MERIWA PROJECT : M 227

RAPID IDENTIFICATION OF SPECIES OF *PHYTOPHTHORA*

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QUARTERLY REPORT NO. 4

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EXECUTIVE SUMMARY

Stephen A. Carstairs was contracted by CALM for 12 months, starting in March 1994, to compare the morphological methods of identifying *Phytophthora* species currently used by the Vegetation Health Service (VHS) at Science and Information Division (SID)/Como with an isoenzyme method on the basis of accuracy, efficiency and cost.

Cellulose acetate gel electrophoresis (CAGE) procedures, specific for the assay of *Phytophthora* proteins, were established. Four enzyme systems (selected for various technical reasons) were found to be sufficient for the purpose of this investigation.

Three hundred and ninety isolates, representing the five species/species groups known from native vegetation in the wild in WA, were compared with 51 standards among the same 5 species imported from California. Among the WA isolates tested 45% were isoenzymically comparable to the Californian standards. The variation observed among the WA isolates was of the same general order as that in 586 isolates (collected from the northern and southern hemispheres) tested by Coffey's team in California. WA isolates of *Phytophthora cinnamomi* however, were more variable than was expected.

The assignment of fifty unknown *Phytophthora* isolates to species by the isoenzyme method was found to be correct except in one case in the taxonomically contentious *cryptogea/ drechsleri* group. Morphological approaches to diagnosis were satisfactory apart from the *cryptogea/drechsleri* group, one misassignment of *Phytophthora megasperma* to *Phytophthora citricola*, and one uncertain *P. cinnamomi* isolate.

The isoenzyme technique was more reliable than the morphological and provided unequivocal information on variability and relationships among isolates and species which was not available in the morphological approach.

The costs of morphological diagnosis vary with :

1. the facility with which the isolates produce the organs essential for diagnosis, which may vary both within species and between them; and
2. the taxonomic rank required by the client, i.e. diagnosis to a) genus, or to b) species.

Routine VHS morphological diagnosis to *P. cinnamomi* or to '*Phytophthora* sp.' is faster (15 days) and cheaper ($5.85) per sample, but is less certain than the fuller morphological or the isoenzyme technique. A fuller morphological diagnosis may require 27.7 to 41 days and cost between $19.00 and $25.00 per sample (with no on-costs other than a 32% administrative charge on salary). Isoenzyme diagnosis required, consistently for all species, 19-20 days and cost $17.00 per sample (with the same on-cost basis).

In these comparisons the minimum savings by diagnosing species, other than *P. cinnamomi*, by isoenzymes are 38.5% in time and 12% in costs.

For locations where long experience shows that the proportions of *Phytophthora* isolates of the various species are dominated by species other than *P. cinnamomi*, eg. Northern Sand Plains and Fitzgerald River National Park, all isolates retrieved should be directly routed to isoenzyme analysis because of the considerable savings.

EXTENDED SUMMARY

Preface

In March 1994 the services of Stephen A. Carstairs, an experienced chemical geneticist, were engaged for 12 months by CALM, acting for MERIWA and an informal consortium from the mining industry which contributed in cash and kind as follows:

	Cash ($)	In Kind ($)
CALM		38,600
MERIWA	23,320	
ALCOA	10,000	
WORSLEY	10,000	
TI WEST	6,000	
RGC	5,000	
WESTERN COLLIERIES	5,000	

The schedule of agreement for contractual services is attached at Appendix 1.

The contractual work was conducted at the Como Laboratories of the Science and Information Division (SID) of CALM; the isoenzyme analyses being performed in a newly equipped isoenzyme laboratory.

The technique used to examine these matters is known as cellulose acetate gel electrophoresis (CAGE). Genotypically distinct variants (called electromorphs) are recognised by their protein banding patterns expressed on electrophoretically charged gels of cellulose acetate. These unique patterns are very much like bar codes and have the same value for information transfer.

This summary details, for each of the contracted services, an account of performance by the contractor and recommendations flowing from the work and from studies by the author additional to the contract.

Performance of contract

The schedule of contract required address of 6 'services'. Each is addressed here in turn:

1. Selection and testing of enzyme systems and establishment of methodology.

   From among the 30 or so enzyme systems commonly used in isoenzyme studies world wide 18 were tested. Of the 13 found suitable, 4 systems (selected for various technical reasons) were found to be sufficient for the purpose of this investigation.

2. Comparison of enzyme profiles of species of *Phytophthora* from WA with a set of standards imported from California.

The 390 isolates used were selected from existing collections of the 5 species/species groups known from native vegetation in the wild in WA. These were compared with 51 standards among the same 5 species imported from California. The variation was broadly comparable for both sets (WA v California). Variation observed among the 180 WA isolates of *Phytophthora cinnamomi* was greater than expected. For other species the high levels of intraspecific variability expected from the literature and local experience were observed.

3. Effectiveness of isoenzymes for identifying species.

Isoenzyme preparations of fifty unidentified Western Australian isolates across the range of 5 species were presented to an experienced operator for matching with standards selected by the author. The assignment of unknown isolates to species was found to be correct except in one case in the taxonomically contentious *cryptogea/drechsleri* group. This is attributable to human error in interpretation of gels. Such error could be avoided with automation of the interpretation step using Polyacrylamide gel electrophoresis but this would require a substantial further investment of capital.

4. Comparison of isoenzyme analysis with morphological methods on the basis of time, accuracy and cost.

The costs of morphological diagnosis vary with :

1. the facility with which the isolates produce the organs essential for diagnosis, which may vary both within and between species; and
2. the degree of certainty of diagnosis required by the client.

