were taken from the leading edge of the colonies, flooded with clarified carrot broth (Erwin and Ribeiro, 1996) in flat bottom flasks and incubated for three days at 17 ^{o}C. The resulting mycelial mats were rinsed thoroughly for 8 hours with deionised water, drained and flooded with 50 ml sterile pond water. These were incubated at 17 ^{o}C in the dark for a further three days before zoospore release was induced with 45 minute intervals at 4 °C in the dark then at room temperature (21-22 ^{o}C) on a light box. Zoospore concentrations were determined using a haemocytometer and standardised to 1 x 10^4 zoospores per ml with sterile pond water. Zoospore suspensions were used within two hours of preparation.

Experimental Design

The experiment was set up as a split-plot design with four blocks applying the inoculation treatment as whole-plot factor. The methodology (detached needles assay *vs.* on plant inoculation) was applied as a sub-plot factor as was the genotype ID as sub-sub-plot factor.

Detached needle assay

Thirty fascicles were collected from each plant, including the controls. Fifteen of these fascicles were exposed to 1 x 10^4 *P. pluvialis* zoospores per ml overnight (18 hours). Controls were exposed to sterile pond water only. Fascicles were placed on trays moistened with wet paper towels and incubated in a controlled environment (17 ^{o}C, 65-70 % relative humidity, 14 h photoperiod) for 10 days. As the inoculated and uninoculated treatments could not be fully randomised due to the risk of cross contamination, the position of paired sets of inoculated and control trays were switched every 24 hours to account for variation within the growth chamber. After 10 days, the needles within each fascicle were separated and lesion length measured.

On-plant inoculations

On the day of inoculation, plants were placed into the growth chamber set with a 14 hour photoperiod, 20/8 ^{o}C temperature and misted for 15 seconds every 10 minutes to maintain

needle wetness. The growth room was fitted with growth lamps with a photosynthetically active spectrum between 400 and 700nm and 181 W/m^2, +/-10%. Overall humidity of the room was maintained above 80% and oscillated to 100% in relation to the misting cycle throughout the course of the trial. Each plant was inoculated with 100 ml of zoospores using a bag on shoot protocol. Each pot was bagged with a zip-lock bag and laid horizontally such that the needles were lying in the inoculum. The bag was crimped and fastened with a rubber band to optimise immersion and left overnight to allow infection to establish. The inoculum was removed while the plants remained bagged for a further 24 hours to favour infection. The plants were arranged in paired blocks (inoculated and H_2O control) on either side of the growth room. As with the detached needle assay, the position of paired sets of inoculated and control plants were switched every 24 hours to account for variation within the growth chamber. After 10 days, fifteen fascicles were harvested from each plant, the needles within each fascicle separated and lesion length measured.

Statistical analysis

A linear mixed effects model (LMM) fitted by restricted maximum likelihood was used to analyse the lesion length data (R version 3.1.2, R Development Core Team 2015, R-package *nlme*, Pinheiro *et al.* 2015). The model contained inoculation treatment, methodology, genotype identity, and their interaction as fixed effects. The nested random term reflected the blocked design and from the highest to the lower levels with the following structure: block/inoculation/method.

The significance of the fixed terms was assessed using a backwards selection procedure based on likelihood ratio tests (Zuur et al. 2009). Graphical model validation tools were used to test the underlying assumptions of variance homogeneity and normality (plots of standardised residuals vs. fitted values and against all explanatory variables to evaluate variance patterns, and quantile-quantile plots to assess the normality criterion). The data showed strong heteroscedasticity, which was modelled using a combination of a constant plus power variance function structure (*varConstPower*, using the fitted values as variance covariate) and a constant variance function with inoculation and genotype identity as grouping factors (*varIdent*).

The significant inoculation $\times$ method interaction was followed up applying a multiple comparison procedure using Tukey contrasts (R-package *multcomp*, Hothorn *et al.* 2008).

Results

Lesion length

As anticipated, significantly larger lesions occurred in plant material inoculated with *Phytophthora pluvialis* compared to the water controls, indicating that inoculation was successful using both methods. The low levels of lesions observed on the control needles in both assays represent background secondary infections with no isolates recovered from the needles plated onto *Phytophthora*-selective media (data not shown). Averaged across genotypes, the baseline level of lesions in the control fascicles of the detached needle assay was lower than that on the plants, with an average of 7 mm baseline lesion presence to 12 mm on the plant (

Figure 1). This indicates an inadvertent selection for healthy needles for the detached needle assay at the start of the trial. However, there is no indication that this impacted the overall trial.

