MERIWA PROJECT : M 227

RAPID IDENTIFICATION OF SPECIES OF *PHYTOPHTHORA*

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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. 4 a work programme for the fifth quarter was outlined.

Work programme for the fifth quarter :

Topic	**Type of Work**	**Time (weeks)**
5-7	Literature search	2.0
5.1	Monitor the pH of soil samples tested by the VHS	0.5
5.2	Test modifications to baiting techniques	6.5
6.1	Develop techniques for the direct isoenzyme assay of bait tissues	2
	MERIWA Report	1
	Total	12

Work in the above 4 topics was to run concurrently.

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

WORK DONE DURING THE FOURTH 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

Topics 5-7

LITERATURE SEARCH

The literature search covers the following subjects :

Mycological Techniques
Phytophthora and Bacteria Physiology
Phytophthora Reproductive Biology
Phytophthora Ecology
Electrophoresis Techniques
and Probability

This search will be ongoing for the duration of project M 227.

Topic 5

5.1 THE pH OF SOIL SAMPLES TESTED FOR *PHYTOPHTHORA*

The baiting technique, as used routinely by the Vegetation Health Service (VHS) of CALM for detecting the presence of *Phytophthora* in soil/plant tissue samples, requires that the fungus produce sporangia, and that the zoospores from these sporangia infect the bait (eg. young lupin leaves and Eucalypt cotyledons). *Phytophthora* species may form sporangia within a wide pH range from 4 to 9 (Ribiero 1983), and Pegg (1977) found that *P. cinnamomi* was rarely recovered from soils with pH below 3.8. The object of this research was to determine whether or not the pH of the soil samples received by the VHS for testing for *Phytophthora* affected recovery of the fungus i.e. to test the null hypothesis: that the mean pH of samples positive for *Phytophthora* is the same as that of negatives.

Procedure

The test samples consisted of 25 soil/plant tissue samples found by the VHS to be infected with *Phytophthora* and 25 samples that were not found to be infected. 10g samples of each of these 50 samples were weighed out into separate vials to which was added 10ml of distilled water (pH 4.5). The soil-water samples were each mixed thoroughly, and the pH was then measured.

Results

The pH of the soils in this study that were positive for *Phytophthora* (pH 4.5 - 7.3) were within the range of pH in which *Phytophthora* produce sporangia (pH 4 - 9; Ribiero 1983), and all but one of the soil samples found to be negative for the fungus were within this range also (Table 1). However, the mean pH of soils positive for *Phytophthora* was higher (5.88) than that of the negatives (5.1), and a two-tailed test showed that for d.f. = 48 the pooled population variance of pH of these samples ($t = 13.22$) was greater than the table value ($t_{.05} = 1.68$). The null hypothesis therefore, that the mean of positive (those found to be infected with *Phytophthora*) samples is the same as the mean of negatives, is rejected with $p < 0.05$.

Discussion

The significant difference in pH between soil infected and those not infected with *Phytophthora* was an unexpected result. This raises the question : was *Phytophthora* not detected in the more acid soils because i) the fungus was absent; or ii) the fungus was present but the acid condition of the soil inhibited sporangial production and zoospore activity, resulting in the fungus going undetected.

Our intention now is to :

1. increase the number of samples in the test study; and if the trend holds,
2. test whether artificial manipulation of the pH of soil samples during baiting affects the recovery of species of *Phytophthora*.

This area of research is not completed and will require a further 1.5 weeks work.

5.2 TEST MODIFICATIONS TO THE BAITING TECHNIQUE

Part of the detection method for the recovery of *Phytophthora* from soil/plant tissue samples is to plate baits (young lupin leaves and Eucalypt cotyledons) showing lesions onto selective agar. Twenty four hours later the lesions are examined microscopically for the presence of *Phytophthora* growing into the agar. My objective was to determine the number of baits that require testing for the presence of *Phytophthora* to be 95% confident of retrieving the fungus from infested samples.

Procedure

The method used here was recommended by Frank Podger and is a modification of that practised by the VHS :

1. *Circa* 300g of soil/plant tissue sample was distributed into each of two (reps.) plastic baiting dishes.
2. *Circa* 300ml of distilled water was added to the baiting dishes.
3. Thirty to fourty W.A. blue lupin baits were then added to the baiting dishes. VHS staff added *Eucalypt* cotyledons also.
4. On the third-fifth day following baiting a number of baits with lesions (column 2 of Table 2 : No. of baits plated) were plated onto selective agar plates.
5. Sixteen to forty hrs later the infected baits were examined for the presence of *Phytophthora* growing out of the baits into the selective agar.
6. Samples that were negative after ten days, were dried, re-wetted, rebaited and then treated as per steps 4 and 5 above (i.e. were double baited).

Results

In samples containing two *Phytophthora* species, one species produced far fewer colonies than the other (column 3 of Table 2).

Phytophthora was not detected in all the lesions from bait tissue. The mean percentage of lesions (per soil/tissue sample) from which *Phytophthora* colonies grew was 40% (mean of values in column 3 of Table 2), and ranged from 8 - 100%.

One to as many as 35 baits required testing to be 95% confident of retrieving *Phytophthora* in these samples. In 34.5% of cases (9 of 26; column 4 of Table 2) too few baits were tested to reach the 95% confidence level of retrieving the fungus.

