PROJECT : M 227 : RAPID IDENTIFICATION OF SPECIES OF PHYTOPHTHORA

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INTRODUCTION

As outlined in the work plan of the 'Application for Research and Development Assistance' to MERIWA, it was anticipated that Stages 1 and 2 would overlap in the first and second quarters.

To be achieved during :

Stage 1

1.1 Cultures were to be prepared.

1.1.1 Sample Size

1.1.2 The Collection

1.2. Methods for culturing the aforementioned isolates were to be determined.

1.3. Methods for analysing the isolates isozymically were to be determined.

1.3.1 Which Method?

1.3.2 Isozyme Protocols

Stage 2

2.1 Isozyme profiles of the local *Phytophthora* species were to be identified.

The following report contains a point by point account of the work done on Project M227 in the first quarter of a four quarter programme. A coverage is given of the progress to date for each of the sections listed above, and any incomplete business arising from those areas of research is described.

WORK DONE DURING THE QUARTER

The primary objective of this research is to identify isozyme banding pattern differences between the local species of *Phytophthora*.

In order to achieve this goal one must first describe the range of banding patterns that occur within each species. After reviewing the literature it was considered appropriate to determine, by statistical analysis of published data, the appropriate sample size needed to describe the banding patterns.

Stage 1

1.1 CULTURES TO BE PREPARED.

1.1.1 Sample Size

Our objective was to determine the number of isolates required to be tested in order to detect, with 95% confidence, all of the 'affective' banding pattern types (to be referred to as 'affective multilocus genotypes' or AMG's) that occur within *Phytophthora* species. AMG's are those multilocus genotypes that occur with a frequency of 5% or more in the population/sample.

Procedure

A literature search of isozyme studies of five *Phytophthora* species, and one species each of four other fungal genera, revealed that researchers examine between 22 and 200+ isolates per species. From these sample sets they determine the frequencies of multilocus genotypes.

From the published multilocus genotype frequencies, those that were 'affective' (in frequency of 5% or more) were identified, and then the sample size required to retrieve them, with 95% confidence, was determined statistically (Figures 1a and 1b).

Results and Conclusions

It was determined that the average number of *Phytophthora* samples required in an isozyme study, to be 95% confident of retrieving all of the AMG's, was 54 (range 46 - 67). This area of research has been completed.

1.1.2 Collection

In the previous section it was shown that 46 - 67 isolates would be required per *Phytophthora* species in order to detect, with 95% confidence, all of the AMG's.

In this section our objective was to acquire, from available *Phytophthora* collections, the isolates required to research the rapid identification technique. It was also important to identify species in which the number of available isolates was less than that recommended for isozyme studies (1.1 Sample Size), so that additional isolates could be sought to increase numbers as necessary.

Included in our study are some *Phytophthora* species that do not occur locally and some *Pythium* species. These *Phytophthora* species were included because we have reference specimens in our collection which feature in published work. The *Pythiums* were included because they sometimes occur in mixed culture with *Phytophthora,* and it will be necessary to be able to distinguish these from *Phytophthora.*

A *Phytophthora* species was considered to be a priority species if it occurred locally.

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

Procedure

The content of ten *Phytophthora* culture collections is presented in Table 1.

Isolates required for the project are subcultured to Corn Meal Agar (CMA) plates and checked for purity. They are 'cleaned up' (freed of contamination) as necessary and put into semi-permanent storage.

Results

The following species are considered to be deficient in number :

P. cactorum, *P. cambivora*, *P. drechsleri*, *P. erythroseptica*, *P. nicotianae* and *P. clandestina.*

Efforts are presently underway to increase the sample sizes of the following priority species : *P. drechsleri*
P. nicotianae

38% of the isolates indicated in Table 1 have been subcultured and cleaned up, where necessary, for the isozyme collection. It is estimated that a further 3 weeks will be required to process the outstanding (62% of Table 1) samples.

1.2. METHODS FOR CULTURING THE ISOLATES

A large amount (10's of mgs) of actively growing hyphae is required for isozyme analysis. Our objective was to assess two culture techniques and several media and select that which was most suitable for our purposes.

Procedure

Phytophthora cinnamomi produces copious amounts of aerial hyphae on Green Pea Agar (GPA). Two isolates of each of six local species were grown on GPA plates and the amount of aerial hyphae they produced was subjectively assessed.

A series of trials was carried out in which standard *Phytophthora* isolates were grown in V8, Corn Meal, Green Pea and synthetic broth in various combinations and concentrations.

Results and Conclusions

25% of the isolates tested on GPA produced less than the desired amount of aerial hyphae. It was therefore decided not to use this technique for growing hyphae.

A wide range of isolates grew well on a V8 + Green Pea + synthetic broth, and we have continued to use this medium. A further weeks' work will be required to finalize the synthetic component of this culture medium.