For the needles infected with *P. pluvialis,* the level of infection varied with both methodology (significant inoculation × methodology interaction) and genotype identity (significant inoculation × genotype ID interactions, Table 2 and Table 3). There was a significant interaction between genotype and inoculation method, indicating that the detached needle assay impacts the level of susceptibility of some genotypes more than others (Table 2). This is readily observed for genotypes 09_19, 23_07, 57_24 and 61_21 which all showed moderate levels of infection in the on-plant assay, but were relatively susceptible in the detached needle assay.

In the inoculated material, lesions on detached needles were 20 % larger compared to intact plants (90 mm vs. 75 mm, respectively;

Figure 1) indicating higher rates of infection with the detached needle assay compared to the on-plant inoculation. Lesion length varied more strongly in the on-plant inoculation (

Figure 1).

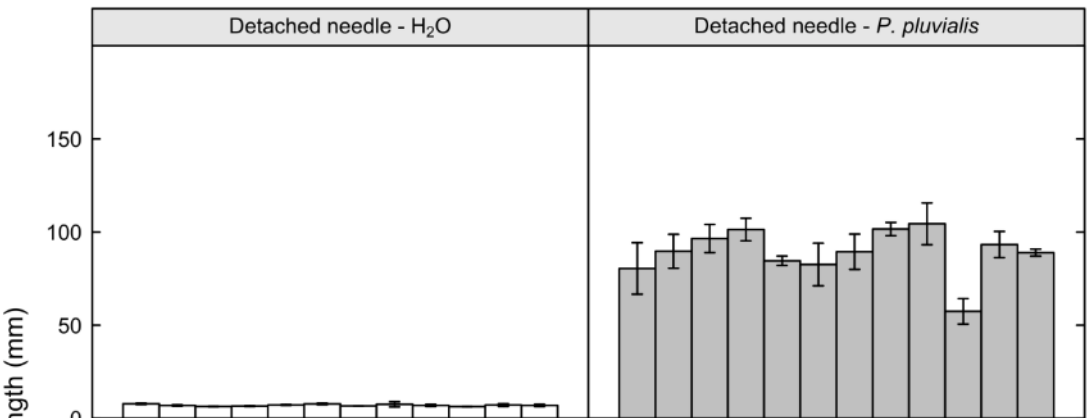

Table 2: Results from model comparison to arrive at the most parsimonious model. Starting with highest interaction, each term is dropped in turn and the respective models are compared using the AIC and likelihood ratio tests until only significant terms remain in the model. Terms that have been statistically significant in a previous round (in the presence of other terms) are subsequently tested again in a new backwards selection round (see third round).
AIC = Akaike's Information Criterion, logLik = log Likelihood, *L* = Likelihood ratio, *DF* = Degrees of freedom of the *L* statistic, *P* = *P*-value.

Figure 1: Average lesion length per fascicle in *Pinus radiata* needles inoculated with *Phytophthora pluvialis* in a detached needle assay (top row) or in an on-plant inoculation (bottom row). Left panels (white bars) show the H_2O control, right panels (grey bars) show the *P. pluvialis* inoculations.

Dropped term	AIC	logLik	*L*	*DF*	*P*	
First round						
None	1169.6	-507.8				
Inoc × method × genotype	1151.5	-509.7	3.92	11	0.972	
Second round						
None	1151.5	-509.7				
Method × genotype	1135.0	-512.5	5.52	11	0.903	
Inoc × genotype	1151.7	-520.9	22.21	1	0.023	*
Inoc × method	1193.4	-531.7	43.89	1	< 0.001	***
Third round						
None	1135.0	-512.5				
Inoc × genotype	1136.4	-524.2	23.34		0.016	*

Inoc × method	1161.5	-526.7	28.44	< 0.001	***

Parameter	DF_{num}	DF_{den}	*F*	*P*	
Intercept	1	154	242393.41	< 0.001	***
Inoc	1	3	1290.87	< 0.001	***
Method	1	6	1088.14	< 0.001	***
Genotype	11	154	5.61	< 0.001	***
Inoc × method	1	6	25.56	0.002	**
Inoc × genotype	11	154	5.18	< 0.001	***

Lesion number

Significantly more lesions occurred in plant material inoculated with *Phytophthora pluvialis* compared to the controls, but this effect varied with both methodology and genotype identity (significant inoculation × method × genotype ID interaction, Table 4 and 5). Across genotypes there were on average 3.2 lesions in the detached needle assay and only 1.7 lesions in the on plant assay (Figure 2). Lesion numbers showed less variation in the detached needle assay compared to the on plant assay (Figure 2). The results of a post-hoc comparison contrasting the two methodologies within treatment and genotype are given in Table 4.