Routine VHS diagnosis to *P. cinnamomi* or to '*Phytophthora* sp.' is faster (15 days, see Table 2) and cheaper ($5.85, see Table 5) per sample, but is less certain than the fuller morphological or the isoenzyme technique. A fuller morphological diagnosis may range from 27.7 to 41 days (Table 2) and cost between $19.00 and $25.00 per sample (with no on-costs other than a 32% administrative charge on salary, see Table 5). Isoenzyme diagnosis required, consistently for all species, 19-20 days (Table 2) and cost $17.00 (Table 5) per sample (with the same on-cost basis). In these comparisons the minimum savings of diagnosing species other than *P. cinnamomi* by isoenzymes are 38.5% in time and 12% in costs.

Concerning the reliability of diagnoses, morphological approaches were satisfactory apart from the *cryptogea/drechsleri* group, one misassignment of *P. megasperma* to *P. citricola*, and one uncertain *P. cinnamomi* isolate. Isoenzyme analysis was accurate except for the single case referred to in point 3 above.

The isoenzyme technique is far more reliable than the morphological and provides unequivocal information on variability and relationships among isolates and species which is not always available in the morphological approach.

5. Variation in local spp. of *Phytophthora* in relation to world-wide variation.

This service as required in the schedule of contractual services is phrased in a form of words which sets an unattainable goal. 'World wide variation' therefore, is taken to mean the variation found in Coffey's extensive Californian collection.The work referred to in service 2 above indicates that variability observed among WA isolates was of the same general order as the set of samples originally obtained by Coffey from donors in a range of countries. Among the 390 WA isolates tested 45% were isoenzymically comparable to isolates in Coffey's collection of 586. The extent to which Coffey's extensive but far from fully comprehensive collection reflects true world-wide variability is unknown.

6. Training of staff at the Vegetation Health Service in the use of isozyme analysis for the identification of species of *Phytophthora*.

Both technicians routinely identifying *Phytophthora* during the contract period have been trained and perform at a highly satisfactory level in the routine protocols of sample preparation and isoenzyme electrophoresis. Complete operation of the isoenzyme method as a diagnostic service for all isolates, including species either not tested here or difficult to assign by traditional taxonomy (ie. those usually designated *Phytophthora* sp.), will require further training, an ongoing familiarity with the technique and freely available access to professional advice.

Recommendations

In areas, such as in the jarrah forest or Stirling Range National Park, where long experience shows that the proportions of *Phytophthora* isolates of various species are likely to be overwhelmingly dominated by *P. cinnamomi* there are considerable advantages to be obtained by continuing use of the cheap simple hyphal morphology stratagem. Those isolates retrieved by baiting or direct isolation and not assignable to *P. cinnamomi* should be passed on for isoenzyme analysis, perhaps with a small percentage of *P. cinnamomi* isolates for verification and quality control.

In locations where experience shows that the proportions of *Phytophthora* isolates of the various species are dominated by species other than *P. cinnamomi*, eg. Northern Sand Plains and Fitzgerald River National Park, all isolates retrieved should be directly routed to isoenzyme analysis because of the considerable savings and for the benefit which will accrue from a better understanding of the identity and knowledge of variability in these morphologicaly more contentious taxa.

The existing data bases of distributions of these various species of *Phytophthora* provide potentially important opportunities for exploring the possibility of interaction among species of these pathogens. Isozyme studies also offer a more rational approach to evaluating differences in the capacity of various taxa to inflict severe and persistent damage on the flora.

INTRODUCTION

In CALM's application for research and development assistance to MERIWA and the mining industry four stages in the work plan for M 227 were recognised:

Stage 1. Prepare cultures, and establish methodology.
Stage 2. Compare isoenzyme profiles of local isolates with Californian standards, and document intra- and interspecific variation among local species of *Phytophthora*.
Stage 3. Teach technical staff how to use the isoenzyme method routinely.
Stage 4. Compare the isoenzyme and morphological methods of identifying local *Phytophthora* species for efficiency and cost effectiveness.

In M 227 Quarterly Report No. 3 a work programme for the fourth quarter was outlined.

Work programme for the fourth quarter :

Stage	Type of Work	Time (weeks)
1.1.2	The *Phytophthora* Culture Collection	0.5
2.1.1	Comparison of Local Isolates with Standards	1
2.1.2	Intraspecific Isozyme Variation	0.5
2.2	Interspecific Differences	1.5
3.1	Introduce Technical Staff to Methodology	0.5
4.1	Comparison of Isozyme & Morphology Techniques for Efficiency and Cost	3
	Final MERIWA Report	3
	Total	10

Work in the above 4 stages was to run concurrently.

What follows summarises progress made in quarters 1 to 3 and gives an account of the progress made in the fourth quarter on each of the sections 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

Stage 1

1.1 CULTURES TO BE PREPARED

1.1.1 Sample Size

This area of research has been **completed**. An account is given in MERIWA M227 Report #1.

1.1.2 The *Phytophthora* Culture Collection

In this section our objective was to acquire, from various collections, the isolates required to research the rapid identification technique. In the third quarter it became apparent that additional Californian cultures (standards) were required with which to compare the range of variants observed in CALM's collection of Western Australian *Phytophthora* isolates.

Those standards were aquired, and no additional standards are required at this stage. CALM's collection of local isolates is a subset of the wider population, and as new taxa or variants are encountered steps will have to be taken to extend the collection of standards where possible.

1.2 METHODS FOR CULTURING *PHYTOPHTHORA* ISOLATES

This area of research has been **completed**. An account is given in MERIWA M227 Reports 1 & 2, and a fuller account will be given in the final report.

1.3 METHODS FOR ANALYSING THE ISOLATES ISOENZYMICALLY

This area of research has been **completed**. From among the 30 or so enzyme systems commonly used in isoenzyme studies worldwide 18 were tested with the CAGE technique. Thirteen of these were found to be suitable. An account is given in MERIWA M227 Reports 1 & 2, and a fuller account will be given in the final report.

