Healthy trees, healthy future
Enabling technologies to combat *Phytophthora* diseases

SCION
forests · products · innovation

Development of a tissue culture approach for analysing *Phytophthora* host-pathogen interactions

Authors: Nari Williams, Cathy Hargreaves and Keiko Gough

Date: June 2015

File Number: HTHF-T007

supported by forestgrowers commodity levy

FOREST OWNERS ASSOCIATION

KEEP KAURI STANDING
KIA TOITU HE KAURI

REPORT INFORMATION SHEET

REPORT TITLE	DEVELOPMENT OF A TISSUE CULTURE APPROACH FOR ANALYSING *PHYTOPHTHORA* HOST-PATHOGEN INTERACTIONS
AUTHORS	NARI WILLIAMS, CATHY HARGREAVES AND KEIKO GOUGH
CLIENT	MBIE / FOREST OWNERS ASSOCIATION
MBIE CONTRACT NO:	C04X1305
SIDNEY OUTPUT NUMBER	
SIGNED OFF BY	NARI WILLIAMS
DATE	JUNE 2015
CONFIDENTIALITY REQUIREMENT	
INTELLECTUAL PROPERTY	

EXECUTIVE SUMMARY

Report Title: Development of a tissue culture approach for analysing *Phytophthora* host-pathogen interactions

Authors: Nari Williams, Cathy Hargreaves and Keiko Gough

The problem

One of the key limitations of host-pathogen challenge studies is analysing sufficient cells which are at a uniform stage of infection and thus analysing a consistent state of host-pathogen responses with comparable levels of both host and pathogen gene and metabolite expression within the sample.

This project

To assess the potential of using a tissue culture system for host-pathogen interaction studies with *Phytophthora pluvialis.* Also, to assess the benefits of tissue culture over our current approach of needle inoculation including the rapid reproduction and uniformity of clonal callus culture, strict control of plant cell growth conditions and improved control of infection timing. Assessments will also be made on the improved efficiencies in experimental work-flows and the potential to compare these across host genotypes and species.

Key Results and Further Work

This work sought to investigate the potential use of *P. radiata* tissue culture for systems-biology comparisons of host-pathogen interactions as needed in the HTHF project. Furthermore, we sought to refine the system. From this limited study there are indications that the level of necrotrophy align with susceptibility of each genotype in needle assays.

Lawns of callus present as a suitable model for investigating host pathogen interactions. The radial growth of *Phytophthora* across the callus enables direct comparison across an infection gradient to study the phases of bio-trophic and necrotrophic host-pathogen interactions.

Development of a tissue culture approach for analysing *Phytophthora* host-pathogen interactions

Nari Williams[1], Cathy Hargreaves[2] and **Keiko Gough[2]**
Forest Protection[1], Forest Genetics[2],
Scion, 49 Sala Street, Private Bag 3020, Rotorua 3046, New Zealand

30 June 2015

Table of Contents

Introduction

One of the key limitations of host-pathogen challenge studies is analysing sufficient cells which are at a uniform stage of infection and thus analysing a consistent state of host-pathogen responses with comparable levels of both host and pathogen gene and metabolite expression within the sample. Some of these difficulties are overcome in the model/ agricultural *Phytophthora* systems including potato, Arabidopsis, tobacco, capsicum and soybean as the plants can be propagated rapidly, produce an abundance of succulent tissue and have rapid disease cycles. All of which are critical factors in being able to perform comparative studies of host-pathogen interactions and abiotic influences. However, even in these model systems, tissue culture has had a role in understanding host-pathogen interactions (REFS).

Tissue culture has been long applied in screening and selection programmes for resistance to *Phytophthora* diseases with responses in callus and suspension cultures shown to correlate with field susceptibility (Tables 1 & 2). Similarly, several studies have investigated compatible and incompatible interactions between plant pathogens and hosts in callus and suspension cultures (Bulman, et al., 2011; Wielgoss, et al., 2015).

One of the significant step-change objectives of the HTHF programme is to establish research platforms in which host-pathogen interactions can be assessed in tree species such that the results of the research can be linked and compared to the foundation of research built up over many years in the model *Phytophthora* patho-systems described above. Utilisation of tissue culture thus presents several advantages in developing model systems for host-pathogen research across a range of tree-hosts and pathogens.