Table 3: ANOVA table of the final model (DFnum = numerator degrees of freedom, *DFden* = denumerator degrees of freedom, *F* = *F*-value, *P* = *P*-value).

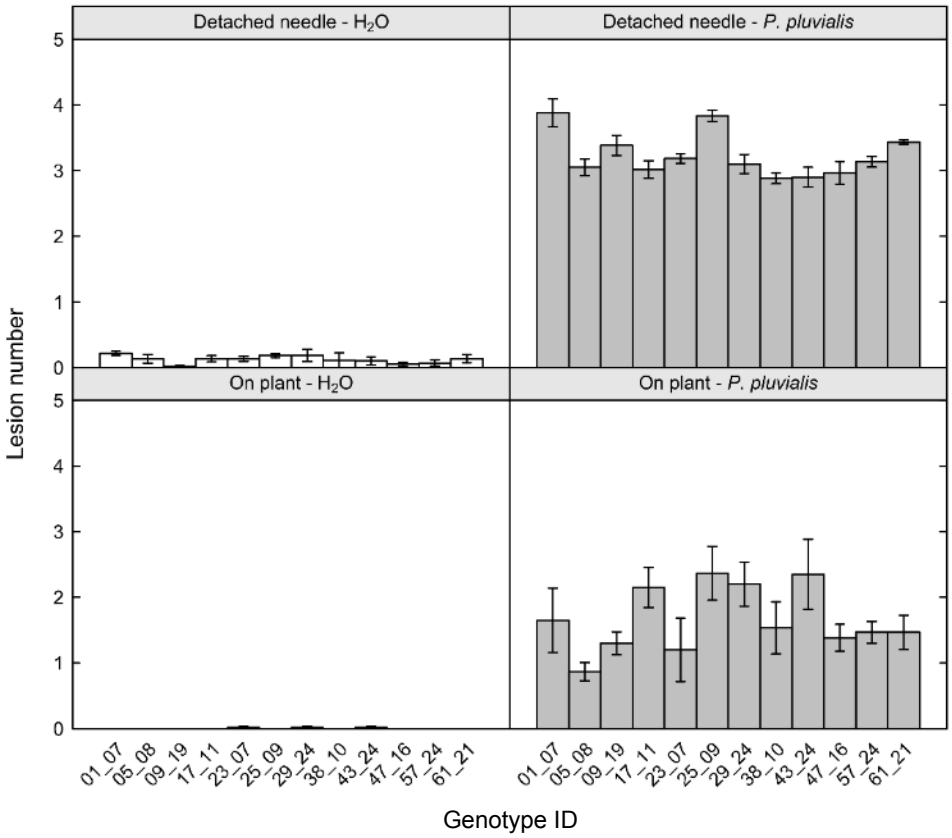

Figure 2: Average lesion number per fascicle in Pinus radiata needles inoculated with Phytophthora pluvialis in a detached needle assay (top row) or in an on plant inoculation (bottom row). Left panels (white bars) show the H2O control, right panels (grey bars)

Table 4: Results from a backwards selection procedure applied to arrive at the most parsimonious lesion number model. AIC = Akaike's Information Criterion, logLik = log Likelihood, L = Likelihood ratio, DF = Degrees of freedom of the L statistic, P = P-value.

Dropped term	AIC	logLik	*L*	*DF*	*P*	
First round						
None	-168.6	139.3				
Inoc × method × genotype	-165.4	126.7	25.15	11	0.008	**

Inoculation method comparison

Plotting the cumulative lesion lengths derived from the detached needle assay against the ones observed in the corresponding genotypes in an on plant inoculation yielded a nonlinear pattern Figure 3. A comparison between a linear regression model and a nonlinear least squares model based on Akaike's Information Criterion (AIC) indicated that the nonlinear model is more appropriate (AIC_{linear} = 94.6, $AIC_{nonlinear}$ = 88.3, the lower the AIC the better the model, a difference in AIC values greater than 5 provides evidence for the superiority of the model with the lower AIC). The parameter estimates are given in Table 5.