Stage 2

2.1 ISOENZYME PROFILES OF *PHYTOPHTHORA* SPECIES

2.1.1 Comparison of Local Isolates with Standards

One of the objectives of this project, as outlined in the 'Application for Research and Development Assistance' to MERIWA, was to compare isoenzyme profiles of local isolates with standards obtained from Californian and ACT collections with the view to establishing the reliability of the electrophoresis technique.

This area of research has been **completed**. Western Australian isolates that had been assigned to *P. cinnamomi* or *P. nicotianae* by the various donors who provided them for this study, produced comparable isoenzyme profiles to Californian standards of the same species (see MERIWA M 227 Reports 2 & 3). However fewer WA isolates of the other four species researched, *P. citricola, P. cryptogea, P. drechsleri* and *P. megasperma,* matched with Californian standards. Of the 390 WA isolates (representing the priority species) evaluated isoenzymically in this study, 45% matched with Californian standards. A subset of these is given in Appendix 2 from which an appreciation may be gained of the extent to which the various WA species matched isoenzymically with the standards.

2.1.2 Intraspecific Isozyme Variation

The primary objective of this project is to identify isozyme banding pattern differences between the local species of *Phytophthora*. To achieve this goal one must first describe the range of banding patterns that occurs within each of the priority species. Given the sample sizes of the various WA species examined in this study (see MERIWA M 227 Reports 2 & 3), a high proportion of the variation that occurs within them was expected to have been identified (see MERIWA Quarterly Report #1 for an account of the effect of sample size in isoenzyme studies of *Phytophthora*).

This area of research has been **completed**. The levels of variation observed among the WA isolates was of the same general order as that in 586 isolates (collected from the northern and southern hemispheres) tested by Coffey's team in California, except in the case of *P. cinnamomi* which was more variable than expected. A fuller account will be given in the final report.

2.2 INTERSPECIFIC DIFFERENCES

This research aims to determine whether or not isoenzymes discriminate between priority *Phytophthora* species. The objective therefore, was to identify loci which were relatively invariant within species, while exhibiting 'fixed' allozyme differences between species, i.e. to identify genes which gave species-specific CAGE bands thereby distinguishing between them. Such allozyme differences may then be used routinely to assign unidentified isolates to species. This method of discriminating between species assumes that sufficiently large sample sizes have been tested (see 1.1.1 Sample Size of MERIWA Quarterly Report #1).

Procedure

Isolates representing much of the range of variation found in the six species tested in this study:

P. cinnamomi	1	electromorph group
P. citricola	3	" groups
P. cryptogea	3	" "
P. drechsleri	2	" "
P. megasperma	3	" groups
P. nicotianae	1	electromorph group

were compared in side-by-side trials with a *P. cinnamomi* standard for the CAGE isoenzyme patterns they produced when tested with a range of enzyme systems. A subset of these systems were then selected for routine diagnosis on the basis of their ability to discriminate between species, technical ease of use, and cost.

Results

This area of research has been **completed**. No one isoenzyme discriminated consistently between all the *Phytophthora* species tested. When the data of four enzyme systems:

Isocitrate Dehydrogenase (IDH);
Lactate Dehydrogenase (LDH);
Malate Dehydrogenase (MDH); and
Phospho-6-glucose Isomerase (PGI)

was used together, the 13 *Phytophthora* electromorph groups tested were distinguished. Further, these enzyme systems were user friendly and cost effective, compared with most others. A fuller account will be given in the final report.

The four enzyme systems identified in this study were subsequently used to compare the isoenzyme and morphological methods for accuracy, speed and cost of identifying *Phytophthora* species (see Stage 4).

Stage 3

3.1 INTRODUCTION OF TECHNICAL STAFF TO ISOZYMES

If it is shown that isoenzymes identify unknown *Phytophthora* cultures to species more efficiently and cost effectively than does the morphological method, our objective is to train CALM staff in the CAGE identification technique such that they will be able to provide this service on a regular basis. Two CALM technicians from the VHS attended individual training sessions in the use of the isozyme laboratory (see MERIWA Quarterly Report # 3). Both technicians have sufficient experience to work in an isozyme laboratory and carry out the various CAGE protocols required for the identification of local *Phytophthora* species.

Because staff changes are a feature of all organizations, it is recommended that professional CALM staff be trained in the use of the CAGE isoenzyme technique in the forthcoming quarters.

Stage 4

4. COMPARISON OF ISOENZYME AND MORPHOLOGY TECHNIQUES FOR ACCURACY, EFFICIENCY AND COST

In this section it was assumed that there was no significant difference in accuracy, efficiency or cost between the isoenzyme and morphology techniques for identifying *Phytophthora* species. Our objective therefore, was to test the hypothesis : that there was no difference between the methods (hereafter referred to as Experiment 1 or Deliberate Experiment). If the techniques do differ then management may decide which method to adopt for routine diagnosis.

Procedure

Listed in Appendix 2 are fifty isolates that were selected at random from the cultures used to describe the isoenzyme variation in the priority species (section 2.1.2 above), which were used to compare the morphological and isoenzyme methods. Sizes classes in each of the 6 species were biased such that the more variable (contentious) species had greater representation.

Two pure cultures of each of the 50 isolates (prepared on corn meal agar (CMA) plates) were given unique identification numbers, and were subsequently referred to as 'unknowns'. One set of 50 unknowns was used to assess the isoenzyme technique for accurracy, efficiency and cost, and the other set was used to assess the morphological.

Two experienced operators recorded the times they took to perform the various tasks required to identify each of the 50 isolates by the respective methods (see Appendix 3). *P. cinnamomi* was assessed with the 'short' morphological method (presence or absence of coralloid hyphae) and 'fuller' morphology (see Newhook *et. al.* 1978).

