MINERALS AND ENERGY RESEARCH INSTITUTE OF WESTERN AUSTRALIA

Report No. 175

RAPID IDENTIFICATION OF SPECIES OF *PHYTOPHTHORA*

Project No. M227 & M254

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(MERIWA)

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RAPID IDENTIFICATION OF SPECIES OF *PHYTOPHTHORA*

Results of research carried out as MERIWA Project No's M227 and M254 in the Department of Conservation and Land Management

by

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November, 1996

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ACKNOWLEDGMENTS

Research project M227/M254 was partly funded, and energetically supported, by the Minerals and Energy Research Institute of Western Australia (MERIWA), and an informal consortium from the mining industry:

ALCOA OF AUSTRALIA Ltd.
WORSLEY ALUMINA Pty Ltd.
TI WEST JOINT VENTURE.
RGC MINERAL SANDS Ltd.
WESTERN COLLIERIES Ltd.

We wish to thank Mr. R. Hannaford and Dr J. Muhling of MERIWA, and Roz Hart, of Hart, Simpson and Assoc., for their contributions to the project. M. Dudzinski, M. Coffey and C. M. Brasier provided us with reference isolates of species of *Phytophthora*.

This project was also partly funded by the Department of Conservation and Land Management (CALM), and the research was conducted at Science and Information Division/Como of CALM. We wish to thank the following people at SID/Como and the Western Australian Herbarium for their wide and varied inputs:

I. Abbott, J. Bartle, S. Bellgard, F. Bunny, T. Butcher, D. Coates, C. Crane, E. Davison, W. Edgecombe, V. Hamley, R. Harper, E. Hopkins, K. Lee, J. Morris, L. Newcombe, N. North, F. Podger, B. Shearer, S. Suffling, R. Tan, F. Tay, D. Ward, J. Webster, C. Wilkinson, M. Williams, A. Wills, A. Wincza, and L. Wong.

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PREFACE

In March 1994 the services of Stephen A. Carstairs were engaged for 18 months by CALM, acting for MERIWA and the following informal consortium from the mining industry:

ALCOA OF AUSTRALIA Ltd.
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Schedule of agreement for Contractual Services

The schedule of contract between MERIWA and CALM (Contract M227/M254) required address of nine services:

- Selection and development of electrophoretic procedures and protocols for identifying species of *Phytophthora*.
- Comparison of isoenzyme profiles of Western Australian species of *Phytophthora* with a set of standards imported from California.
- Comparison of the variation in local species of *Phytophthora* in relation to world-wide variation.
- Evaluation of the effectiveness of isoenzymes for identifying species of *Phytophthora*.
- Comparison of isoenzyme analysis with morphological methods on the basis of accuracy, time and cost.

- Testing modifications to *Phytophthora* baiting techniques, and comparing by cost and time the efficiency of retrieving *Phytophthora* by traditional and modified methods.

- Testing new procedures for directly assaying bait tissues for *Phytophthora* by isoenzyme analysis, and comparing time and cost with the present method.

- Testing ways of establishing the intensity of field sampling necessary to achieve given levels of certainty of detecting *Phytophthoras* if present.

- Training staff at the Vegetation and Health Service (VHS) of CALM in the use of isoenzyme analysis for the identification of species of *Phytophthora*.

Each service is addressed here in turn.

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.

SUMMARY

Detection and isolation of species of *Phytophthora* from field samples requires between one and two weeks. In this time a sample may be determined to be:

negative for all *Phytophthora* species;
positive for *Phytophthora cinnamomi* and negative for other *Phytophthora* sp;
negative for *P. cinnamomi* and positive for other *Phytophthora* sp; or
positive for *P. cinnamomi* and positive for other *Phytophthora* sp.

Identification of species of *Phytophthora,* other than *P. cinnamomi*, by traditional morphological means may take between four weeks and several months. Our objective was to develop isoenzyme procedures and protocols which would discriminate between Western Australian field species of *Phytophthora*, and to compare the isoenzyme method of identification with the traditional method by accuracy, turn-around time and cost per sample.

The procedures and protocols for isoenzyme identification of species of *Phytophthora* were developed (see Service 1), and when compared for accuracy of diagnosis the isoenzyme method of identification was found to be far superior to the traditional method (see Service 4). Further, the isoenzyme method decreased by *circa* 10% the turn-around time of identification of Phytophthoras, relative to the time required to identify them by the traditional morphological method. By identifying Phytophthoras by the isoenzyme method rather than the traditional morphological methods, management may expect to realise faster and more accurate identifications, at no additional cost (see Service 5).

Direct isoenzyme assay of baits to detect species of *Phytophthora* in field samples is possible, but was not cost effective (see Service 6).

In order to minimise the spread of disease-causing species of *Phytophthora* during exploration and mining, companies are required to determine the presence or absence of these pathogens within their exploration tenements. Assessments of the

Phytophthora status of wooded sites in the field usually starts with a visual evaluation by a skilled interpreter, who looks for symptoms of disease caused by *Phytophthora*. Where visual interpretation of a site's *Phytophthora* status requires further confirmation, soil and plant tissue is usually retrieved from 3 suspect locations in the site, and these are bulked together to make a *circa* 1 kg field sample for laboratory analysis. Our objective was to determine how many field samples from a site need to be assessed to achieve a given degree of certainty of detecting *Phytophthora* if it is present in some of the samples from the site. We determined that by assessing 11 soil/plant material samples per site for *Phytophthora*, we might expect to detect it 80% of the time in sites that are infested with the fungus (see Service 8).