When taking the lesion lengths from the on plant inoculation as a reference, the observed nonlinearity suggests that the detached needle assay tends to overestimate the cumulative length of smaller lesions and underestimates the cumulative length of larger lesions.

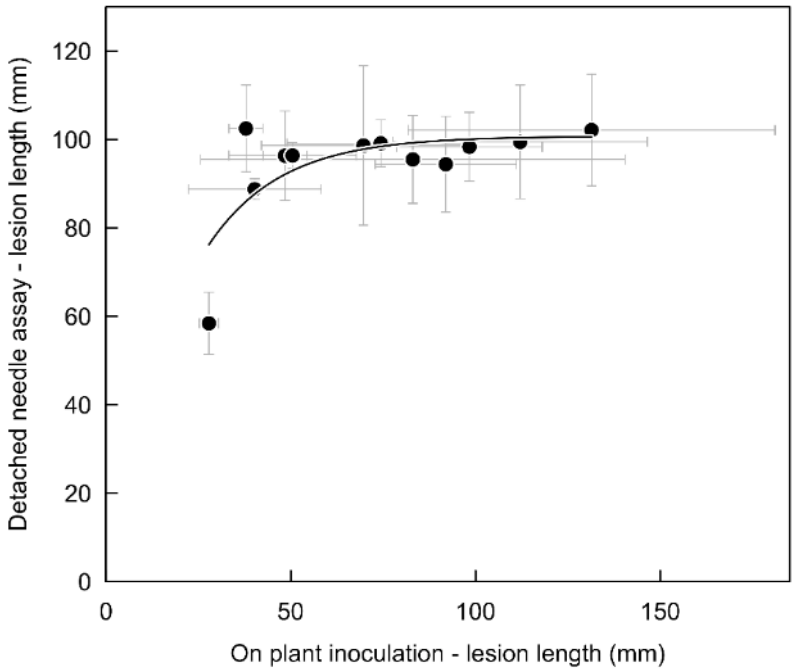

Figure 3: Cumulative lesion lengths obtained from detached needle assay as a function of the cumulative lesion lengths derived from an on plant inoculation approach. The data shown is confined to the *Phytophthora*-inoculated samples. Error bars denote standard errors of the mean for the lesion length observed in on plant inoculations (x-direction) and the lesion length seen in the detached needle assay (y-direction), *n* = 4 blocks (water control excluded).

Table 5: Results from the asymptotic nonlinear regression model. LRC = logarithm of the rate constant.

Parameter	Estimate	SE	*t*	*P*	
Asymptote	100.73	3.68	27.35	< 0.001	***
LRC	-2.98	0.19	-15.39	< 0.001	***

Response variable comparison

Plotting lesion length vs. lesion number also showed a statistically significant nonlinear (sigmoid) relationship (confirmed by a likelihood ratio test comparing a linear regression model with a nonlinear four-parameter logistic model, $\chi^2 = 160.75$, $df = 2$, $P < 0.001$). The model shows that lesion length rapidly increases with lesion number but levels off at around two lesions. That means samples with two or more lesions are likely to display lesions with a cumulative length around the model's upper asymptote, which translates into the average maximum lesion length (*c.* 91 mm, see Table 6).

