Needle disease strategy Research Programme

SCION forests · products · innovation

Minimum *Phytophthora pluvialis* zoospore concentration for red needle cast infection *in planta*

Beccy Ganley, Peter Scott and Martin Bader

Date: June 2015

Report: ND-T006

supported by forestgrowers commodity levy

FOREST OWNERS ASSOCIATION

PUBLIC REPORT INFORMATION SHEET

Report Title	Minimum *Phytophthora pluvialis* zoospore concentration for red needle cast infection *in planta*
Authors	Beccy Ganley, Peter Scott and Martin Bader
ISBN No	
Scion Publication No.	
Sidney Output Number	56054
Signed off by	Lindsay Bulman
Date	June 2015
Copyright	
Intellectual Property Category	

Disclaimer

The opinions provided in the Report have been provided in good faith and on the basis that every endeavour has been made to be accurate and not misleading and to exercise reasonable care, skill and judgment in providing such information and opinions. Neither New Zealand Forest Research Institute Limited, trading as Scion ("Scion") nor any of its employees, officers, contractors, agents or other persons acting on its behalf or under its control accepts any responsibility or liability in respect of any opinion provided in this Report by Scion.

EXECUTIVE SUMMARY

Minimum *Phytophthora pluvialis* zoospore concentration for red needle cast infection *in planta*

Beccy Ganley, Peter Scott and Martin Bader

June 2015

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 a detached needle assay has been investigated but has not been tested *in planta.* 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 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 planta*. 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. The average lesion length and average lesion number were analysed using a generalised nonlinear least-squares models and pair-wise comparisons between clones were performed.

Key Results

All three clones were able to be successfully infected with *P. pluvialis in planta*. An exponential correlation between disease and zoospore concentration was observed with high *P. pluvialis* zoospore concentration required to achieve high levels of infection. There was a significant difference in infection between clones.

For both lesion length and number there is a threshold concentration between 200 and 2000 zoospores per ml where infection became exponential. 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 zoospore concentrations, the impact of inoculation at concentrations of 200 zoospores per ml or below is minimal.

Implications of Results for Client

Research into the control of the disease red needle cast (RNC) caused by the foliar pathogen *P. pluvialis*, 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 helps researchers validate findings and maximise operational screening of products being tested. This research is a first step towards determining a zoospore concentration threshold for *in planta* artificial inoculations.

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 it is unlikely any product would provide near total control. However, the significant difference in lesion length at concentrations below 200 zoospores per ml suggests inoculum density has an impact on disease severity and it is likely that reducing inoculum density would result in effective control.

Further Work

Results from this *in planta* assay and from previous zoospore concentration experiment using detached needle assays need to be analysed and compared to validate the detached needle assay results as well as the minimum zoospore concentrations required for infection.

This *in planta* assay has further reduced the threshold concentration where exponential infection occurs. In the detached needle assay the threshold was found to be between 5000 to 200 zoospores per ml and this has been reduced to between 2000 to 200 zoospores per ml. Further testing is required to further narrow this range, testing concentrations between 200 to 2000 zoospores per ml would be recommended.

Further testing and research into the physiological differences between host material of different ages and on different age needles is also 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.

Finally, understanding dose response to zoospore inoculation would also be recommended. In particular, comparing the effect of one high concentration inoculum exposure to multiple, low concentration inoculum exposures, to further understand the effect of zoospore concentration on disease expression,

Minimum *Phytophthora pluvialis* zoospore concentration for red needle cast infection *in planta*

Beccy Ganley, Peter Scott and Martin Bader
Scion

June 2015

Table of Contents

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. Although younger material (three years and younger) can be artificially infected, disease symptoms in young material in the field 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]). The two artificial inoculation methods developed are a detached needle assay, where needles are removed from the plant and inoculated in zoospore suspensinos, and an *in planta* assay where needles are inoculated while they are attached to the plant. The detached needle assay uses a smaller volume of inoculum and thus, allows a larger number of plant hosts to be screened. Concentrations of between $5x10^3$ - $1x10^4$ zoospores per ml have been used for inoculations. However, the lower limit for statistically significant infection using these artificial inoculation methods is unknown. In a detached needle assay completed in 2014 testing a range of zoospore concentrations, a high *P. pluvialis* zoospore concentration was required to achieve high levels of infection and the degree of infection at low inoculum concentration was strongly influenced by host clone (Ganley, Scott and Bader unpublished data[2]). It was also demonstrated that while infection could occur at all concentrations, there was no significant difference in infection up to concentration of 200 zoospores per ml.