1.3 METHODS FOR ANALYSING THE ISOLATES ISOZYMICALLY

1.3.1 Which Method?

Our objective was to compare two internationally accepted isozyme methods to determine which gave the best results, and which was the most cost effective and user friendly.

Procedure

Two methods compared were the Poly Acrylamide Gel Electrophoresis (PAGE) and the Cellulose Acetate Gel Electrophoresis (CAGE) techniques. A standard set of samples and assays was used, and the two methods were assessed for the quality of results they produced, cost and user friendliness.

Results and Conclusions

The PAGE and CAGE methods both gave comparable results and good resolution of bands in standard trials. The CAGE method was superior, however, in the following respects :

1. It gave a semi permanent record of results whereas the PAGE method did not.
2. It was more cost effective, and faster to run than the PAGE method.
3. It was the more user friendly.

The CAGE method was therefore used in subsequent tests.

1.3.2 Development of Standard Isozyme Protocols

Our objective was to develop standard isozyme protocols for *Phytophthora*. Four protocols were identified :

1. Protein extraction
2. Electrophoresis
3. Assay techniques
4. Photographic record of results

Procedure

The fungal isozyme literature was reviewed, and the various protocols used by researchers were compared.

The literature review revealed that Tris-HCl was the most commonly used protein extraction buffer. Concentrations ranged from 0.05-0.2 M and pH ranged from 7.0-9.0. 0.1 M Tris-HCl buffers at pH 7.2, 7.7, 8.2 and 8.7 were compared with *Phytophthora* and assay standards.

Very few CAGE electrophoresis running buffers were found published in the fungal isoenzyme literature. Consequently SAC's TEM buffer (developed for plants), and a new electrophoresis buffer, SAC's TC, were compared with *Phytophthora* and assay standards.

25 enzyme assay systems were used widely in the fungal isoenzyme literature . While the substates required to test all of these systems have been ordered, not all were available for assessment in the first quarter. Preliminary tests have been carried out on 18 assay systems.

Lighting, film and exposure conditions were determined for photographing CAGE gels.

Results and Conclusions

Extraction buffers in the pH 8.2-8.7 range gave the best results. Two more days' work are required in this area of research to test assays for which substrates have not yet been available.

Both the TEM and TC electrophoresis buffers gave highly resolved bands and good signal. No further research is required in this area, and both buffers will be used in subsequent tests.

Of the 18 assay systems subjected to preliminary investigation, 8 have been standardized for routine use. For an example of the banding quality achieved in these 8 systems, refer to Figure 2. This area of research requires a further 2 weeks' work for its completion.

Standard photographic protocols have been developed for CAGE gels, and no further reasearch is required in this area. For an example of the photographic quality achieved, refer to Figure 2.

Stage 2

2.1 ISOZYME PROFILES OF THE *Phytophthora* SPECIES

As stated earlier our primary objective is to identify isozyme banding pattern differences between the local species of *Phytophthora*. A preliminary investigation, using one of the 8 standardized assay systems, was initiated to examine the level of variation between some of the local species.

Procedure

One sample each of *Phytophthora cinnamomi* (Pc) and 4 other local *Phytophthora* species were compared for their Glucose Phosphate Isomerase (GPI) protein.

Results and Conclusions

Four distinct banding patterns (allozymes) were observed (Figure 2). Pc had the most negatively charged (i.e. the most mobile) GPI protein, distinguishing it from the other 4 species. Species 3 and 4 also had unique bands, but GPI did not distinguish between Species 2 and 5.

While we do not as yet have a measure of the variation within species, the between species variation observed in Figure 2 was considered to be very promising. This area of research is scheduled for completion in the third quarter.

PROGRAMME FOR THE NEXT QUARTER

The second quarter is of 13 weeks duration. The work to be done in this quarter is scheduled as follows :

Stage	Type of Work	Time (Weeks)
1.1.2	The *Phytophthora* Collection	3
1.2	The Culture Medium	1
1.3.2	Isozyme Protocols	2.5
2.1	Isozyme Profiles of *Phytophthora* species	6*
	Second MERIWA Report	0.5
	Total	13

* The isozyme profiles of *Phytophthora* species are scheduled for completion in the third quarter.

Work to be completed in Stages 1 and 2 are to run concurrently.

Final Comment

All work areas are progressing in accordance with the programme set out in the research application to MERIWA. No major obsticles have been encountered at this stage, and none are foreseen for the second quarter.

TABLE 1. A summary of the *Phytophthora* isolates available for this research, and the various collections from which they have (will have) been received.