Table 1: Applications of tissue culture to the selection of resistance to *Phytophthora* species

Host	*Phytophthora* species	Tissue inoculation, culture filtrate, metabolite screening	Reference
Avocado (*Persea americana*)	*P. cinnamomi*	Inoculation	(Phillips, et al., 1991)
Jarrah (*Eucalyptus marginata*)	*P. cinnamomi*	Inoculation	(McComb, et al., 1987)
Loblolly pine (*Pinus taeda*), Shortleaf pine (*Pinus echinata*) and Virginia pine (*Pinus virginiana*)	*P. cinnamomi*	Inoculation	(Jang, et al., 1991)
Alfalfa (*Medicago sativa*)	*P. megasperma*	Inoculated	(Miller, et al., 1984)
Cowpea (*Vigna unguiculata*)	*P. vignae*	Inoculated	(Bateman, et al., 1989)
Rough Lemon (*Citrus jambhiri* Lush.)	*P. parasitica*	Filtrate	(Virk, et al., 2011)
Apple (*Malus domestica*)	*P. cactorum*	Filtrate	(Rosati, et al., 1990)
Potato (*Solanum tuberosum*)	*P. infestans*	Filtrate	(Behnke, 1980)

Table 2: Applications of tissue culture for the investigation of *Phytophthora* host-pathogen interactions

Host	*Phytophthora* species	Reference
Tobacco (*Nicotiana tabacum*)	*P. nicotianae*	(Perrone, et al., 2000)
Loblolly pine hybrids (*P. echinata* Mill.) & Virginia pine (*P.virginiana* Mill.)	*P. cinnamomi*	(Jang, et al., 1990)

Tissue culture is ideal for a systems-biology approach to investigating host-pathogen interactions. Benefits of tissue culture over our current approach of needle inoculation include the rapid reproduction and uniformity of clonal callus culture, strict control of plant cell growth conditions and improved control of infection timing. There are also the improved efficiencies in experimental work-flows and the potential to compare these across host genotypes and species. Preliminary work by the Scion team and established protocols for callus production in apple, provide confidence that tissue culture will provide a system in which host-pathogen interactions in all three host species can be compared in parallel.

Objective

To assess the potential of using a tissue culture system for host-pathogen interaction studies with *Phytophthora pluvialis.*

General Materials and Methods

Plant material - Pinus radiata *clones*

Pine needle meristem cultures were initiated from the mother-plants of eight genotypes based on the HTHF selection of susceptible and resistant genotypes. Needles were harvested from mother plants in October 2014 and meristem cultures initiated.

Inoculum preparation

Phytophthora pluvialis zoospore inoculum was prepared in accordance with Scion's standard protocol. In brief, isolates of *P. pluvialis* were grown 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 in flat bottom flasks and incubated for three days at 17 ^{o}C (Erwin, et al., 1996). 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.

Mycelium cultures were prepared by sub-culturing fresh isolates from the leading edge of cultures and incubating for 3 days in the dark at 17 ^{o}C prior to inoculation.

Identifying the optimal approach to inoculating *P. radiata* calli

Two basal media and several approaches were tested to identify a practical and reproducible means of inoculating *P. radiata* callus which would be amenable to host-pathogen interaction studies. The tissue culture media tested were GLITZ and EDM with two plates of each genotype x media prepared, plus non-inoculated controls. From these plates the basal media and callus grown on these tested with the following inoculation approaches:

1. Inhibition of *P. pluvialis* mycelial growth on the basal media
2. Inhibition of zoospore germination on the basal media
3. Transfer of individual calli to a microtitre plate to be independent of the basal media followed by inoculation with 2 ml of zoospore suspension (3 calli per genotype)
4. Transfer of *P. radiata* calli to mycelial masses of *P. pluvialis.*
5. Direct inoculation of the tissue culture plate with a mycelium plug adjacent to *P. radiata* calli.
6. Inoculation of the tissue culture plate with zoospore suspension

Please note, these studies were observational based and were not fully replicated as the intent was to assess the practicality, sterility and host-pathogen interactions achieved with each method to quickly narrow down the approaches that have potential going forward.

Observations

1) *Phytophthora pluvialis* mycelium grew on both GLITZ and EDM media with few indications of inhibition. In the presence of callus, *Phytophthora* growth was observed to be more consistent on GLITZ than EDM.