Isoenzyme profiles of the 50 unknowns used in the isoenzyme trial were prepared by the author and one of the aforementioned operators. These were presented to a third experienced operator for matching with standards determined in section 2.2 above. Three 'distractors' (isolates which had isoenzyme patterns similar to some of the study isolates) were included with the standards.The times required to perform these tasks were also recorded.

Results

4.1 Accuracy : All of the *P. cinnamomi* unknowns used in this study were correctly assigned by the operators to species on the coralloid hyphae they produced (Table 1). One of the operators was not confident about assigning MJS 171 to *P. cinnamomi* on

coralloid hyphae alone. Two isolates did not produce all of the structures required to complete the fuller morphological identification.

The 9 *P. citricola* isolates used in this study produced all of the structures required to complete the fuller morphological identification, and all were correctely assigned to *P. citricola* with this identification technique (Table 1).

Only 2 of the 12 *P. cryptogea* isolates used in this study produced all of the structures required to complete the fuller morphological identification. These 2 were correctly assigned to *P. cryptogea* with this identification method; the other 10 *P. cryptogea* unknowns were also correctly assigned although their morphological data sets were incomplete (Table 1).

Two of the 10 *P. drechlseri* isolates used in this study produced all of the structures required to complete the fuller morphological identification, however, only one of these was correctly assigned to *P. drechsleri* with this identification method (Table 1) while the other was misassigned to *P. cryptogea.* Of the 8 isolates that did not produce all structures, 2 were correctly assigned to *P. drechsleri* and 6 were misassigned to *P. cryptogea* (Table 1).

Eight of the 9 *P. megasperma* isolates used in this study produced all of the structures required to complete the fuller morphological identification. These 8 were correctly assigned to *P. megasperma* with this identification method (Table 1). The isolate that did not produce all of the structures was misassigned to *P. citricola.*

The 3 *P. nicotianae* isolates used in this study produced all of the structures required to complete the fuller morphological identification, and all were correctly assigned to *P. nicotianae* with this method (Table 1).

All but one of the isolates used in this study were correctly assigned to species by the isoenzyme patterns they produced (Table 1). One of the *P. drechsleri* isolates was incorrectly assigned to a distractor (*P.* aff. *drechsleri* #5) which had similar isoenzyme patterns.

4.2 Efficiency

4.2.1. Time from receipt of soil/plant tissue sample to diagnosis.

The VHS requires 15 days to routinely bait soil/plant tissue samples and identify *P. cinnamomi* by its coralloid hyphae (Table 2). Diagnosis to *P. cinnamomi* by this method is highly reliable (see Table 1 and the section on accuracy above). The VHS required 24.2 to 94.2 days to identify species other than *P. cinnamomi* by the fuller morphological method.

In Experiment 1 it was assumed that 14 days are required to isolate and purify *Phytophthora* isolates. Our operators required therefore 27.7 to 41 days to assign unknowns (other than *P. cinnamomi*) to species (Table 2). After 80 days (at which

time the experiment was terminated) 17 of 22 isolates in the contentious *P. cryptogea/drechsleri* group had not produced all of the structures required for the fuller morphological identification.One *P. megasperma* isolate had not produced all structures after 74 days (Table 2).

Unknowns were correctly assigned to *P. cinnamomi* by their isoenzyme patterns in 19.6 days (Table 2). This method of identifying unknowns as *P. cinnamomi* was not as efficient, time-wise, as the shorter coralloid hyphae morphology technique (15 days). The coralloid hyphae technique was 31% more efficient than the isoenzyme technique.

Unknowns of species other than *P. cinnamomi* were assigned to species by the isoenzyme method in 19.5 days (Table 2) and 20.5 days for one *P. drechsleri* isolate. This method of identifying unknowns (other than *P. cinnamomi*) to species was more efficient, time-wise, than the fuller morphological method (27+ days). The isoenzyme method, if adopted to identify species other than *P. cinnamomi*, would result in a 42+ percent time saving to management.

4.2.2. Time operators are actively engaged in diagnosing.

Operators required less time (13.0 minutes) to identify unknowns to *P. cinnamomi* by the shorter coralloid hyphae method than by the fuller morphology method (57.7minutes) or the isoenzyme method (36.6minutes) (Table 3). The coralloid hyphae technique was 182% more efficient than either of the other techniques for identifying *P. cinnamomi.*

Operators required 53.0 to 57.9 minutes to assign unknowns (other than *P. cinnamomi*) to species by the fuller morphological technique, compared with 36.0 to 39.4 minutes by the isoenzyme technique (Table 3). If the isoenzyme method is adopted for assigning unknowns (other than *P. cinnamomi*) to species, management would realize a 34+ percent saving in operator time.

4.3 Cost : Listed in Table 4 is the materials required, and the costs per isolate ($), to assign unknown *Phytophthora* isolates to species.

Least cost ($1.87) is incurred if soil/plant tissue samples are analysed and diagnosed only to either *P. cinnamomi*, positive for *Phytophthora* (these being species other than *P. cinnamomi*) or negative for *Phytophthora.*

If a specific identification is required, for species other than *P. cinnamomi*, the fuller morphological method would cost less ($2.85) materially than the isoenzyme method ($5.53).

The actual (real or total) cost is the sum of the cost of materials and the cost of the operator's time (to which a 32% administration loading has been added) :

material cost + operators time (minutes) x cost per minute ($) x admin. loading (32%).

Listed in Table 5 are the calculations (using the above algorithm) of actual cost, per isolate, of diagnosing the *Phytophthora* status of soil/plant tissue samples.

Least real cost ($5.85) is incurred if samples are analysed and diagnosed to either *P. cinnamomi*, positive for *Phytophthora* (these being species other than *P. cinnamomi*) or negative for *Phytophthora*.

If a specific identification is required, for species other than *P. cinnamomi*, the isoenzyme method would cost less ($16.55 to $17.60) to perform than the fuller morphological method ($19.08 to $20.58), in real terms.