When soil/plant material samples are returned to the laboratory their *Phytophthora* status may be determined by "baiting". Baiting involves mixing the soil/plant tissue samples into a slurry with water and then adding to the mixture young, rapidly-expanding (growing) plant tissues, e.g. *Lupinus angustifolius* seedlings and *Eucalyptus sieberi* cotyledons, or "baits". The plates are then plated on *Phytophthora* selective agar, and pure cultures of the fungi are obtained for identification. Our objective was to determine how many baits need to be assessed to achieve a given degree of certainty of detecting *Phytophthora* if it is present in some of the baits from a soil/plant tissue sample. We found that by assessing the *Phytophthora* status of 28 baits from a baited sample, assessors may be 95% confident of detecting the fungus if it is present in some of the baits (see Service 7.1).

When a field sample is being assessed for *Phytophthora* it is usually baited for a set period, say up to 10 days, or until lesions form on the seedlings or cotyledons provided as baits. If *Phytophthora* is not retrieved from baits exposed to a sample for the set period, the sample is deemed to be negative for *Phytophthora*. Some field samples may contain more than one species of *Phytophthora*. Our objective was to determine whether substantially more Phytophthoras are recovered from baits that have been exposed to field samples from day 1-to-5 and days 6-to-10 combined, relative to assessing baits for days 1-to-5 only. We determined that by assessing the combined baits for days 5 and 10, substantially more Phytophthoras were recovered from field samples, and that the number of samples found to be positive was substantially increased relative to assessing baits after 5 days only (see Service 7.3).

Some species of *Phytophthora* may go undetected in samples which contain more than one species of *Phytophthora* because either their inoculum levels are low, or they are not able to access the baits in competition with other species of *Phytophthora*, or in competition with Pythiums and with other pathogenic fungi. Our objective was to determine whether or not the pH of the baiting mixture affected the competitive abilities of *P. cinnamomi* and *Phytophthora citricola* in combination. We determined that at pH 5.0 and 5.5 *P. citricola* was better able to access baits than *P. cinnamomi*, whereas at pH 4.5 and below, *P. cinnamomi* was able to outcompete *P. citricola* for baits. We propose that it may be possible to manipulate baiting conditions to optimise the recovery of *Phytophthora* relative to that of species of *Pythium* and other pathogenic fungi by altering pH or by the inclusion of Hymexazol™ in the baiting solution.

Our endeavour has been to increase the sensitivity of detecting species of *Phytophthora* in samples retrieved from exploration tenements, to reduce the number of "false negatives" (samples deemed to be negative for *Phytophthora* when *Phytophthora* is actually present) by improving baiting techniques, and to determine the levels of sampling that are necessary to achieve given degrees of certainty of detecting *Phytophthora* when it is present. As a consequence of this research, mining companies may expect that the *Phytophthora* status of their tenements will be more accurately assessed.

We have also endeavoured to increase the accuracy of identification of species of *Phytophthora* while reducing the turn-around time required to detect and identify them in field samples retrieved from exploration tenements. As a consequence of this research, mining companies may expect faster turn-around times of assessment of field samples, and increased accuracy of identification of species of *Phytophthora* at no extra cost.

Service 1. Selection and development of electrophoresis procedures and protocols for identifying species of *Phytophthora.*

Protein electrophoresis, or isoenzyme analysis, is a commonly used and cost-effective research tool in taxonomy. As routine identification of unknown isolates of *Phytophthora* is essentially a taxonomic exercise, it follows that protein electrophoresis may be a viable alternative to the classical alpha (morphological) taxonomic procedures employed by laboratories around the world for this purpose.

Isoenzymes are enzymes which differ in molecular form, and yet are specific for the same substrate(s) (Market and Moller 1959). Isoenzymes are visualised when crude protein extracts from tissues are subjected to electrophoresis in a matrix (gel or membrane), and the matrix is then transferred to solutions containing enzyme-specific stains. Information about any one isoenzyme thus visualised is usually encoded according to its mobility, and information about several isoenzymes may be transformed into multi-locus genotypes for the individuals analysed. Alternatively the information may be 'bar coded'.

Effective development of techniques for electrophoresis of isoenzymes requires that many aspects of the procedure be contemporaneously optimised. One is faced, for example, with a choice of electrophoretic matrixes and with various technical demands including: preparation of the tissue(s), choice of extraction procedure and extraction buffer, and the problem of identifying which enzymes can be electrophoretically resolved.

Crude protein extracts may be separated electrophoretically in several types of gel including: agarose, polyacrylamide gel electrophoresis (PAGE) and starch gel; or in cellulose acetate membranes (i.e. CAGE).

- Agarose gel electrophoresis lacks the resolving power of the other media and is seldom used for studying enzyme polymorphism (Wendel and Weeden 1989).