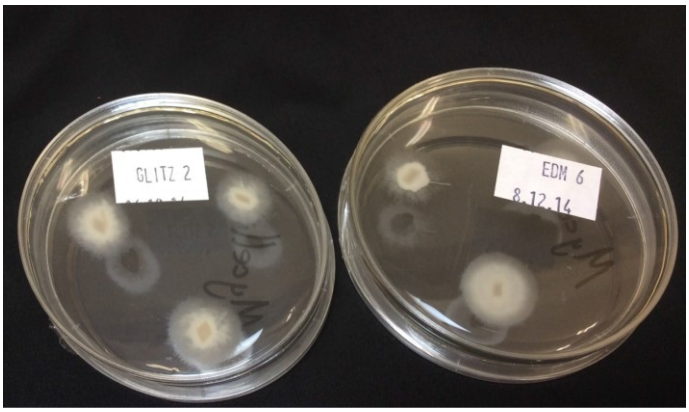


Figure 1: Three isolates of *Phytophthora pluvialis* grown on GLITZ (left) and EDM (right) tissue culture media.

2) Zoospores germinated on both GLITZ and EDM, however bacterial contamination and low zoospore titres were observed to be an issue.
3) Inoculation of *P. radiata* calli in microtitre plates resulted in few interactions between *Phytophthora* and the pine cell masses. A suspension or finer colonies of plant cells might be better.
4) Cell masses transferred to mycelial mats resulted in a close interaction between the *Phytophthora* and *P. radiata* cell masses. Haustoria and infection pegs were found (Figure 2 and Figure 3) which shows infection potential but this was found to be a very fiddly way of handing cells and it would be difficult to quantify a stage of infection. A more streamline and quantifiable means of inoculation is required.

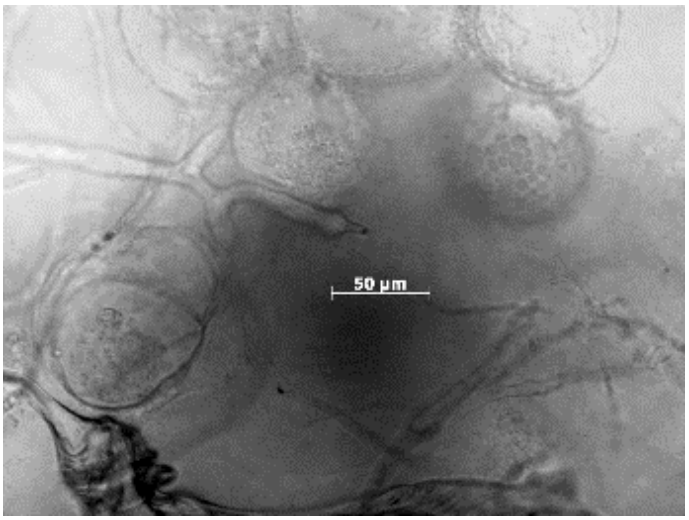


Figure 2: *Phytophthora* penetration peg. (Ignore scale bar - settings wrong)

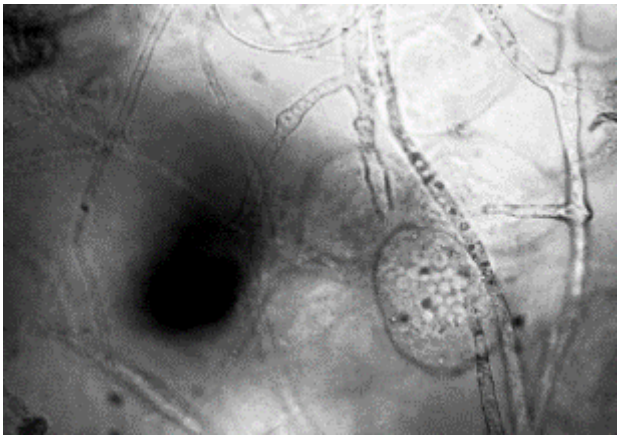


Figure 3: *P. pluvialis* haustoria penetrating into plant cell

5) Direct inoculation with mycelial plugs *Phytophthora pluvialis* resulted in calli being overgrown by the *Phytophthora* and turning necrotic (black) within 6 days. The level of necrosis was observed to differ between *P. radiata* genotypes(Table 3).

