MERIWA PROJECT : M 227

RAPID IDENTIFICATION OF SPECIES OF *PHYTOPHTHORA*

Research Scientist : STEPHEN A CARSTAIRS

Research Supervisor : MIKE STUKELY

QUARTERLY REPORT NO. 6

JULY - SEPTEMBER 1995

INTRODUCTION

In CALM's March 1995 application for research and development assistance to MERIWA and the mining industry the 'Work plan' recognised three research topics for project M 227 :

Topic 5 Test modifications to *Phytophthora* baiting techniques.

Topic 6 Test new procedures for directly assaying bait tissues for *Phytophthora* by isoenzyme analysis.

Topic 7 Review the literature and consider ways of establishing the intensity of field sampling necessary to achieve given levels of certainty of detecting *Phytophthora* if present.

In M 227 Quarterly Report No. 5 a work programme for the sixth quarter was outlined.

Work programme for the sixth quarter :

Stage	Type of Work	Time (weeks)
5.1.1	Monitor the pH of soil samples tested by the VHS	0.5
5.1.2	Test pH against recovery of *Phytophthora* from samples	1.5
5.2	Test modifications to baiting techniques	5.5
6.2	Isoenzyme assay of species mixes	3.0
7	Examining the question of appropriate field sample Nos.	1.5
	MERIWA Report	1.0
	Total	13

Work in the above 4 topics was to run concurrently.

What follows is an account of the progress made in the sixth quarter on each of the topics listed above. New business arising from those areas of research is also discussed.

WORK DONE DURING THE SIXTH QUARTER

In M227 quarterly Report No. 1 a *Phytophthora* species was considered to be a priority species if it occurred locally in the CALM estate.

Priority Species : *Phytophthora cinnamomi*
P. citricola
P. cryptogea
P. drechsleri
P. megasperma
P. nicotianae

5.1.1 Monitor the pH of soil samples tested by the Vegetation Health Service

This area of research is completed.

5.1.2 Test pH against recovery of *Phytophthora* from samples

This area of research is completed and the outcomes will be presented in the final report.

5.2 Test modifications to baiting techniques

5.2.2 RECOVERY OF SPECIES OF *PHYTOPHTHORA* FROM BAITS HARVESTED ON TWO OCCASIONS (FIVE OR SIX DAYS APART) FROM THE SAME SOIL SAMPLES

A standard procedure for recovering *Phytophthora* from soil/plant tissue samples is to bait the samples with young rapidly expanding live plant material (baits) for the zoospores of *Phytophthora* (Ribeiro 1978). After several days the baits are transferred to selective agar plates and the Phytophthoras are grown out. In MERIWA Quarterly Report No. 5 Section 5.2.1 it was reported that large numbers of baits (up to 28) having lesions require testing to attain 95% confidence level of detecting species of *Phytophthora*. Once a sample has proven to be positive for a species of *Phytophthora* it is seldom tested further. The aim of this investigation was to determine whether or not the species of *Phytophthora* recovered from baits harvested after ten days of baiting, concur with those recovered from baits harvested after baiting samples for four or five days.

PROCEDURE

Twenty seven soil samples were assessed. The baiting procedure adopted was one recommended by Dr F. D. Podger (pers. com.) and is a modification of that practised by the Vegetation Health Service (VHS) of CALM:

1. *Circa* 300g of soil/ plant tissue sample was distributed into each of two plastic baiting dishes.

2. *Circa* 300ml of distilled water was added to the baiting dishes.

3. Thirty to forty W.A. blue lupin pinnae and an equal amount of *Eucalyptus sieberi* cotyledons were then added to the baiting dishes.

4. Four to five days later eight to twelve baits having lesions were transferred to selective agar plates, and fresh baits were added to the dishes.

5. Sixteen to forty hours after being plated baits were examined for *Phytophthora* growing out of them and into the selective agar.

6. At day ten a second harvest of baits from the same sample trays were treated as in steps 4 and 5 above.

Identification of species of *Phytophthora* other than *P. cinnamomi* was determined isozymically.