- A valuable property of polyacrylamide gels is their transparency which allows for densitometric quantification of product, however PAGE is not as efficient as some of the other procedures for the purpose of rapid identification of large numbers of individuals (Wendel and Weeden 1989).

- Starch gel electrophoresis (Smithies 1955) has been preferred for most studies involving the analysis of large numbers of isolates of *Phytophthora* (Old *et al.* 1984; Tooley *et al.* 1985; Old *et al.* 1988; Nygaard *et al.* 1989; Oudemans and Coffey 1991a; Oudemans and Coffey 1991b; Oudemans *et al.* 1994).

- Oudemans and Coffey (1991a) noted that CAGE was superior to starch gel electrophoresis for resolving some isoenzymes of three species of *Phytophthora*, and subsequently concluded that CAGE had potential as a rapid diagnostic method for identifying unknown isolates of *Phytophthora*.

This section focuses on the selection and development of electrophoretic techniques which produce isoenzyme markers which discriminate between species of *Phytophthora*. Emphasis is placed on Cellulose Acetate Gel Electrophoresis (CAGE) of enzymes because this method has a broad and relatively inexpensive commercial support base, making it efficient and cost effective for isoenzyme laboratories. Further, CAGE is also very efficient for applications, such as routine identifications, which require scoring several isoenzymes for large numbers of individuals. Many of the technical aspects of CAGE have been published elsewhere (Hebert and Beaton 1993), however these were directed toward isoenzyme analysis of plants and animals. Consequently the aim of this section is to provide a reference source of CAGE procedures for public and private interests who have a need for identifying species of *Phytophthora*. The CAGE procedures for Phytophthoras that follow are described as 'instructions' for use as an operating manual.

1.1 Equipment Required for CAGE and its Cost.

A large initial capital outlay is required to establish a facility capable of performing CAGE identifications of unknown isolates of *Phytophthora*. Thereafter maintenance and upkeep costs of the facility would compare favourably with most research laboratories. The equipment required to perform CAGE and its current cost (1995) is presented in Table 1.1.

Table 1.1 Capital cost (1995) of establishing a facility for CAGE identification of isolates of *Phytophthora*.

Item		Value ($)
Balance (3 decimal place)		2300
Camera and Tripod		525
Computer and Software		2500
Freezer Unit (Upright)		669
Fume Hood		5000 +
Gel Incubation Cabinet		300
Light Box		300
Liquid Nitrogen Dewar		1500
Magnetic Stirrer - Hot Plate		600
Micro Centrifuge (Tommy HF 120)		400
Micro Pipette (5-50 µl)		350
pH meter		200
Electrophoresis Power Supply		1370
Refrigerator		302
Available from Helena laboratories:		
Item	Cat. No.	Value ($)
Titan Alignment Base	4094	536
Titan Bufferizing Tanks	5093	82
Titan III Carry Rack	5110	74
Titan III Gel Plates	3066	789
Titan Sample Applicator	4090	551
Titan Sample Well Plate	4096	124
Titan Zip Zone Chamber with Heat Shield	1283	536

1.2 Crude Protein Extract Preparation.

Isoenzyme electrophoresis was performed on crude proteins extracted from the mycelium of isolates of *Phytophthora.* The various protocols required and procedures one must follow to produce these protein extracts were developed in the course of our investigation, and are outlined below.

1.2.1 Medium for Culturing Mycelium of Species of *Phytophthora.*

Starting with pure cultures of *Phytophthora* on Corn Meal Agar (CMA) plates, a variety of methods may be used for growing up the mycelium required for CAGE. A plug of inoculum from a CMA plate may be transferred to a Pea Agar plate for growing up mycelial mats. Alternatively *Phytophthora* mycelium may be grown up in Campbell's V8 juice broth. The former method requires that *Phytophthora* isolates produce copious quantities of mycelium on Pea Agar plates, which is not always the case. Several researchers (e.g. Old *et al.* 1984; Nygaard *et al.* 1989; Oudemans and Coffey 1991a) have used the latter method to 'grow up' the mycelium of a range of species of *Phytophthora,* and a modified recipe which incorporates Campbell's V8 juice, green peas and glucose was adapted for this project.

Culture medium for producing the mycelium required for CAGE

Ingredients:

Campbell's V8 juice	250 mL
Frozen green peas	300 g
D-Glucose	5 g
Calcium carbonate	2 g
Distilled water	3250 mL

The solid ingredients and V8 juice are added to a Waring blender with 250 mL of distilled water. These ingredients are then blended intermittently for five minutes or until the mixture has a fine texture. The mixture is then transferred to an autoclavable container and autoclaved at 121°C for five minutes to dissolve the solids. When cool the mixture is centrifuged at 7000 rpm for three minutes. The clear

portion is collected, bulked and made up to three litres with distilled water. Five ml aliquots of this broth are then added to 1 ounce McCartney bottles and these are autoclaved at 121°C for twenty minutes. The McCartneys of broth may then be stored in the refrigerator until required.