In 96% of cases (25 of 26; column 5 of Table 2) the lesions of at least 28 baits required testing to reach 95% confidence of retrieving *Phytophthora* from these samples. Even at this level of testing false negatives are expected 1 in 20 times.

Discussion

These results emphasize the need to test large number of baits having lesions (n=28), when screening for *Phytophthora,* to attain 95% confidence levels of detecting the fungus i.e. to keep the number of false negatives down to 5% or less. Because of the large differences in the size of the baits used (lupin and Eucalypt) and variation in lesion sizes, these analyses should be taken to be indicative and not definitive.

This area of research is not completed and will require a further 5.5 weeks work.

Recommendations

1. 40+ lupin baits should be added to each baiting tray.

2. The entire lesions of baits should be plated onto selective agar on the day lesions are first observed. This would require that each baiting dish be assessed for the presence of lesions on day 3 and each day thereafter to day 10.

3. For each sample the entire lesions of 28 or more baits, where available, should be plated to selective agar (2 plates at 14 baits per plate).

6.1 DEVELOP TECHNIQUES FOR DIRECT ISOENZYME ASSAY OF BAITS

Techniques for the direct isoenzyme assay of *Phytophthora* infested baits have been developed and tested successfully with *P. cinnamomi* and one taxa in the *P. citricola* complex. This area of research is **completed** to the point where it can be applied in the case of infection by single species if *Phytophthora*.

This researchs' extension :

6.2 ISOENZYME ASSAY OF SPECIES MIXES

requires further development. Three weeks are required for this research.

Acknowledgments

My thanks to Jan Webster for help in the detection of *Phytophthora* and Frank Podger for helpful discussions in the development of research aims.

References

Pegg, K G 1977. Soil application of elemental sulphur as a control of *Phytophthora cinnamomi* root and heart rot of pineapple. Aust J Exp Ag Anim Husb 17:859-865.

Ribiero, OK 1983. Physiology of asexual sporulation and spore germination in *Phytophthora*. In : ***Phytophthora*** *its biology, taxonomy, ecology, and pathology.* (Eds. DC Erwin, S Bartnicki-Garcia, and PH Tsao). An American Pathological Society publication. pp 392.

PROGRAMME FOR THE FORTHCOMING QUARTER

The July - September 1995 quarter is of 13 weeks duration. The work to be done in this quarter is scheduled as follows :

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 soils	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

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. **pH of 25 soil/plant tissue samples found to be infected with *Phytophthora* and 25 samples found not to be infected.**

Sample No.	pH of +ves(x_i)	$(x_i - \bar{X})^2$	Sample No.	pH of -ves(x_j)	$(x_j - \bar{X})^2$
1	5.6	0.081	26	5.9	0.640
2	4.7	1.402	27	5.5	0.160
3	5.4	0.234	28	5.0	0.010
4	5.7	0.034	29	5.1	0.000
5	5.4	0.234	30	5.0	0.010
6	6.2	0.100	31	5.3	0.040
7	6.5	0.379	32	5.5	0.160
8	5.8	0.007	33	5.9	0.640
9	4.9	0.968	34	6.4	1.690
10	5.5	0.147	35	4.8	0.090
11	7.1	1.479	36	4.5	0.360
12	7.3	2.005	37	5.4	0.090
13	4.9	0.968	38	5.0	0.010
14	4.5	1.915	39	5.0	0.010
15	5.6	0.018	40	4.9	0.040
16	6.8	0.839	41	5.5	0.160
17	5.2	0.468	42	6.3	1.440
18	7.0	1.245	43	5.2	0.010
19	6.8	0.839	44	4.1	1.000
20	6.3	0.173	45	3.8	1.690
21	6.3	0.173	46	4.1	1.000
22	6.3	0.173	47	4.7	0.160
23	5.7	0.034	48	4.1	1.000
24	5.9	0.000	49	4.4	0.490
25	5.7	0.034	50	6.1	1.000

TABLE 2. **Percentage of plated lesions (Lupin/Eucalypt) from VHS soil/plant tissue bait trays which *Phytophthora* grew from (column 3), the observed confidence level of retrieving *Phytophthora* from those plated lesions (column 4), and the estimated # of baits requiring testing to be 95% confident of retrieving the fungus (column 5).**

SAMPLE No.		No. OF BAITS PLATED	% COLONIES PER BAIT	CONFIDENCE OF DETECT (%)	No. BAITS FOR 95% CONF.
1	Sp. 1	10	10	65	28
	Sp. 2	10	50	99.9	4
2	Sp. 1	8	12.5	65	23
	Sp. 2	8	87.5	99.9	1.5
3	Sp. 1	8	12.5	65	23
	Sp. 2	8	25	90	11
4	Sp. 1	12	83	99.9	1.6
	Sp. 2	12	17	89	16
5	Sp. 1	10	40	99.9	6
	Sp. 2	10	50	99.9	4
6		9	44	99.9	5
7		8	12.5	65	23
8		9	100	99.9999	1
9		24	16.6	98.3	17
10		29	79	99.9999	2
11		13	31	99.9	8
12		16	37.5	99.9	6
13		15	40	99.9	6
14		17	47	99.9	5
15		13	31	99.9	8
16		14	93	99.9999	1.2
17		10	10	65	28
18		12	8	63	35
19		11	27	99.9	9
20		6	16.6	66.5	17
21		9	55.5	99.9	4