Knowing the minimum zoospore concentration where statistically significant infection occurs will allow researchers to substantiate findings from artificial inoculation assays, contribute to our understanding of the minimum zoospore levels required for field infection to occur and the biology of the organism. Furthermore, this information can be used for risk assessments to determine the minimum zoospore levels required for infection. The objective of this study was to determine the minimum *P. pluvialis* zoospore concentration required to get infection *in planta*.

Materials and Methods

Plant material

Three different *Pinus radiata* clones were used in this study, with ten ramets used per clone. The same clones and ramets used in the 2014 detached needle minimum zoospore concentration assay (Ganley, Scott and Bader unpublished data[2]) were used for this experiment Clones A and C were from grafted material prepared in 2012 from buds taken from mature trees in the field. Clone B (ortet 6/3) was from cuttings prepared in 2012 from an ortet sown in 2011. The susceptibility of clone A against *P. pluvialis* was unknown. 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]). Clone C has been tested

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

[2] Ganley B, Scott P and M Bader. (2014) Minimum zoospore concentration for red needle cast infection. Scion report output 53748.

[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

previously against *P. pluvialis* and is known to be susceptible. All plant material was propagated and maintained at Scion under standard nursery conditions.

In planta 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 4014n, 4015n, 4016n, 4017n, 3901n and 3902n. Zoospore suspensions produced from each isolate were combined and diluted to form 20, 200, 2000 and 5000 zoospores per ml. Autoclaved pond water was used for the control.

On 9 April 2015 the plants were inoculated with zoospore suspensions. Within two hours of inoculum being produced, 30 ml of inoculum for the four different zoospore concentrations or pond water were attached to branches in plastic bags (Figure 1). Each tree was inoculated with all four treatments and a control. The only exceptions to this were one ramet for clone B that was not inoculated with the 20 zoospores per ml concentration, and another clone B ramet that was not inoculated with the 200 zoospores per ml concentration; in both cases this was due to a lack of available branches on the plants for inoculation. The needles were left in the inoculum for 20 hours outside. The following day, the plastic bags were removed and the plants were left outside for a further 11 days to allow lesion development.

Figure 1. *In planta* inoculation.
Four different zoospore concentrations of *Phytophthora pluvialis* inoculum and a pond water control were attached to five different branches per plant in plastic bags. The needles were left in the inoculum for 20 hours outside before the plastic bags were removed.

cast. Scion report output 51311.

Phytophthora pluvialis lesion assessments

Ten fascicles per treatment, per ramet, were assessed after 11 days. The ten fascicles were randomly removed from the treated area of each branch. The number of needles present in each fascicle, 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 lesion length per fascicle was calculated and afterwards pseudo-replication was removed by averaging lesion length across fascicles resulting in a data set with mean lesion length per ramet. This was followed by a graphical data exploration that showed the anticipated exponential increase, thus guiding the subsequent data analysis towards a nonlinear regression approach.

Data analysis

Generalised nonlinear least-squares models were applied to analyse mean lesion length and mean lesion number using R version 3.1.2 (R Development Core Team 2014, R-package *nlme,* Pinheiro *et al.* 2015). For the analysis of lesion length, an exponential model was used (since a logistic model failed to converge):

$$y = y_0 \, e^{(x/b)}$$

where y_0 = the response level when the predictor is zero, b = parameter related to the speed of exponential changes.
The mean lesion numbers were fitted using a three-parameter logistic model (a model comparison between an exponential and the logistic model favoured the latter):

$$y = \frac{Asym}{1 + e^{\frac{(xmid - x)}{scal}}}$$

where *Asym* = Asymptote, *xmid* = x value at the inflection point of the curve, *scal* = numeric scale parameter.

Both models contained lesion length as response variable and the logarithm of the zoospore concentration (log + 1) as predictor. 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 an exponential variance structure using the log zoospore concentration as variance covariate for the lesion length model (*varExp*, R-package *nlme*) and a power function including a constant using the fitted values as variance covariate for the lesion number model (*varConstPower*, R-package *nlme*). To test for differences between clones we fitted a parameter model (containing the same variance structure), allowing parameter estimates to vary with clone identity. This parameter model was compared to the more general model assuming common parameter estimates across clones using a likelihood ratio test (Ritz & Streibig 2008).