1.2.2 Culturing and Harvesting the Mycelium.

Care should be taken to ensure, as much as possible, that all cultures of mycelium for CAGE are maintained in the same physiological condition. The physiological state of the growing mycelium and environmental conditions (e.g. differences in batches of V8 juice or frozen peas) may influence the electrophoretic band patterns produced. Ways in which the influences may be manifested range from changes in the intensity of banding to the appearance or disappearance of bands (Wendel and Weeden 1989). Therefore, should the bar code of an isolate otherwise conform to that of a recognised species except for the presence or absence of a band(s), the isolate should be regrown and its isoenzymes reassessed.

The conditions used to grow *Phytophthora* mycelium for electrophoresis vary among researchers (Old *et al.* 1984; Nygaard *et al.* 1989; Oudemans and Coffey 1991a). Adequate amounts of mycelium for CAGE are produced in McCartney bottles of broth inoculated with 0.75 x 0.75 cm CMA plugs of inoculum of *Phytophthora* and incubated on their sides in the dark at 24°C for *circa* 64 hours. These conditions produced consistently good results in a range of taxa of *Phytophthora* recovered from the south-west of Western Australia.

Harvest your mycelium in a laminar flow cabinet. Free the 64 hr old wad of mycelium from its inoculum plug and transfer it to 10 mL of distilled water for a minute to remove residual broth. One rinse in distilled water gave us the same quality of CAGE bands as two rinses. After rinsing the wad of mycelium in distilled water blot it on filter paper to remove excess water, and then transfer it to a labelled 1.5 mL stoppered microcentrifuge tube. Store these tubes with their mycelium in a cool water bath.

Recommendation

1.1 It is recommended that plant pathology services which detect and identify Phytophthoras adopt as standard procedure the practice of accumulating, on CMA, unidentified pure cultures of species of *Phytophthora* other than *Phytophthora cinnamomi* through the week. Inoculum plugs of the cultures should then be added to McCartney bottles of Pea/V8 juice broth on Friday afternoons, and the mycelium grown from them should be harvested on the following Monday mornings (*circa* 64 hrs later) in readiness for CAGE. In so doing the services will be maximising the use of down-time on weekends.

1.2.3 Extraction of Crude Proteins from Mycelium of *Phytophthora.*

Without elaborate protein purification or concentration steps, Oudemans and Coffey (1991a) obtained intensely staining and highly resolved CAGE bands from the mycelium of *Phytophthora*. *Phytophthora* mycelium seems to be relatively free of secondary compounds which act to reduce or destroy extracted enzymes, and so simple enzyme extraction buffers (see Old *et al.* 1984) produce good results. The importance of the grinding buffer used to prepare the crude proteins extracted from mycelium, however, should not be underrated. No single extraction buffer can be expected to be optimally effective in protecting all enzymes as has been demonstrated in several experiments designed to test the effects of different extraction buffers on isoenzyme expression (Kelley and Adams 1977; Wilson and Hancock 1978; Soltis *et al.* 1980).

Altering pH and buffer concentrations led us to develop a simple and effective enzyme extraction buffer for Phytophthoras:

Buffer Used to Extract Enzymes from the Mycelium of *Phytophthora*

75 mM Tris
Buffer the Tris to pH 8.2 with citric acid
Add Mercaptoethanol at a rate of 1 µl/mL of buffer

The buffered Tris is stored in the refrigerator as a stock solution, and five-mL portions are made up with Mercaptoethanol for every day use.

In order to obtain extracts with high enzyme activity, one requires the optimum tissue (mycelium)-to-enzyme extraction buffer ratio. Forty-five microlitres of extraction buffer added to micro centrifuge tubes containing the harvested mycelium was usually sufficient for the amount of mycelium (*circa* 20-30 mg) recovered after 64 hours of incubation in broth, and seemed to be the optimum tissue-to-buffer ratio.

The first step in the homogenising process requires that the mycelium samples be snap frozen in liquid nitrogen. The micro centrifuge tubes with mycelium and extraction buffer are briefly immersed in liquid nitrogen, and then transferred to a freezer. The frozen mycelium samples may now be macerated manually with a 6 mm diameter brass rod which has been tapered and rounded at the end, or with the rod attached to an electric screw-driving device. The objective is to homogenise the tissue and buffer quickly so as not to warm the extract. Once homogenised centrifuge the protein extracts for half to one minute at *circa* 12,000 rpm to pellet the hyphal wall debris. Seven to eight µL aliquots of supernatant from the micro centrifuge tubes may then be added to Titan Sample Well Plate wells. The date, Sample Well Plate number, and well position of each sample of crude protein extract are then recorded in a daily record sheet.

Recommendation

1.2 **In daily record sheets note the date, Sample Well Plate number, and well position of each isolate of *Phytophthora* tested by CAGE.**

1.3 CAGE

1.3.1 Gel Plates and Gel Soaking and Electrode Buffers

The Vegetation Health Service of CALM uses the Super Z-12 applicator kit and 76 mm x 76 mm Titan III cellulose acetate plates to perform CAGE. With plates of this size, well 1 and well 12 may on occasion be distorted and difficult to interpret. To overcome this occasional problem we reduced the number of samples to be assessed to ten or eleven and loaded these in the centre of the acetate plate.

The buffer used to prime cellulose acetate plates, the soaking buffer, was the same as that used for the electrode or 'running' buffer. Comparisons between the CAGE results produced by buffers differing in pH or in buffer concentration led to the development of a single soaking and electrode buffer which was suitable for CAGE of a wide range of *Phytophthora* enzymes.