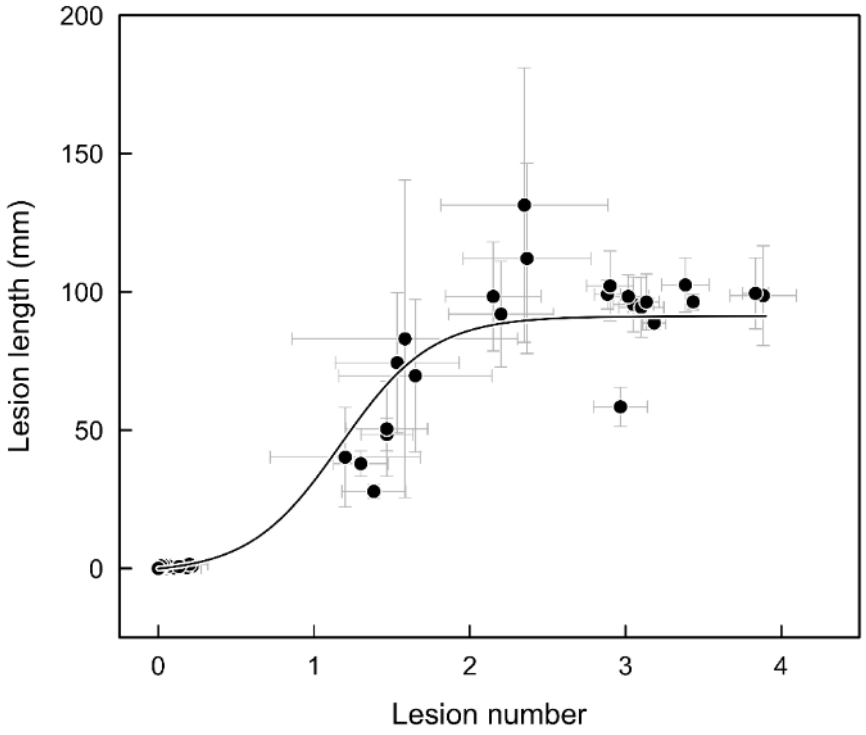

Figure 4: Cumulative lesion length as a function of the lesion number. Error bars denote standard errors of the mean for the lesion number (x-direction) and the lesion length (y-direction), n = 8 blocks.

Table 6: Results from the four-parameter logistic nonlinear regression model. LRC = logarithm of the rate constant.

Parameter	Estimate	SE	*t*	*P*	
Lower asymptote	-1.75	0.40	-4.35	< 0.001	***
Upper asymptote	91.21	5.45	16.72	< 0.001	***
Inflection point	1.17	0.04	29.17	< 0.001	***
Scale	0.29	0.02	15.43	< 0.001	***

Discussion

The main aim of this study was to compare the level of disease incidence using two inoculation methods for screening *P. radiata* for susceptibility to infection by *P. pluvialis,* comparing symptom development between the two methods. On-plant inoculations resulted in fewer RNC lesions and lower lesion lengths than the detached needle inoculation assay with greater variation in infection levels in on-plant assays. Considering these observations, both assays have numerous advantages and disadvantages that need to be weighed up when applying them to screen for responses to infection by *P. pluvialis* depending on the objectives of the assay.

Inoculation by the detached needle assay provides a relatively quick, reliable and quantifiable indicator of potential susceptibility of *P. radiata* to red needle cast. In this limited study, several genotypes developed substantial needle lesions in the detached needle assay but fewer symptoms from the on-plant inoculations when exposed to the same nominal spore load. The fact that the lesion lengths resulting from the detached assay topped out for many of the genotypes at 100 mm per fascicle may indicate that it may be better to assess lesion lengths earlier to capture quantitative differences in the rate of lesion formation before this upper asymptote is reached.

Longer lesions may also resulted from a higher effective inoculum dose, a narrower focal point of inoculum within the tube and environmental conditions which favoured infection, Unlike intact plants, detached needles also provide a wound for pathogen entry. However, this is not believed to be a significant factor as the majority of the lesions were observed to develop 2-3 cm from the base of the needle, corresponding with the top of the inoculum during the overnight exposure (data not shown). In terms of screening for resistance to *Phytophthora* infection, the high disease pressure of the screening assay has a potential advantage of selecting for higher levels of disease resistance, especially when supported by both assays, as for genotype 47_16.

Considering the classical pathology disease triangle, high disease pressure is to be expected with *in vitro* screening, as the detached needle assay favours infection in all three aspects of disease development: host, pathogen and environment (Francl, 2001). The assay exposes host tissues to a level of *P. pluvialis* zoospores shown through repeated trials to consistently produce lesions. *Phytophthora* zoospores characteristically aggregate at the surface of the water and are therefore more highly concentrated at a single point of infection in the detached needle assay where the surface area of the inoculum is restricted in the inoculation tube. Incubation in moist chambers at 17^{o}C provides an environment which favours infection and lesion formation by *P. pluvialis* on *P. radiata.* Finally, the detachment of the needles limits the plant's mechanisms of response to those that can be regulated within senescing tissue. The high disease pressure has an advantage of identifying genotypes with higher than average resistance to infection.