In order to perform pair-wise comparisons between clones, contrasts were set up manually by pooling two clone identities in turn. Then parameter models were run for all

pair-wise clone combinations and each was compared to the parameter model allowing separate parameter estimates for each clone using likelihood ratio tests. The Benjamini & Hochberg (1995) method was used to adjust *P*-values for multiple testing (R-package *multcomp*, Hothorn *et al.* 2008).

Results

All three clones were able to be successfully infected with *P. pluvialis* (Figures 2 and 3) using the *in planta* 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 the nonlinear model assuming common parameter estimates among clones and the parameter model allowing separate parameter estimates for each clone was significant (L = 12.65, df = 4, P = 0.013) indicating that there were clone-related differences in response to the different zoospore concentrations tested (see Table 1., Appendices for clone-specific parameter estimates). Specifically, only Clone A and Clone B were found to differ significantly (L = 12.34, df = 2, P = 0.006) in their response to zoospore concentration (see Table 2., Appendices for pair-wise comparisons).

Lesion length showed an exponential relationship with zoospore concentration (Figure 2). These results indicate that somewhere between 200 and 2000 zoospores per ml there is an inoculum threshold that influences lesion length. Similar to the detached needle assays, infection was still able to occur *in planta* at the lowest zoospore concentration, although the length of lesions that developed were smaller than in the other treatments.

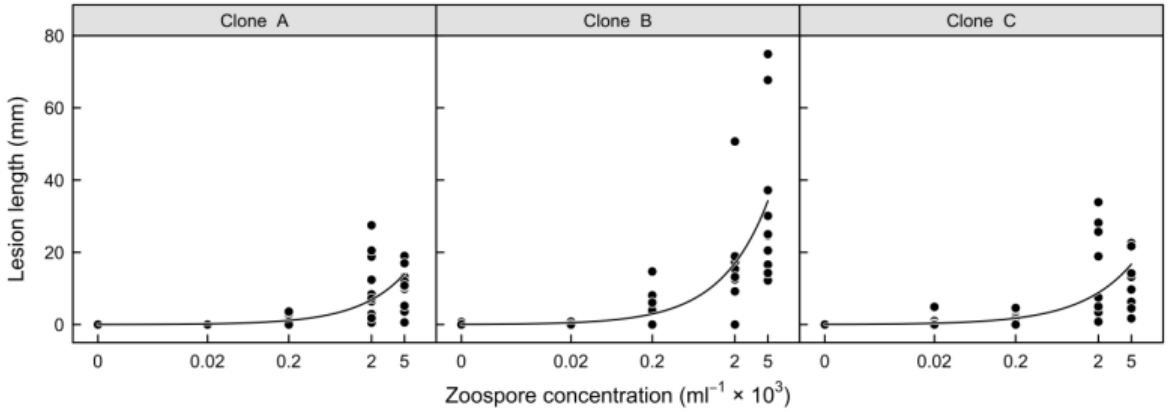

Figure 2 Average lesion length per fascicle in ramets from three *Pinus radiata* clones that were either uninoculated (control) or inoculated with four zoospore concentrations of *Phytophthora pluvialis.* Note that the zoospore concentration is displayed on a logarithmic axis. n = 10 ramets.

Lesion number

Similar to the lesion length data, the comparison between the nonlinear model assuming common parameter estimates among clones and the parameter model allowing separate parameter estimates for each clone showed that there were significant differences between some clones (P < 0.001) (see Table 3., Appendices for clone-specific parameter estimates). For lesion number, the response of Clone A to the different zoospore

concentrations differed significantly from the responses seen in Clone B and C (Figure 3; see Table 4., Appendices for pair-wise comparisons). In Clone A the number of lesions reached a plateau point, whereas in Clone B and Clone C it maintained an exponential phase.