Table 3: Observations of *P. pluvialis* and *P. radiata* calli 6 days post inoculation

RNC Susceptibility	Genotype	GLITZ		EDM	
		P. pluvialis	*P. radiata*	*P. pluvialis*	*P. radiata*
Resistant	1	High	Moderate	High	low
	2	High	High	Low	moderate
	3	High	High	High	moderate
	4	High	Moderate	Low	moderate
Susceptible	1	Hgh	High	High	High
	2	High	High	High	Moderate
	3	Hgh	Moderate	Low	Moderate
	4	High	High	Low	Moderate

6) Inoculation of the tissue culture plate with a zoospore suspension resulted in the plates being overcome with bacterial contamination. This is not greatly surprising as non-sterile zoospore production technique was used. However we will have difficulty producing zoospores of sufficient titre and timing it to callus formation using the sterile protocol which typically produces less zoospores. Inundating the calli with zoospores is also limited in that it does not allow the point or direction of infection to be controlled which may be a problem if targeting cells at a given point of infection and a specified range of infection stages.

Conclusions

From these preliminary tests the following observations and conclusions were drawn:

1. *Phytophthora* growth not apparently inhibited on either of the tissue culture medium used.
2. Inoculation of *P. radiata* callus enables close interaction between plant cells and tissue culture cell lines.
3. Differences in health of cell lines and necrotrophy was observed on two different tissue culture media with more 'healthy' growth of plant cells on GLITZ without *Phytophthora* but more necrotrophy with *Phytophthora*.
4. Pursuit of zoospore inoculation for this system will require higher zoospore titres under strictly sterile conditions. Zoospore inoculation is limited in the approach used here as it is difficult to monitor the progression of the infection.
5. Mycelium inoculation looks promising, but when inoculating individual calli it will be more difficult to define inoculation period.
6. Definitely showing great promise and worth pursuing in establishing a systems biology platform for studying host-pathogen interactions across *P. radiata* clones.

Several attempts were made to establish and inoculate flask cultures of suspended tissue cultures as this has been used for *Phytophthora infestans*-potato interaction studies. However, we found that the suspension cultures did not produce healthy *P. radiata* callus so this approach has not been pursued further.

Next Steps:

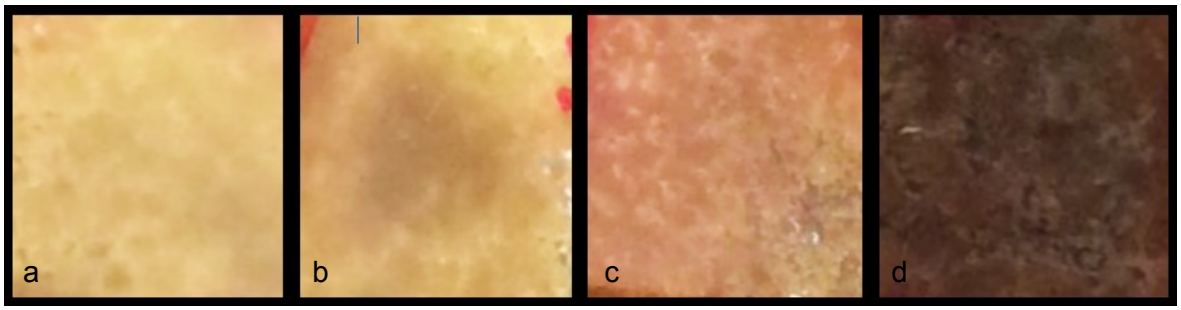
We have decided that many of the limitations in assessing the timing of infection could be overcome by establishing a lawn of tissue culture.

Establishment and inoculation of callus lawns

Tissue culture lawns were established for each of the eight *P. radiata* genotypes and inoculated with 5 mm agar plugs from the leading edge of 3 day old cultures of *P. pluvialis, P. kernoviae, P. multivora* and *P. cactorum.*

Phytophthora pluvialis colonies were observed to radiate from the inoculation plug in much the same fashion as regular growth on agar plates. This was associated with increased necrotrophy of the tissue culture callus over time with susceptible callus being completely necrotrophic 8 days post inoculation (Figure 4).

Figure 4: Stages of infection of *P. radiata* callus with *P. pluvialis*: a) Day 0 prior to inoculation; b) 2 days post inoculation; c) 4 days post inoculation; d) 8 days post inoculation.



Inoculation with three further species of *Phytophthora* (*P. kernoviae, P. multivora* and *P. cactorum*) resulted in differential infection of *P. radiata* callus in association with both genotype and *Phytophthora* species.