The Soaking/Electrode Buffer

		g/2 L distilled water
75 mM	Tris	18.165
13.3 mM	Glycine	2.0
6.4 mM	L-Histidine	2.0
3.8 mM	DL-Aspartic Acid	1.0
3.0 mM	L-Glutamic Acid	1.0
2.5 mM	Magnesium Chloride	1.0

Buffer the solution to pH 7.4 with citric acid. Note the similarity between this buffer and the enzyme extraction buffer.

Up to twelve gel plates may be loaded into a Titan III Carry Rack and simultaneously primed with soaking buffer. Place the carry rack with gel plates into the bottom section of a Titan Bufferizing Tank, attach the upper section and fill it with soaking buffer. The soaking buffer transfers slowly into the bottom section and primes the plates without lifting the acetate layer off the mylar backing. Soak the plates for five to ten minutes before using them. The plates may remain in the soaking buffer for several hours or days (Hebert and Beaton 1993) if kept refrigerated.

1.3.2 The Electrophoresis Chamber.

Titan Zip Zone Chambers may be used with the Titan III cellulose acetate plates. Removing the lids of these chambers will break the electrical circuit, which is an added safety feature of these chambers. Add electrode buffer to the left (cathode) and right (anode) reservoirs of the chamber until the platinum electrodes are completely submerged. Paper wicks, one placed on top and running the full length of each of the two inner partitioning walls, are folded over so that two thirds of the wicks are immersed in the electrode buffer. House the Zip Zone chambers in a refrigerator when in use to keep the running temperature down, and to inhibit bacterial or fungal

growth in the electrode buffer. The wicks should be checked every day to see that they are moist and that they have not been damaged. Change the wicks and electrode buffer after ten runs, or if bacteria or fungi show up in the electrode buffer.

To start a run, rest gel plates across the wicks thereby completing the electrical circuit.

1.3.3 Loading the Samples.

Remove a primed cellulose acetate plate from the Bufferizing Tank and blot it dry between two sheets of absorbent paper. Label the mylar surface with the date, sample well plate number and the 'stain' to be overlayed. Place the plate mylar side down on a Titan Alignment Base and position it such that all of the samples will be applied to the acetate surface about one third along its length. Draw aliquots of enzyme extract from the Well Plate wells into a Titan Sample Applicator, and apply the extracts to the acetate surface of the gel plate.

Once loaded place the gel plates, acetate surface down, on the wicks in the Zip Zone Chamber. As most of the enzymes which interest us are negatively charged, ensure that the end of the plate to which the extracts were applied rests on the wick immersed in the cathode reservoir. In so doing the isoenzymes will have the greater distance to migrate toward the anode end during electrophoresis.

1.3.4 Electrophoresis Conditions.

Ensure that you have 'good contact' between the gel plates and wicks. Remove any air pockets between them, and carry out electrophoresis at 8 mA per gel plate for 15 minutes.

1.4 Stains and Staining the Gel.

Isoenzymes may be detected after electrophoresis with specific activity stains. Wendel and Weeden (1989) give a good account of the range of stains found to be useful for revealing plant isoenzymes. Once overlayed with the staining solution the substrates

and other reagents react with the enzyme under study. Visualisation is usually based upon the precipitation of indicator dyes (e.g. MTT) which become coloured zones only where the enzymes are active.

1.4.1 Stains.

Our objective was to identify simple, and therefore cost-effective, specific activity stains which when used routinely would discriminate between taxa of *Phytophthora*. The published literature revealed that 21 such stains (Table 1.2) produced electrophoretically resolved isoenzymes in three starch gel electrophoresis studies of species of *Phytophthora* (Old *et al.* 1984; Nygaard *et al.* 1989; Oudemans and Coffey 1991a). In this study twenty of these stains, plus TPI, were tested with the CAGE technique (Table 1.2). Of these, ten stains consistently produced quickly staining and highly resolved zones of activity (isoenzymes). Of these ten, four stains:

Glucose-6-PO_4 Isomerase	GPI
Isocitrate Dehydrogenase	IDH
Lactate Dehydrogenase	LDH; and
Malate Dehydrogenase	MDH

were chosen to routinely identify taxa of *Phytophthora*. Details of these stain recipes are listed on pages 12 and 13.

Make all your reagent stocks up in 20 mL batches and store them in amber dropper bottles in the refrigerator. Under these conditions most of the reagents will be stable for two to three weeks. Your biggest concern with these stocks will be degradation by bacteria. When this occurs, isoenzyme signal will be low and band resolution will be poorer than usual.

Recommendation

1.3 It is recommended that the isoenzyme (banding) patterns derived from CAGE with GPI, IDH, LDH and MDH stains be used to distinguish between taxa of *Phytophthora*.

Table 1.2 Selection of stains for CAGE identification of species of *Phytophthora* based on stains reported to be useful in fuller starch electrophoresis studies.