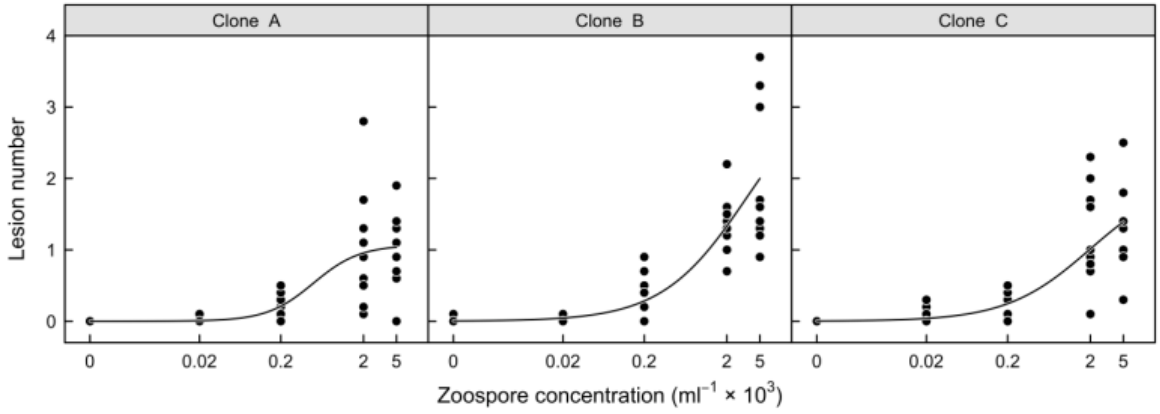

Figure 3 Average lesion number per fascicle in ramets from three *Pinus radiata* clones that were either uninoculated (control) or inoculated with four zoospore concentrations of *Phytophthora pluvialis.* Note that the zoospore concentration is displayed on a logarithmic axis. *n* = 10 ramets.

Similar to lesion length, there appears to be an inoculum threshold between 200 and 2000 zoospores per ml that influences the number of lesions.

Discussion

This experiment shows that, *in planta,* zoospore concentration influences the number and length of lesions produced. Although lesions occurred at all concentrations tested, high levels of *P. pluvialis* zoospore inoculum were required to achieve high levels of infection. Somewhere between 200 and 2000 zoospores per ml there is a concentration threshold where infection becomes exponential, and in one case a plateauing of this exponential phase was observed. The plateauing of lesion number in Clone A, versus the exponential response in Clones B and C indicates a significant difference in host response to high levels of inoculum. For Clone A, zoospore concentration may have less impact on disease expression than in Clones B and C.

The results of this study were similar to the detached needle assay, where infection with 200 zoospore per ml and below was significantly less than at higher zoospore concentrations[2]. Although both studies used the same plant material and similar zoospore concentrations, the 50 zoospore per ml concentration used in the detached needle assay was replaced with 200 zoospore per ml in the *in planta* assay. This means generalisations can be made between the experiments but a direct statistical analysis of the two methods will be completed independently of this report. Overall, both experiments showed infection could occur at all concentrations and the use of zoospore concentrations less than 200 zoospore per ml is not recommended.

In this study there was a significant difference in lesion development between the clones, which reflects the variation in resistance to *P. pluvialis.* Clone A appeared to be the most resistant of the three, consistent with what has been observed previously (Ganley, Scott

and Bader unpublished data[4]; Graham et al., unpublished data[5]). Clone B appeared to be the most susceptible. Any variations in susceptibility of this clone between the detached needle and *in planta* assays could be a reflection on the assay type or age-related host physiological differences, as the assays were performed a year apart. It is anticipated that the biggest age-related physiological differences would be for clone B, which are cuttings and are likely undergoing greater rates of physiological change, versus grafted material for clone A and C, which both had reproductive structures. Regardless of the results, further testing against a larger number of host genotypes would be required to confirm this.

Recommendations and future directions

This experiment show that needles can be infected at very low zoospore concentrations but there is an inoculum threshold between 200 and 2000 zoospores per ml that influences lesion length and the number of lesions. Based on these results, it is recommended that zoospore concentrations of 200 or less zoospores per ml should not be used for artificial inoculation experiments. The effect of zoospore concentrations above 5000 zoospores per ml on *in planta* disease expression is unknown.

In this experiment, the influence of zoospore concentration on lesion length and number was tested *in planta* on clones that had previously been tested using a detached needle assay. Further work is required to directly compare the impact of the same zoospore concentrations used in both the detached needle and *in planta* assays on disease development.

Further testing and research into the differences between of host material of different ages, as well as different aged needles is required to elucidate any influence this has on disease progression and expression. Physiological differences may influence results of the *in planta* and detached needle assays. Although both assays were performed on the same plant host, they were done over a year apart.