Discussion

This work sought to investigate the potential use of *P. radiata* tissue culture for systems-biology comparisons of host-pathogen interactions as needed in the HTHF project. Furthermore, we sought to refine the system. From this limited study there are indications that the level of necrotrophy align with susceptibility of each genotype in needle assays.

Lawns of callus present as a suitable model for investigating host pathogen interactions. The radial growth of *Phytophthora* across the callus enables direct comparison across an infection gradient to study the phases of bio-trophic and necrotrophic host-pathogen interactions.

This system presents a range of benefits for the HTHF programme:

- A clean assay system that is predominantly free of endophytes and contaminating micro-organisms.
- Controlled tissue production
- Ability to accurately control growth and environmental conditions
- Model system that can be directly compared to other plant systems
- Good potential for comparing with kauri and apple in directly parallel inoculation experiments
- Greater capacity for comparing between isolates and across genotypes.
- Not reliant on zoospore production
- Baseline conditions for metabolite and RNA reference library establishment.

Acknowledgements

This work was funded by MBIE (CO4X1305), the Forest Growers Levy Trust (Administered by the Forest Owners Association) and the Radiata Pine Breeding Company. *Pinus radiata* clonal material was sourced from the Radiata Pine Breeding Company and maintained by Scion's Tissue Culture laboratory.

References

Bateman, K., Hinch, J., Ralton, J., Clarke, A., Mckenzie, J., Imrie, B., & Howlett, B. (1989). Inheritance of resistance of cowpea to Phytophthora vignae in whole plants, cuttings and stem callus cultures. *Australian journal of botany, 37*(6), 511-517.

Behnke, M. (1980). General resistance to late blight of Solanum tuberosum plants regenerated from callus resistant to culture filtrates of Phytophthora infestans. *TAG Theoretical and Applied Genetics, 56*(4), 151-152.

Bulman, S., Candy, J. M., Fiers, M., Lister, R., Conner, A. J., & Eady, C. C. (2011). Genomics of biotrophic, plant-infecting plasmodiophorids using in vitro dual cultures. *Protist, 162*(3), 449-461.

Erwin, D. C., & Ribeiro, O. K. (1996). *Phytophthora Diseases Worldwide*. Minnesota: APS Press.

Jang, J., & Tainter, F. (1990). Cellular responses of pine callus to infection by Phytophthora cinnamomi. *Phytopathology, 80*(12), 1347-1352.

Jang, J., & Tainter, F. (1991). Optimum tissue culture conditions for selection of resistance to Phytophtora cinnamomi in pine callus tissue. *Plant cell reports, 9*(9), 488-491.

McComb, J. A., Hinch, J., & Clarke, A. E. (1987). Expression of filed resistance in callus tissue inoculated with *Phytophthora cinnamomi*. *Phytopathology, 77*, 346-351.

Miller, S. A., Davidse, L., & Maxwell, D. P. (1984). Expression of genetic susceptibility, host resistance, and nonhost resistance in alfalfa callus tissue inoculated with Phytophthora megasperma. *Phytopathology, 74*(3), 345-348.

Perrone, S., Bui, F., Sutherland, M., & Guest, D. (2000). Gene-for-gene specificity expressed in planta is preserved in cell cultures of Nicotiana tabacum inoculated with zoospores of Phytophthora nicotianae. *Physiological and Molecular Plant Pathology, 57*(5), 235-242.

Phillips, D., Weste, G., & Hinch, J. M. (1991). Resistance to Phytophthora cinnamomiin callus tissue derived from three avocado cultivars. *Canadian Journal of Botany = Revue canadienne de botanique, 69*(9), 2026.

Rosati, P., Mezzetti, B., Ancherani, M., Foscolo, S., Predieri, S., & Fasolo, F. (1990). In vitro selection of apple rootstock somaclones with Phytophthora cactorum culture filtrate. *Acta Horticulturae (Netherlands)*.

Virk, G. S., & Nagpal, A. (2011). In vitro selection of calli of Citrus jambhiri Lush. for tolerance to culture filtrate of Phytophthora parasitica and their regeneration. *Physiology and Molecular Biology of Plants, 17*(1), 41-47.

Wielgoss, A., & Kortekamp, A. (2015). Comparison of PR1 expression in grapevine cultures after inoculation with a host-and a non-host pathogen. *VITIS-Journal of Grapevine Research, 45*(1), 9.