Enzyme	Stains Found Useful in Starch Studies			Stains Tested in this Study	Stain Giving Useful CAGE isoenzymes	Stain Selected for *Phytoph.* ID
	1	2	3			
Acid Phosphatase (ACP)		+		+		
Aconitate Hydratase (ACO)	+	+	+	+		
Adenylate kinase (AK)			+	+		
Aldolase (ALD)	+			+		
Diaphorase (DIA)		+	+			
Esterase	+			+		
Fructose-biphosphatase			+	+		
Fumarate hydratase (FUM)		+		+	+	
Glucose-6-Phosphate dehydrogenase (G6PD)		+	+	+		
Glucose-6-Phosphate isomerase (GPI)	+	+	+	+	+	+
Glutamate dehydrogenase (GDH)	+			+		
Hexokinase (HEX)	+		+	+	+	
Isocitrate dehydrogenase (IDH)		+	+	+	+	+
Lactate dehydrogenase (LDH)	+	+	+	+	+	+
Malate dehydrogenase (MDH)	+	+	+	+	+	+
Malate dehydrogenase NADP (ME)		+	+	+	+	
Mannose Phosphate isomerase (MPI)	+		+	+	+	
Menadione Reductase (MNR)	+			+		
Phosphogluconate dehydrogenase	+	+	+	+	+	
Peptidase (PEP)	+		+	+		
Superoxide Dismutase (SOD)	+		+	+		
Triose-phosphate isomerase (TPI)				+	+	

1. Old *et al.* 1984
2. Nygaard *et al.* 1989
3. Oudemans and Coffey 1991a

1.4.2 Staining the Gels.

At the end of the electrophoresis run remove the gel plates from the Zip Zone chamber, blot them dry of electrode buffer and lay them acetate-surface-up on a perspex incubation tray in your fume hood. Pour the staining solution over the surface of the gel plate. Allow it to stand for *circa* 90 sec, or until the staining solution sets, then transfer the gel plate(s) to a dark incubating chamber while the isoenzymes stain up.

Once the bands have developed to desired intensity remove the agar overlay and transfer the gel plate to a weak hydrochloric acid (*circa* 0.7%) or acetic acid (*circa* 2%) stop bath for 2 min. Follow this with two 2 min rinses in water baths, then blot the gel plates and lay them out to dry.

Stains used to distinguish between species of *Phytophthora*

Twenty-five mL plastic vials are best for preparing the stains. Prepare the stains in a fume hood while the enzyme samples are being subjected to electrophoresis. Stains applied as agar overlays are convenient, safe and cost effective.

1. Glucose-6-Phosphate Isomerase (GPI)

		No. of plates to be stained		
Stock	Concn.	1	2	3
Tris HCl, pH 7.5	0.1 M	1	2	3 mL
Fructose-6-Phosphate	*	0.75	1.0	1.25 mL
NAD	*	0.75	1.0	1.25 mL
MTT	*	4	6	8 drops
PMS	*	2	4	6 drops
Agar	2.75%	2.5	4.0	5.5 mL
Glucose-6-Phosphate Dehydrogenase		15	20	25 µL

* See the chemical list in Appendix 1.

2. Isocitrate Dehydrogenase (IDH)

		No. of plates to be stained		
Stock	Concn.	1	2	3
Tris HCl, pH 7.5	0.1 M	1	2	3 mL
DL-Isocitric Acid	*	0.75	1.0	1.25 mL
NADP	*	0.75	1.0	1.25 mL
$Mg\ Cl_2$	*	4	5	6 drops
MTT	*	4	6	8 drops
PMS	*	2	4	6 drops
Agar	2.75%	2.5	4.0	5.5 mL

* See the chemical list in Appendix 1.

3. Lactate Dehydrogenase (LDH)

		No. of plates to be stained		
Stock	Concn.	1	2	3
Tris HCl, pH 7.5	0.1 M	1	2	3 mL
DL-Lactic Acid	*	0.75	1.0	1.25 mL
NAD	*	0.75	1.0	1.25 mL
MTT	*	4	6	8 drops
PMS	*	2	4	6 drops
Agar	2.75%	2.5	4.0	5.5 mL

* See the chemical list in Appendix 1.

4. Malate Dehydrogenase (MDH)

		No. of plates to be stained		
Stock	Concn.	1	2	3
Tris HCl, pH 7.5	0.1 M	1	2	3 mL
DL-Malic Acid	*	0.75	1.0	1.25 mL
NAD	*	0.75	1.0	1.25 mL
$Mg\ Cl_2$	*	4	5	6 drops
MTT	*	4	6	8 drops
PMS	*	2	4	6 drops
Agar	2.75%	2.5	4.0	5.5 mL

* See the chemical list in Appendix 1.

1.5 Storing Stained Gel Plates

Once dry, the plates may be conveniently stored in 170 x 97 mm clip-seal plastic bags. Label the plastic bags with the same information entered in the daily record sheets. Catalogue the packets of gels and store them in the dark. Stored in this manner they will keep for several years.

1.6 Interpreting the Banding Patterns on the CAGE Plates.

Wendel and Weeden (1989) give a comprehensive coverage of the art of correctly interpreting banding patterns on starch gels, and so only a brief introduction to the subject is given here.