Finally, understanding dose response to zoospore inoculation would also be recommended. In particular, how individual, high concentration inoculum exposure compared to multiple, low concentration inoculum exposures effects the severity and incident of disease. The effect of zoospore concentration and dose on disease expression would be beneficial for understanding disease progression and outbreaks, but also will give insights into how control methods could reduce disease targeted reductions in inoculum production.

Acknowledgements

The authors would like to thank Catherine Banham and Shannon Hunter for their assistance in producing zoospores, and others members of the red needle cast scoring team for their contributions to this project. This projected was co-funded by the Ministry of Business, Innovation, and Employment and the Forest Growers Levy Trust.

[4] Ganley B, Scott P and M Bader. (2014) Minimum zoospore concentration for red needle cast infection. Scion report output 53748.
[5] Graham, N, Li, Y, and B Ganley. 2014. Screening RPBC Elites for RNC susceptibility. Scion report output 53570.

References

Benjamini Y & Hochberg Y (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B* 57, 289–300.

Dick, M., Williams, N., Bader, M., Gardner, J., & Bulman, L. (2014). Pathogenicity of *Phytophthora pluvialis* to *Pinus radiata* and its relation with red needle cast disease in New Zealand. New Zealand Journal of Forestry Science, 44(1), 1-12. doi:10.1186/s40490-014-0006-7

Hothorn T, Bretz F & Westfall P (2008). Simultaneous Inference in General Parametric Models. *Biometrical Journal* 50, 346-363.

Pinheiro J, Bates D, DebRoy S, Sarkar D and R Core Team (2015). nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-119,
URL: http://CRAN.R-project.org/package=nlme.

R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.

Zuur, A.F., E.N. Ieno, N.J. Walker, A.A. Saveliev, and G.M. Smith.
2009. Mixed effects models and extensions in ecology with R. New York. Springer.

Appendices

Table 1 Parameter estimates from the exponential nonlinear regression model for the lesion length data. *** $P \leq 0.001$.

Parameter	*Estimate*	*SE*	*t*	*P*	
y_0 (Clone A)	0.018	0.018	1.00	0.317	
y_0 (Clone B)	0.052	0.028	1.87	0.064	
y_0 (Clone C)	0.044	0.030	1.44	0.151	
b (Clone A)	1.280	0.207	6.18	< 0.001	***
b (Clone B)	1.311	0.125	10.52	< 0.001	***
b (Clone C)	1.433	0.199	7.19	< 0.001	***

Table 2 Results from pair-wise clone comparisons for lesion lengths based on likelihood ratio tests between parameter models for all pair-wise clone combinations and the parameter model allowing separate parameters for each clone. *df* = degrees of freedom, *L* = likelihood ratio statistic, $P_{adj.}$ = *P*-values adjusted for multiple comparisons. ** $P \leq 0.01$.

Test	*df*	*L*	$P_{adj.}$
Clone A – Clone B	2	12.34	0.006 **
Clone A – Clone C	2	1.28	0.674
Clone B – Clone C	2	0.79	0.674

Table 3 Parameter estimates derived from the three-parameter logistic nonlinear regression model for the lesion number data. ***$P \leq 0.001$; ** $P \leq 0.01$.

Parameter	*Estimate*	*SE*	*t*	*P*
Asym (Clone A)	1.07	0.19	5.52	< 0.001 ***
Asym (Clone B)	3.47	2.42	1.44	0.153
Asym (Clone C)	2.02	1.10	1.84	0.068
xmid (Clone A)	6.21	0.54	11.51	< 0.001 ***
xmid (Clone B)	8.16	1.62	5.05	< 0.001 ***
xmid (Clone C)	7.59	1.43	5.32	< 0.001 ***
scal (Clone A)	0.65	0.20	3.18	0.002 **
scal (Clone B)	1.18	0.27	4.42	< 0.001 ***
scal (Clone C)	1.16	0.29	3.99	< 0.001 ***

Table 4 Results from pair-wise clone comparisons for lesion numbers based on likelihood ratio tests between parameter models for all pair-wise clone combinations and the parameter model allowing separate parameters for each clone. *df* = degrees of freedom, *L* = likelihood ratio statistic, $P_{adj.}$ = *P*-values adjusted for multiple comparisons. *** $P \leq 0.01$.

Test	*df*	*L*	$P_{adj.}$
Clone A – Clone B	3	64.62	< 0.001 ***
Clone A – Clone C	3	41.56	< 0.001 ***
Clone B – Clone C	3	7.03	0.071