If the isoenzyme method is adopted for assigning unknowns (other than *P. cinnamomi*) to species, management would realize a 13+ percent cost saving.

Discussion

Our objective was to compare the morphological with the isoenzyme technique on accuracy, efficiency, and cost.

As any *Phytophthora* species is considered to be a threat to plant communities in Western Australia, the same management approach is adopted for all. Managers therefore may not require diagnosis beyond presence or absence of *Phytophthora* or *P. cinnamomi* in their samples. The shorter hyphal morphology method is satisfactory for identifying *P. cinnamomi*, and is the most efficient and cost effective option ($5.85). However this method is less reliable for distinguishing some other Phytophthoras from Pythiums.

Research for management purposes therefore may require that diagnosis be taken to specific level. Outcomes of research where diagnosis has been taken to specific level are the recognition of the association of several *Phytophthora* species with pine mortalities in the Donnybrook Sunklands plantations (Chevis and Stukely 1982), recognition that plant deaths in native vegetation of the Northern Sand Plains and Fitzgerald River National Park are more often associated with *Phytophthora* species other than *P.cinnamomi,* and that while *P. cryptogea* has been associated with the deaths of a range of W.A. native plant species *P. drechsleri* may not be (Appendix 2).

The shorter coralloid hyphae method for identifying *P. cinnamomi* was efficient, highly reliable (although some isolates are atypical eg. MJS 171) and inexpensive. For species other than *P. cinnamomi* the fuller morphological method was not as accurate as the isoenzyme method for distinguishing between the homothallic (self compatible) species tested (*P. citricola* and *P. megasperma*) or some heterothallic (requiring a sexually compatible partner) species (*P. cryptogea* and *P. drechsleri*). Not only was it less reliable, the fuller morphological method was also less efficient and cost more than the isoenzyme method. Where long experience has shown that the proportions of the isolates of the various species are dominated by species other than *P. cinnamomi*, eg. Northern Sand Plains and Fitzgerald River National Park, all isolates retrieved should be directly routed to isoenzyme analysis because of the considerable savings.

References

Chevis, H. W. and M. J. C. Stukely (1982). Mortalities of young established radiata pine associated with *Phytophthora* spp. in the Donnybrook Sunkland plantations in Western Australia. Aust. For., 45 : 193-200.

Newhook, F.J., G. M. Waterhouse and D. J. Stamps (1978). Tabular key to the species of *Phytophthora* de Bary. Commonw. Inst., Mycol. Pap. **143**. 20 pp.

PROGRAMME FOR THE FORTHCOMING QUARTER

The April - June 1995 quarter is of 12 weeks duration. The work to be done in this quarter is scheduled as follows :

Stage	Type of Work	Time (weeks)
	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	Develop techniques for the direct isoenzyme assay of bait tissues	2
	MERIWA Report	1
	Total	12

Final Comment

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

Table 1. Accuracy of assignment of *Phytophthora* isolates to species determined by morphological characters and by isoenzyme characters.

	Number of Isolates								
Phythophthora[#1] *spp*	Tested	That produced Coralloid Hyphae	That produced Sporangia	That produced Oogonia	Assigned correctly on complete morphological data	Assigned correctly on incomplete morphological data	Misassigned on morphological data	Assigned correctly by isoenzymes	Misassigned by isoenzymes
P. cinnamomi WA[#2]	7	7	6	5	5	2[#3]	0	7	0
P. citricola[#4]	9				9	N/A	0	N/A	0
citricola KAL	1	0	1	1	N/A	N/A	N/A	1	0
citricola NSP	4	0	4	4	N/A	N/A	N/A	4	0
citricola JF	4	0	4	4	N/A	N/A	N/A	4	0
P. cryptogea	10	0	10	2	2	8	0	10	0
cryptogea BUN	1	0	1	0	0	1	0	1	0
cryptogea ENE	1	0	1	0	0	1	0	1	0
P. drechsleri WA	9	0	9	2	1	2	6	8	1[#5]
P. aff. *drechsleri* WA	1	0	1	0	0	1	0	1	0
P. megasperma[#6]	9				8	N/A	1	N/A	0
megasperma BAU	2	0	1	2	N/A	N/A	N/A	2	0
megasperma NSP	3	0	3	3	N/A	N/A	N/A	3	0
megasperma FRNP	4	0	4	4	N/A	N/A	N/A	4	0
P. nicotianae WA	3	0	3	3	3	N/A	0	3	0
Total	50	7	48	30	28	15	7	49	1

[#1] The taxonomic affinities given are those of the donors on their judgement of morphological conformity to original descriptions (see Appendix 2). No holotypes or isotypes were available for analysis in this study.

[#2] Distinctive groups of electromorphs were recognised in *P. citricola*, in *P. cryptogea*, in *P. drechsleri* and in *P. megasperma*, but not in *P. cinnamomi*, or *P. nicotianae*. The locations of geographic origin are: KAL=Kalbarri; NSP=Northern Sand Plain; JF=Jarrah Forest; FRNP=Fitzgerald River National Park; BUN=Bunbury; BAU=Baudin plantation; ENE=Eneabba.

[#3] One of the two operators was not confident about assigning isolate MJS 171 to *P.cinnamomi* on hyphal morphology.

[#4] Three electromorph groups were recognised among the 9 isolates assigned to *P.citricola.*

[#5] Operator read gel to *P.* aff. *drechsleri* #5 bar code instead of to closely releated *P.drechsleri.*

[#6] Three electromorph groups were recognised among the 9 isloates assigned to *P. megasperma.*

Table 2. Comparisons of time to diagnose, and diagnostic reliability for unidentified isolates among five species of *Phytophthora* according to the VHS and Experiment 1 approaches to diagnosis.