When enzymes are visualised with a substrate-specific stain one or more bands may develop on the CAGE plate, and the resulting banding pattern represents the electrophoretic phenotype. The phenotype will vary in complexity depending upon:

- the enzyme being assayed;
- the organism under study; and
- the tissue used.

For some purposes, such as for the rapid identification of species (or taxa), the number of CAGE bands observed, and their relative migrations may be sufficiently informative (see Oudemans and Coffey 1991a, and Goodwin *et al.* 1995). Most studies however, require that electrophoretic phenotypes be translated into genotypes for the underlying loci (isoenzymes).

The number of bands that develop on a gel is determined by several factors:

- the number of coding genes;
- the allelic states (homozygous or heterozygous) of the encoding genes;
- the quaternary structure of the protein products; and
- the subcellular compartmentalisation of the encoding genes.

In some cases the phenotype will be simple, consisting of a single invariant band in all samples, while in others it may display 'complex phenotypes' of multiple bands encoded by several genes (Wendel and Weeden, 1989).

The simplest electrophoretic phenotype would involve a single region of staining, i.e. an isoenzyme, with variant electromorphs (allozymes) observed for different individuals. Such was the case for GPI as reported for species of *Phytophthora* by three researchers using starch gel electrophoresis (Table 1.3). CAGE plates of Western Australian Phytophthoras also revealed only a single staining region (Table 1.3, and Figure 1.1a). For rapid identification purposes the relative mobilities of GPI allozymes may be used to discriminate between taxa, or the allozymes may be coded alphabetically. The most negatively charged, and therefore most rapidly migrating, allozyme of a locus is designated the 'a' allozyme, and sequentially slower migrating allozymes are encoded 'b', 'c' etc.

Table 1.3 The number of isoenzymes reported in three starch gel electrophoresis studies, and that revealed by CAGE in Western Australian isolates of *Phytophthora*.

Study	No. of Isoenzymes (Zones of activity)			
	GPI	IDH	LDH	MDH
Starch electrophoresis Old *et al.* 1984.	1	N/A	2	2
Nygaard *et al.* 1989	1	N/A	1 or 2	1 or 2
Oudemans and Coffey 1991a	1	2	2	2
CAGE. This Study.	1	1 or 2	1 or 2	2, 3, or 4

Electrophoretic phenotypes may appear to be complex when there is more than one active gene for a substrate-specific stain. Oudemans and Coffey (1991a) reported that when they stained starch gels of Phytophthoras for NADP-dependent IDH, two putative loci were revealed (Table 1.3). CAGE plates of Western Australian Phytophthoras stained for IDH consistently produced a single staining region for isolates of *P. cinnamomi*, and two staining regions in other taxa (Table 1.3, Figure 1.1b). Newcombe and Carstairs (1996) argued that of the two NADP dependent IDH enzymes in CIT 3 *P. citricola*, the most anodally migrating enzyme corresponded in action to what has been described for cytosolic-specific NADP-IDH in plants (Randall and Givan, 1981; Ni *et al.* 1987; Wendel and Weeden, 1989), and that the slower migrating enzyme behaved as do plastid specific NADP-IDH enzymes in plants. The two NADP IDH active zones produced by CIT 3 *P. citricola* isolates may therefore be interpreted as being two independent loci. For rapid identification purposes the relative mobilities of the two IDH loci of this and other taxa may be used, or the loci may be coded numerically. The most anodally migrating locus is designated number one, and sequentially more slowly migrating loci are encoded 2, 3 etc.

Figure 1.1 and Table 1.4 give a diagrammatic representation of the process of scoring loci (numerical) and allozymes (alphabetical) to derive a genotypic interpretation of the electrophoretic phenotypes of individuals of *Phytophthora*.

Figure 1.1 Examples of designating (scoring) the isoenzymes and allozymes on developed CAGE plates. Designations were based on relative mobilities of enzymes after electrophoresis. a). A CAGE plate stained for GPI. One locus (GPI-1) and three alleles are depicted in this plate. b). A CAGE plate stained for NADP dependent IDH. Two loci (IDH 1 and 2) are depicted in this plate. IDH-1 is monomorphic and IDH-2 is polymorphic. Refer to Table 1.4 for genotypic interpretations of electrophoretic phenotypes of a) and b).