RESULTS AND DISCUSSION

Six biological species* of *Phytophthora* were recovered from the twenty seven samples tested in this study (Table 1). From column four of Table 1 it is seen that for each species except the two biological species of *P. cryptogea* , there were soil samples which contained the Phytophthoras and which were not identified as such in the first harvest of baits. The percent increase in recovery of the various species of *Phytophthora* ranged for 0% for *P. cryptogea* 1 and *P. cryptogea* 2, which were recovered in lowest frequency overall, to 66.7% for *P. drechsleri*. The second harvest increased by 28.6% the rate of recovery of isolates of *Phytophthora* relative to the first harvest (Table 1).

A Wilcoxon signed rank test for the paired difference between the expected and observed increase in recoveries due to a second harvest of baits, was used to test the hypothesis that the second harvest substantially increased recovery, where a substantial increase would be 7.5% or more, of species of *Phytophthora* from soil samples relative

* Biological species: cultures of *Phytophthora* which share the same genetic identity, as determined isozymically.

to the first harvest. For the one-tailed test with alpha = 0.10 and n = 6, T_o = 18. In testing the null hypothesis T+ = 18, which is the same as the critical value. It was therefore concluded that this sample provided sufficient evidence to support the hypothesis that a second harvest of baits substantially increased (i.e. > 7.5%) recovery of *Phytophthora* relative to the first harvest.

When the species of *Phytophthora* recovered from samples in the second harvest were compared with those recovered in the first (Table 2), it was found that on twenty one occasions they were the same. On nine occasions (30%) the species were not the same. On one occasion *P. cinnamomi* was recovered in the second harvest of baits while only *P. citricola* 3 was recovered in the first. If it were not for the second harvest of baits, this soil sample would have been classified as negative for *P. cinnamomi*. That the species of *Phytophthora* recovered in the second harvests did not always concur with those recovered in the first, is to be expected given that the second harvest of baits increased substanially the recovery rates of most species found in these soil samples.

As mentioned earlier, once a sample has proven to be positive for a species of *Phytophthora,* it is seldom tested further. Before management decides as to whether or not it will implement changes to procedures for recovering *Phytophthora* from samples, some points need to be considered. Firstly, as many as twenty eight baits from any one harvest may require testing to attain the 95% confidence level of detecting species of *Phytophthora* in that harvest (MERIWA Quarterly Report No. 5). In this study eight to twelve baits per harvest were assessed. Failure to detect some Phytophthoras in the first harvest therefore, may have been due to insufficient baits being assessed. Alternatively it may be that the various species of *Phytophthora* in a sample attain competitive advantage for baits at different times during the period a sample is being baited. Repeating the experiment and assessing twenty eight baits per harvest might resolve this issue.

A second consideration is the cost of a second harvest of baits. Technicians would be required to invest an additional *circa* twenty two minutes to process a second harvest of baits from a sample given that it has already proven to be positive for *Phytophthora*. This is equivalent to a 76% increase in operator time for these samples. There would also be almost a 100% increase in the cost of consumables.

Should management decide that it is necessary to continue to test a sample (i.e. process a second harvest of baits), given that from an assessment of the first harvest of baits the sample proved to be positive for *Phytophthora*, then a twelve day programme for double harvesting samples, such as that presented in Table 3, is recommended. This programme maximises the use of down-time on weekends, and according to it thirteen to fifteen days are required from when a sample is received until it has been given a complete assessment.

REFERENCE

Ribeiro, O.F. 1978. A source book of the genus *Phytophthora*. Strauss and Cramer. Germany.

5.5.3 EFFECT OF A SECOND BAITING ON RECOVERY OF SPECIES OF *PHYTOPHTHORA* FROM SOIL

Palzer (1976) increased recovery of *Phytophthora cinnamomi* from soil and root samples by 26 to 142% when he subjected samples to a second baiting two weeks after the first baiting event. In his experiment Palzer baited the samples for two days with the root radicles of young *Lupinus angustifolius* seedlings.

The aim of this investigation was to test the hypothesis that baiting soil samples for ten days followed by a second ten day baiting period substantially increases recovery, where a substantial increase would be 7.5%, of species of *Phytophthors* from soil samples relative to a single ten day baiting event only.

PROCEDURE

Three hundred and forty seven soil samples were tested. Inorganic matter predominated in these samples.

The baiting procedure adopted was that recommended by Dr F.D. Podger (pers. com.), and has been described in the previous section. After the first ten day baiting period, excess liquid was drained off the soil samples, and they were allowed to dry for five days before being baited for a second ten day period.