In contrast, on-plant inoculation have lower effective inoculum pressure at the needle surfaces than for the detached needle assay as the inoculum is spread over a greater surface area. While similar studies have shown that on-plant inoculations more closely approximate susceptibility in the field (Hansen and others, 2005), they are also subject to higher variability, require higher volumes of inoculum and are more difficult to quantify as infection can occur at any point on the needle. Based on these observations, further investigation may be warranted to improve the consistency of the on-plant inoculation method.

The physiological condition of needle tissues associated with needle age and/or position in the canopy, along with micro-climatic conditions, affects disease expression (Denman and others, 2005). In this trial we targeted fully formed needles approximately 15 cm down the stem of the plant to target needles of similar age across the trial.

The inoculum density of 1 x 10^4 zoospores per ml was in the mid-range of similar screening studies for other plant host and *Phytophthora* species which have ranged from 5 x 10^3 (Tooley and others, 2004), to 6 x 10^4 (Hansen and others, 2005) and 2–4 x 10^5 zoospores per ml (Denman and others, 2005). There is an innate challenge with quantifying the effective inoculum with zoospores, due to the time-line of encystment, short motility period of the spores and tendency for spores to encyst upon contact with solid surfaces and through agitation. The target inoculum density of 1 x 104 zoospores per ml used in this study aims to favour infection in the detached needle assay, however higher inoculum densities may improve the consistency of on-plant inoculations. The challenge then being to produce sufficient volumes and concentrations for parallel inoculations.

The use of in vitro inoculations to infect tissues or seedlings to indicate the potential susceptibility/resistance of mature trees in forest conditions is rightly questionable. However, classical screening for resistance to plant pathogens commonly begins with a primary screen that places plant genotypes under high disease pressure (Butcher and others, 1984; Fazio and others, 2009; McComb and others, 1991; Smit and Labuschagne, 2004; Utkhede and Quamme, 1988). In the case of screening for canopy-borne species of *Phytophthora,* detached leaf and needle assays have been widely applied for screening plant material for susceptibility to infection (Denman and others, 2005; Hansen and others, 2005; Ireland and others, 2012; Jinek and others, 2011; Nyassé and others, 2002; Tahi and others, 2006). In these cases, rapid exclusion of susceptible genotypes is an advantage. For some purposes, such as the assessment of defined treatment responses, these differences may not matter. For instance, where the treatment effect is the dominant factor in the response. This has been shown to be the case for RNC within chemical control trials, where treatments have been clearly differentiated with detached needle assays (Rolando and others, 2014a; Rolando and others, 2014b; Rolando and others, 2014c). We do acknowledge that in vitro screening may be less useful in differentiating more subtle differences in susceptibility due to multi-genic effects.

At this stage, the results of both inoculation methods are informative for identifying genotypes that potentially have higher levels of RNC resistance than others screened in the same trial. While both methods resulted in infection of the host material, the response of mature plants in the field is yet to be correlated. This is something we hope to clarify in future trials as the Elite Clone populations having been planted out across multiple field sites in 2013 and 2014. Consideration should also be given to the link between juvenile plants and mature trees in the field where numerous factors may contribute to the effective susceptibility of plants in the field, including not only the susceptibility of the host tissue, but also the inoculum potential of the genotype and perpetuation of the pathogen life cycle to enable the build-up of inoculum within the forest. Inoculum potential on each host was not assessed as part of this trial.

This trial was carried out on 12 genotypes due to limitations in the volume and concentration of zoospore inoculum required for parallel detached needle and on-plant inoculations. Further up-scaling of this assay for controlled inoculations is limited by the capacity of zoospore production and time frames in which the inoculum needs to be applied before zoospore encystment. Exposing a wider range of potted genotypes should therefore consider aerosol spore application exposure to natural inoculum at the peak of RNC symptom expression, rather than relying on inoculum production (Hansen and others, 2015).

Recommendations and Conclusions

The detached needle assay is a useful tool for high throughput screening the susceptibility of plant tissues to infection by *Phytophthora.*

The detached needle assay provides a high level of disease pressure compared to on-plant inoculations.

For breeding and screening purposes, the detached needle assay provides a means of identifying genotypes with a higher likelihood of resistance to RNC. It is recommended that where appropriate, this assay is used to narrow down selections for repeated on-plant and in-field inoculations and/or exposure to natural inoculum wherever possible.

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