Diagnostic Approach	Species		Number of isolates tested	Time (Days) using[#1] Hyphal morphology	Full morphology	Incomplete morphology	Isoenzyme	Reliability[#2]
A. Routine VHS[#3]								
1. short morphological[#4]	*P. cinnamomi*		122	15(0.4)	not applicable (N/A)	N/A	N/A	H
	Other species		29	N/A	N/A	N/A	N/A	U
2. "full"[#5] morphological	*P. cinnamomi*		16	N/A	?[#6]	N/A	N/A	VH
	P. citricola		8	N/A	30.5(5.2)	N/A	N/A	H
	P. cryptogea/drechsleri		9	N/A	94.2(7.8)	N/A	N/A	U
	P. megasperma		12	N/A	24.2(1.9)	N/A	N/A	H
	P. nicotianae		1	N/A	?	N/A	N/A	H
B. Deliberate Experiment								
3. Morphological[#7]	*P. cinnamomi*	Group A	5	14	39.4(1.7)	N/A	N/A	100
		Group B	2	14[#8]	N/A	67(21)	N/A	H
	P. citricola		9	N/A	27.7(1.8)	N/A	N/A	100
	P. cryptogea/ drechsleri[#9]	Group A	5	N/A	41.0(4.9)	N/A	N/A	23
		Group B	17	N/A	N/A	80(3.6)	N/A	U
	P. megasperma[#10]	Group A	8	N/A	31.8(3.2)	N/A	N/A	89
		Group B	1	N/A	N/A	74	N/A	U
	P. nicotianae		3	N/A	35.3(3.2)	N/A	N/A	100
4. Isoenzyme	*P. cinnamomi*		7	N/A	N/A	N/A	19.6(0.3)	100
	P. citricola		9	N/A	N/A	N/A	19.5(0.2)	100
	P. cryptogea/ drechsleri	Group A	21	N/A	N/A	N/A	19.5(0.2)	95
		Group B	1	N/A	N/A	N/A	20.5	U
	P. megasperma		9	N/A	N/A	N/A	19.5(0.0)	100
	P. nicotianae		3	N/A	N/A	N/A	19.2(0.3)	100

#1 Time in days elapsed (standard errors in brackets) from receipt of sample by Vegetation Health Service (VHS) to written advice of diagnosis.

#2 Reliability for routine (VHS) diagnosis is the estimate of staff judgement based on long familiarity. It is given as very high (VH), high (H), or unreliable (U). For the data of Experiment 1 the reliability is quantitative; for morphology it is here expressed as the proportion correctly diagnosed. For isoenzyme it is given as inverse of proportion misassigned, but see also text p. p.

#3 All VHS methods are morphological.

#4 Two to three day old hyphae emerging from baits plated onto selective agar are examined microscopically.

#5 The VHS "full" diagnosis involves production of sporangia and oogonia but is not as complete as that required for full taxonomic treatment (see Newhook *et al* 1978).

#6 Time in days calculated from VHS records (5/93 to 1/95) by Carstairs. Records were not available for *P. cinnamomi* and *P. nicotianae* in VHS records so given as :?.

#7 This was conducted by 2 experienced VHS staff and included calculations of time to complete hyphal morphology (for *P. cinnamomi* alone) as well as time to complete a full morphology (for all 5 speoeis). Both approaches started at the point where a clean isolate was available on corn meal agar. The figures have been adjusted by addition of 14 days to accomodate the baiting, isolation and cleaning of cultures.

#8 Two isolates among seven failed to produce all traits. Of these two isolates (Group B), one failed to produce both sporangia and oogonia, and the second failed to produce oogonia.

#9 In *P. cryptogea/drechsleri* 5 isolates (Group A) produced oogonia and 17 isolates (Group B) failed to produce oogonia .

#10 In *P. megasperma* 8 isolates (Group A) produced sporangia and one isolate (Group B) failed to produce sporangia in soil extract.

Table 3. Time (in minutes) that operators are actively engaged in operations required to diagnose unidentified isolates among five species of *Phytophthora* according to four approaches to diagnosis.

Diagnostic Approach	Species		Number of isolates tested	Time (Minutes) using Hyphal morphology	Full morphology	Incomplete morphology	Isoenzyme
A. Routine VHS							
1. short morphological	*P. cinnamomi*		122	13.0	N/A	N/A	N/A
	Other species		29	13+[#1]			
2. "fuller" morphology	*P. cinnamomi*		16	13.0	72.0	?	N/A
	P. nicotianae		1	N/A	72.0	?	N/A
	Other species		28	N/A	72.0	?	N/A
B. Deliberate Experiment							
3. Morphological	*P. cinnamomi*	Group A	5	13.0	55.7(0.6)	N/A	N/A
		Group B	2	15.5	N/A	48.0(5.5)	N/A
	P. citricola		9	N/A	53.0(0.3)	N/A	N/A
	P. cryptogea/ drechsleri	Group A	5	N/A	57.9(1.8)	N/A	N/A
		Group B	17	N/A	N/A	52.3(0.6)	N/A
	P. megasperma	Group A	8	N/A	53.2(0.3)	N/A	N/A
		Group B	1	N/A	N/A	46.8	N/A
	P. nicotianae		3	N/A	55.0(0.0)	N/A	N/A
4. Isoenzyme	*P. cinnamomi*		7	N/A	N/A	N/A	36.6(0.6)
	P. citricola		9	N/A	N/A	N/A	37.0(1.0)
	P. cryptogea/ drechsleri	Group A	21	N/A	N/A	N/A	39.4(1.1)
		Group B	1	N/A	N/A	N/A	36.5
	P. megasperma		9	N/A	N/A	N/A	36.0(0.5)
	P. nicotianae		3	N/A	N/A	N/A	36.5(0.9)

[#1] Greater time spent searching unsuccessfully for coralloid hyphae and diagnosis to *Phytophthora* sp.