Identification of species of *Phytophthora* other than *P. cinnamomi* was determined isozymically.

RESULTS AND DISCUSSION

Seven biological species of *Phytophthora* were recovered from the 347 soil samples tested (Table 4). The rate of recovery of these species ranged from 0.29% (*P. citricola* 1) to 9.22% (*P. citricola* 2) in the first baiting. In the first baiting *P. cinnamomi* was recovered from 6.92% of the samples, and in total 31.99% of the samples were positive for one or some Phytophthoras in the first baiting (Table 4).

From column three of Table 4 it is seen that for each species except for the two biological species of *P. cryptogea*, there were soil samples which contained the Phytophthoras but which were not identified as such in the first ten days of baiting. The second baiting increased the number of soil samples found to be positive for the various species of *Phytophthora* by zero for *P. cryptogea* 1 and *P. cryptogea* 2, which were recovered in low frequencies in the first baiting, to 5 for *P. citricola* 2 and *P. drechsleri*, which were recovered in considerably higher frequencies in the first baiting (Table 1).

A Wilcoxon signed rank test for the paired difference between the expected and observed increase in recoveries due to a second baiting of samples that were negative in the first baiting, was used to test the hypothesis that a second baiting substantially

increased recovery, where a substantial increase would be 7.5% or more, of species of *Phytophthora* from soil samples relative to the first baiting. For the one-tailed test with alpha = 0.10 and n = 7, T_o = 23. In testing the null hypothesis T+ = 22, which is less than the critical value. It was therefore concluded that this sample did not provide sufficient evidence to support the hypothesis that a second baiting substantially increased (i.e. > 7.5%) recovery of *Phytophthora* relative to the first.

Double baiting soil samples may identify more samples as positive for *Phytophthora,* albeit an insubstantial amount, however the benefits of increasing recovery rates of *Phytophthora* from soil samples by double baiting may be offset by the concomitant inefficiency of the process relative to single baiting. Samples may be determined to be negative for *Phytophthora* in thirteen to fifteen days by single baiting alone (see the previous section). In contrast, double baiting would require 28 days for the same sample to be deemed to be negative. In addition to the delay to diagnosis, but less importantly, double baiting would increase laboratory costs by *circa* 65%.

Because the important issue is to know whether or not the source of the sample is positive for *Phytophthora*, increasing the intensity of sampling at the source may be a more efficient approach to assessment than increasing the amount of resources invested in diagnosing individual samples. This requires testing.

Interestingly, species that were recovered in highest frequencies in the first baiting seemed to be recovered in highest frequencies in the second baiting also (Table 4). Regression analysis of the recovery rates of the various species of *Phytophthora* in the first baiting, and the subsequent recovery rates of the Phytophthoras on rebaiting those samples that were negative for *Phytophthora* in the first baiting , i.e. the values in column 3 of Table 4 were regressed against those values in column 2, indicated that there was a positive (r = 0.713) and significant (p = 0.044) relationship between the two recovery rates, i.e. species that were recovered in highest frequencies in the first baiting of the soil samples were also recovered in highest frequency in samples rebaited.

As the relationship between frequency of samples positive in the second baiting and that positive in the first was significant, and assuming that all of the species of *Phytophthora* behaved the same during the baiting process, it may be possible to predict the expected number of samples that would be positive on rebaiting (y) those samples that were negative for *Phytophthora* in the first baiting, from the observed number of samples positive (x) for *Phytophthora* in the first baiting. The expression being:

$$y = 0.076 + 0.127x \quad \ldots\ldots 1$$

which describes the linear relationship between the recovery rates of the various species of *Phytophthora* in the first baiting, and the subsequent recovery rates of the Phytophthoras on rebaiting those samples that were negative for *Phytophthora* in the

first baiting. The predictive value of expression 1, however, is not expected to extend beyond 10% samples positive for a species of *Phytophthora* in the first baiting. To increase the utility of the predictor, sample sets with 20+% samples positive for Phytophthoras should be included in its determination.

The % confidence (z) that a sample that was negative for *Phytophthora* in the first baiting will also be negative on rebaiting, may also be expressed in terms of x (the observed number of samples positive for *Phytophthora* in the first baiting). The relationship between z and x is expressed by:

$$z = 100 - \left[\frac{0.076 + 0.127 \frac{x}{N}}{100 - \frac{x}{N}} \right] \qquad \text{.....2}$$

where N is the number of samples tested.