Species	DCE	MS	VHS	MC	MD	FB	SEB	GH	PW	RH	Tot
P. cactorum	5			1							6
P. cambivora	4			5							9
*P. cinnamomi	125	100	10	10	3			35			283
*P. citricola	12	22	16	2		72					124
P. clandestina									5		5
*P. cryptogea	5	10	33	3	13				1		65
*P. drechsleri	7		3		2					5	17
P. erythroseptica	1			1							2
*P. megasperma	12	7		8			78			15	120
*P. nicotianae	13		10	4	11				2	1	41
Pythium sulcatum								40			40
Pythium spp.	30										30
TOTAL	214	139	72	34	29	72	78	75	8	21	742

	Collection Housed	Curator
DCE	CALM/Como	Elaine Davison
MS	"	Mike Stukely
VHS	"	Francis Tay
MC	"	Elaine Davison
MD	CSIRO/Canberra	Mark Dudzinski
FB	CALM/Como	Felicity Bunny
SEB	CALM/Como	Stan Bellgard
GH	Murdoch University	Giles Hardy
PW	WA Ag Dept	Peter Wood
RH	Shenton Park WA	Hart, Simpson & Assoc.

* Priority species (i.e. those occurring in native vegetation in WA)

Figure 1. **The relationship between sample size and the probability of retrieving all 'affective' multilocus genotypes (AMG's). From data published for 5 *Phytophthora* species and one species each of 4 other genera.**

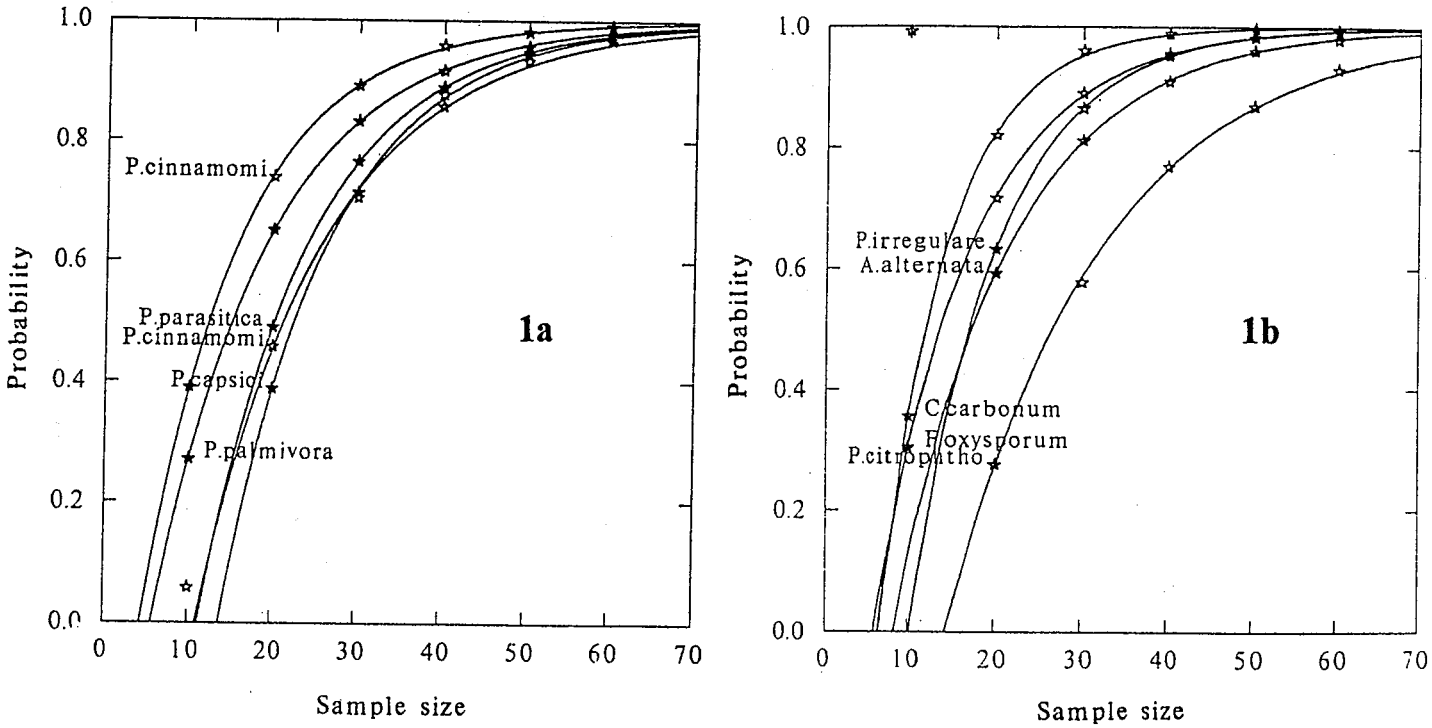

Figure 2. CAGE photograph of Glucose Phosphate Isomerase (GPI) proteins of *Phytophthora cinnamomi* (Pc) and 4 other local *Phytophthora* species. The Pc protein was the most mobile, differentiating it from the other 4 species.