Table 4. **The cost ($) of materials to identify *Phytophthora* spp. by each of three stratagems: Hyphal morphology to *P. cinnamomi* or to *Phytophthora* sp. (genus only); comprehensive 'fuller' morphology to species; and isoenzymes to species and electromorphs.**

Materials	+/-*Phytophthora* spp. (Hyphal morphology)	Full Morphology I.D	Isoenzyme I.D.
Antibiotic agar[#1]	0.22	0.44	0.44
CMA[#2]		0.42	0.21
V8[#3]		0.21	
Trays[#4]	0.24	0.24	0.24
Baits[#5]	0.25	0.25	0.25
Alcohol[#6]	0.76	0.76	0.76
Incidentals[#7]	0.40	0.53	0.53
CAGE Plate			0.21
Stains and Buffers			1.54
Liquid nitrogen			0.70
Incidentals			0.65
Totals per identification	**1.87**	**2.85**	**5.53**

[#1] Antibiotic agar NARPH for isolation from bait
[#2] CMA for preparation for sporangia
[#3] V8 for induction of oogenia
[#4] Trays for raising baits and baiting soils
[#5] E. sieberi seed for baits
[#6] Alcohol sterilization
[#7] Incidentals - Tissues,slides,stains,coverslips,mountants,hand towels,razor blades etc., gloves, cellulose acetate gel, electrophoretic plates.

Table 5. **Computation of costs for diagnosis to : *P. cinnamomi* by hyphal morphology; to *Phytophthora* species by the VHS using the 'fuller' morphological method; to *Phytophthora* species in experiment 1 by the 'fuller' morphological treatment and by the isoenzyme method.**

Species and stratagem		Operation Time (minutes)		# cost per minute		Administration loading		Materials	Total
P. cinnamomi	hyphal morphology VHS	13.0	x	0.232	x	1.32	+	1.87	5.85
	fuller morphology VHS	72.0	x	0.232	x	1.32	+	2.85	24.90
	Experiment 1 (Group A)	55.7	x	0.232	x	1.32	+	2.85	19.91
	Isoenzymes	36.6	x	0.232	x	1.32	+	5.53	16.74
P. citricola	fuller morphology VHS	72.0	x	0.232	x	1.32	+	2.85	24.90
	Experiment 1	53.0	x	0.232	x	1.32	+	2.85	19.08
	Isoenzymes	37.0	x	0.232	x	1.32	+	5.53	16.86
P. cryptogea/drechsleri	fuller morphology VHS	72.0	x	0.232	x	1.32	+	2.85	24.90
	Experiment 1	57.9	x	0.232	x	1.32	+	2.85	20.58
	Isoenzymes Group A	39.4	x	0.232	x	1.32	+	5.53	17.60
P. megasperma	fuller morphology VHS	72.0	x	0.232	x	1.32	+	2.85	24.90
	Experiment1	53.2	x	0.232	x	1.32	+	2.85	19.14
	Isoenzyme	36.0	x	0.232	x	1.32	+	5.53	16.55
P. nicotianae	fuller morphology VHS	72.0	x	0.232	x	1.32	+	2.85	24.90
	Experiment 1	55.0	x	0.232	x	1.32	+	2.85	19.69
	Isoenzymes	36.5	x	0.232	x	1.32	+	5.53	16.71

Appendix 1. AGREEMENT FOR CONTRACTUAL SERVICES

SERVICES

1. **CONTRACTOR :**

MR STEPHEN A. CARSTAIRS

2. **SERVICES :**

Research the use of the technique of isoenzyme analysis for the identification of species of *Phytophthora* and provide a detailed report with particular reference to :

a) Selection and testing of enzyme systems; establishment of methodology.

b) Comparison of isoenzyme profiles of species of *Phytophthora* from WA with standard isolates fron California.

c) Effectiveness of isoenzyme analysis for identifying *Phytophthora* species.

d) Comparison of isoenzyme analysis with traditional morphological methods of *Phytophthora* identification on the basis of time, accuracy and cost.

e) Variation between local isolates of species of *Phytophthora*, in relation to world-wide variation.

f) Training of staff of Vegetation Health Service in the use of the isoenzyme analysis for the identification of species of *Phytophthora*.

3. **PERIOD :**

Six (6) months, with an option of renewal for a further six (6) months.

Commencing 14 March 1994. To be completed by March 1995.

Appendix 2. *Phytophthora* isolates used in experiment number 1 indicating donor and laboratory of origin[#1], Coffey's electrophoretic equivalent[#2], host plant, geographic origin and compatibility type.

Species	WA Isolates	Coffey Isolate Equivalents	Host plant	Origin	Compatibility Type[#3]
P. cinnamomi	MJS 123	CINN 4	*Pinus radiata*	Jarrahwood Plantation	?
	MJS 147	-	Soil	Molloy Island	?
	MJS 171	CINN 5	*Banksia* sp.	Wanneroo	A2
	MJS 285	CINN 4	*Eucalyptus marginata*	Jarrahdale	?
	DCE 108	CINN 4	*B. menziesii*	Gosnells	S
	DCE114	CINN 4	*Conostephum pendulus*	Gosnells	A2
	DCE 230	CINN 4	*E. marginata*	Gordon Block	A2
P. nicotianae	VHS 3375		?	Bunbury District	A2
	VHS 3627		*E. ficifolia*	Perth	?
	DCE 17	-	?	?	
P. citricola	MJS 287	-	*E. erythrocorys*	Perth	H
	MJS 168	CIT 3	*P. radiata*	Baudin Plantation	H
	MJS 207	CIT 3	*P. radiata*	Pot trial	H
	DCE 220	CIT 3	Sandalwood	Narrogin	H
	DCE 2233	CIT 3	Woodchip heap	Manjimup	H
	VHS 1723		?	-	H
	FB E 20		?	-	H
	FB F 17		?	-	H
	FB N 40		?	-	H

[#1] MJS: M J Stukely CALM; DCE: E Davison CALM; VHS: Vegetation Health Service CALM; FB: F Bunny CALM; SEB: S E Bellgard CALM; TH: T Hill CALM; HSA: Hart Simpson & Associates.