As with expression 1, the predictive value of expression 2 is limited. Notwithstanding this, the method for predicting the % confidence one has that a sample that was negative for *Phytophthora* in the first baiting will also be negative on rebaiting, has been established.

REFERENCE

Palzer, C. 1976. Zoospore Inoculum Potential of *Phytophthora cinnamomi*. Ph. D Thesis. University of Western Australia.

6.2 Isoenzyme assay of species mixes

This area of research is completed and the outcomes will be presented in the final report.

7 Examining the question of appropriate field sample Nos.

This area of research is completed and the outcomes will be presented in the final report.

Acknowledgments

My thanks to Larissa Newcombe, Nola North and Janet Webster for help in the detection of *Phytophthora*; to Ian Abbott, Francis Tay and Matt Williams for comments on the report; and Frank Podger for helpful discussions in the development of research aims.

PROGRAMME FOR THE FORTHCOMING QUARTER

The October - November 1995 quarter is of 9 weeks duration. The work to be done in this quarter is scheduled as follows :

Stage	Type of Work	Time (weeks)
	Prepare the final report	9

Final Comment

All work areas are progressing in accordance with the research applications to MERIWA, and no obstacles to progress have been encountered at this stage.

Table 1. Recovery of species of *Phytophthora* from twenty seven soil samples from which baits were harvested on day 4-5 and again on day 10.

Species	No. samples from which the same species of Phyt. was recovered on both harvestings	No. samples +ve for a Phyt. 1st harvest, and -ve for that Phyt. 2nd harvest	No. samples -ve 1st harvest, and +ve for same Phyt. 2nd harvest	% increase in recovery due to 2nd harvest
P. cinnamomi	9	0	1	11.1
P. citricola 2	5	2	4	57.1
P. citricola 3	4	3	1	14.3
P.cryptogea 1	1	0	0	0
P.cryptogea 2	1	0	0	0
P. drechsleri	1	2	2	66.7
TOTALS	21	7	8	28.6

Table 2. Recovery of species of *Phytophthora* when baits from the same soil samples were harvested and assessed on two separate occasions, five days apart.

Species recovered 2nd harvest	Species recovered 1st harvest					
	P. c	P. cit 2	P. cit 3	P.cryp 1	P.cryp 2	P.drech
P. cinnamomi	9		1			
P. citricola 2		5	3			2
P. citricola 3			4			1
P. cryptogea 1				1		
P. cryptogea 2					1	
P. drechsleri		2				1

Table 3. Twelve day double harvest programme for recovering species of *Phytophthora* from soil / plant tissue samples.

What are A1, B1?

Day no.	Week Day	Activities for samples A1 *et. seq.*	Activities for samples B1 *et. seq*
-2	Wed	Receive samples A1 *et. seq.*	
-1	Thurs	Receive samples A1 *et. seq.*	
0	Fri	Bait A1 samples	
1	Sat		
2	Sun		
3	Mon		
4	Tues	First harvest of baits	
5	Wed	First harvest of baits	Receive samples B1 *et. seq.*
6	Thurs	Examine baits harvested day 4	Receive samples B1 *et. seq.*
7	Fri	Examine baits harvested day 5	Bait B1 samples
8	Sat		
9	Sun		
10	Mon	Second harvest of baits	
11	Tues		First harvest of baits
12	Wed	Examine baits harvested day 10	First harvest of baits

Table 4. Recovery of species of *Phytophthora* from 347 soil samples subjected to double baiting.

Species	**X**: No. samples +ve 1st baiting (%).	**Y**: No. samples -ve 1st baiting, +ve 2nd baiting (%).	% increase in *Phytophthora* recovery due to 2nd baiting.
P. cinnamomi	24 (6.92)	1 (0.29)	4.19
P. citricola 1	1 (0.29)	1 (0.29)	~~200.0~~ 100
P. citricola 2	32 (9.22)	5 (1.44)	15.62
P. citricola 3	31 (8.93)	4 (1.15)	12.9
P. cryptogea 1	3 (0.86)	0 (0.0)	0.0
P. cryptogea 2	2 (0.58)	0 (0.0)	0.0
P. drechsleri	18 (5.19)	5 (1.44)	27.78
Totals	111 (31.99)	16 (4.61)	14.41