[#2] Sometimes an individual electromorph, sometimes a group of closely related electromorphs.

[#3] ?: compatability type not determined; S: sterile; H: homothallic.

Appendix 2. (cont.) *Phytophthora* isolates used in experiment number 1 indicating donor and laboratory of origin[#1], Coffey's electrophoretic equivalent[#2], host plant, geographic origin and compatibility type.

Species	WA Isolates	Coffey Isolate Equivalents	Host plant	Origin	Compatibility Type[#3]
P. megasperma	MJS 216	-	Soil	Baudin Plantation	H
	MJS 217	-	Soil/*P. radiata*	Baudin Plantation	H
	SEB 250	D(AC)	*Allocasuarina campestris*	FRNP	H
	SEB 251	D(AC)	*Xanthorrhoea platyphylla*	FRNP	H
	TH 7	D(AC)	*B. prionotes*	North Sand Plain	H
	SEB 218	-	*Isopogon formosus*	FRNP	H
	SEB 243	-	*B. media.*	FRNP	H
	VHS 3594	-	*B. baxteri*	FRNP	H
	HSA(DCE 441)	-	*B. attenuata*	Minyolo Brook	H
P. cryptogea	VHS 3352	E	-	FRNP	A2
	VHS 3437	E	*B. attenuata*	Jarrahdale	A2
	VHS 3499	E	Soil	Woodarda Track	A2
	VHS 3606	E	*Daviesia* sp.	FRNP	A2
	VHS 3617	E	*Dryandra* sp.	FRNP	A2
	MJS 80	E	*P. radiata*	Jarrahwood Plantat'n	A2
	MJS 105	E	*P. radiata*	Jarrahwood Plantat'n	A2
	MJS 125	E	*P. radiata*	Jarrahwood Plantat'n	A2
	DCE 154	E	-	-	-
	DCE 456	E	*B. attenuata*	Cervantes Road	A2

[#1] MJS: M J Stukely CALM; DCE: E Davison CALM; VHS: Vegetation Health Service CALM; FB: F Bunny CALM; SEB: S E Bellgard CALM; TH: T Hill CALM; HSA: Hart Simpson & Associates

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Appendix 2. (cont.) *Phytophthora* isolates used in experiment number 1 indicating donor and laboratory of origin[#1], Coffey's electrophoretic equivalent[#2], host plant, geographic origin and compatibility type.

Species	WA Isolates	Coffey Isolate Equivalents	Host plant	Origin	Compatibility Type[#3]
P. cryptogea cont.	VHS 3631	-	*X. preissii*	Bunbury District	?
	DCE 458	-	Water source	Eneabba	?
P. drechsleri	HSA 1655	-	Bait	Cataby	?
	HSA 1924	-	Bait	Jurien	?
	HSA 1925	-	Bait	Jurien	?
	HSA 1935	-	Bait	Jurien	?
	HSA 1943	-	Bait	Jurien	?
	HSA 1944	-	Bait	Jurien	?
	HSA 1948	-	Bait	Jurien	?
	VHS 3514	-	Bait	FRNP	?
	DCE 457	-	Bait	Cooljarloo	?
	VHS 3360	-	-	-	?

[#1] MJS: M J Stukely CALM; DCE: E Davison CALM: Vegetation Health Service CALM; FB: F Bunny CALM; SEB: S E Bellgard CALM; TH: T Hill CALM; HSA: Hart Simpson & Associates.

[#2] Sometimes an individual electromorph, sometimes a group of closely related electromorphs.

[#3] ?: compatability type not determined; S: sterile; H: homothallic.

Appendix 3. RECORD SHEETS USED TO RECORD THE TIME TAKEN BY OPERATORS TO PERFORM THE VARIOUS TASKS REQUIRED IN EXPERIMENT 1 TO IDENTIFY UNKNOWN *PHYTOPHTHORA* ISOLATES TO SPECIES.

Time Taken to Identify *Phytophthora* sp. Using Isozyme Bands

Isolate:_____

Date started (date sample was subbed into V8/Pea broth) : ____/____/____

Task	Time started	Time finished	Total time day	hrs	mins
1. Subbing from CMA to V8/Pea broth					
2. Growth in V8/Pea broth					
3. Harvest mycelium from broth to vials					
4. Add buffer and store in liquid nitrogen					
5. Grind sample for crude protein extract					
6. Load unknown + standard samples into tray					
6.1					
6.2					
7. Make up buffers and substrates (eg NAD)					
7.1					
7.2					
8. Load samples (+ standards), run, stain and dry					
8.1					
8.2					
9. Diagnose on the light box, return to 6.					
9.1					
9.2					

Date finished: ____/____/____

***Phytophthora* sp:**_____

Total time to identify the isolate:_____days

Total number of people hours:_____mins

Time Taken to Identify *Phytophthora* sp. Using Morphological Characters

Isolate: _____

Date started (date sample was subbed onto V8) : ____/____/____

Task	Time started	Time finished	Total time day	hrs	mins
1. Botryose hyphal swellings					
2. Subbing from CMA onto V8					
3. Growth on V8					
4. Subbing from V8 onto soil extract					
5. Time for sporangia to be produced					
6. Measurement of sporangia					
7. Mating- time to set up matings					
8. Time for production of mature oogonia					
9. Measurement of oogonia					
10. Checking tables etc for final identification					

Date finished: ____/____/____

***Phytophthora* sp:**_____

Total time to identify the isolate:_____days

Total number of people hours:_____mins