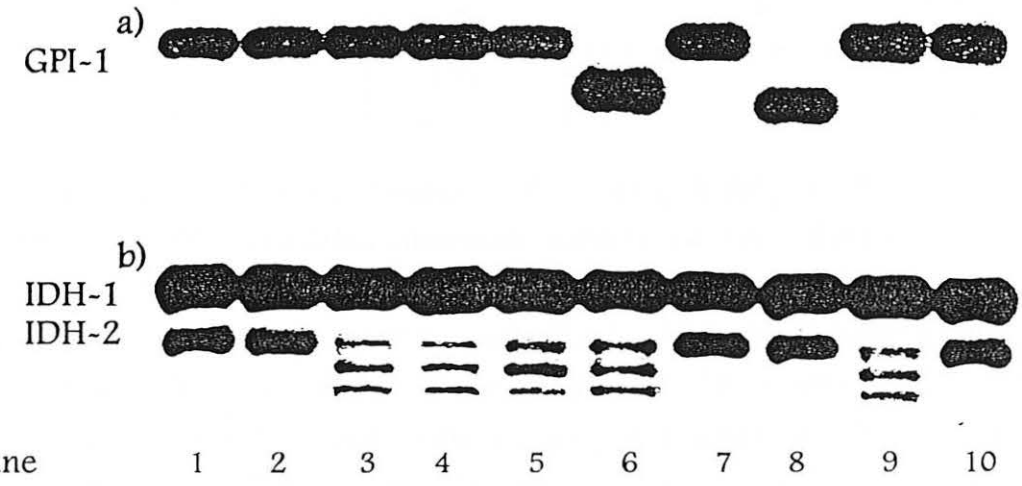

Table 1.4 Genotypic interpretation of electrophoretic phenotypes of a) GPI-1 and b) IDH-1 and 2, depicted in Figure 1.1.

Lane	1	2	3	4	5	6	7	8	9	10
a) GPI-1	aa	aa	aa	aa	aa	bb	aa	cc	aa	aa
b) IDH-1	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa
IDH-2	aa	aa	ab	ab	ab	ab	aa	aa	ab	aa

The genotypes of several loci may be combined to give multi-locus-genotypes for individuals. Individual 1 in lane 1 of Figure 1.1, for example, would have a multi-locus-genotype of aa, aa and aa for loci GPI-1, IDH-1 and IDH-2 (Table 1.4), while the multi-locus-genotype of individual 6 (lane 6 of Figure 1.1) would be bb, aa and ab for the same loci (Table 1.4). By combining the information derived from GPI, IDH, LDH, and MDH isoenzymes, a multi-locus-genotype(s), or a profile, may be constructed for each taxon of *Phytophthora*.

Service 2 Comparison of isoenzyme profiles of Western Australian species of *Phytophthora* with reference cultures imported from California, the UK, and the Australian Capital Territory.

Using the key of Newhook *et al.* (1978) to identify species of *Phytophthora,* Hardy and Sivasithamparam (1988) reported recovering the following Phytophthoras:

Phytophthora cactorum;
Phytophthora cinnamomi;
Phytophthora citricola;
Phytophthora cryptogea;
Phytophthora drechsleri;
Phytophthora megasperma;
Phytophthora nicotianae var. *nicotianae;* and
Phytophthora nicotianae var. *parasitica*

from container-grown plants in nurseries in Western Australia.

Newhook's *et al.* (1978) classification distinguishes two varieties of *P. nicotianae* (var. *nicotianae* and var. *parasitica*) based on oogonium diameters and chlamydospore characters. Expecting significant genetic divergence between the two varieties, Oudemans and Coffey (1991b) compared them isoenzymically and found only limited variation among their sample of isolates, and consequently reported no evidence of genetic divergence. This finding was in agreement with that of Ho and Jong (1989) who found no evidence for distinguishing between the varieties based strictly on morphological comparisons, and Forster *et al.* (1990) who could not distinguish between them using Restriction Fragment Length Polymorphism (RFLP) analysis of mitochondrial (mt) DNA.

Like *P. nicotianae*, *P. cactorum* and *P. cinnamomi* are morphologically and isoenzymically distinct species (Oudemans and Coffey 1991a). The other species referred to by Hardy and Sivasithamparam (1988) however, are extremely variable and probably represent artificial taxonomic groupings into which several unrelated taxa have been placed. For more information refer to Oudemans *et al.* (1994) for *P. citricola*, Mills *et al.* (1991) and Forster and Coffey (1993) for *P. megasperma*.

In this study we restricted our investigation to Phytophthoras recovered from the field in Western Australia. Although type specimens of species in the genus *Phytophthora* were not available to us, the field isolates we examined fell into six taxonomic groupings:

Phytophthora cinnamomi;
Phytophthora citricola;
Phytophthora cryptogea;
Phytophthora drechsleri;
Phytophthora megasperma; and
Phytophthora nicotianae

based on morphological characters used by Newhook *et al.* (1978).

To give an appreciation of how the isoenzyme profiles of Western Australian Phytophthoras compare with the profiles of imported reference isolates that have been reported on by M. Coffey's group in California, Hansen and Maxwell (1991), and Old *et al.* (1984), we will be presenting two case studies. The first is a study of *P. cinnamomi*, a morphologically distinct species, and the second examines the isoenzyme profiles of *P. megasperma*, which is considered to be variable in both morphology (Hansen and Maxwell 1991), and mt DNA (Forster and Coffey 1993).

2.1 Comparison of the isoenzyme profiles of Western Australian isolates of *Phytophthora cinnamomi* with those of *P. cinnamomi* reference cultures (standards) imported from California and the Australian Capital Territory.

Introduction

P. cinnamomi is placed in Group 6 of Waterhouse's (1963) scheme as it produces non-papillate sporangia and forms oospores with amphigynous antheridia in paired culture. According to Waterhouse (1963) *P. cinnamomi* may be identified by the distinctive coralloid hyphae it produces. Consequently, all *P. cinnamomi* determinations made by the VHS, for example, are based on hyphal morphology, with confirmation of identification of occasional isolates by a fuller morphological assessment. To assess the reliability of determinations of local Western Australian isolates of *P. cinnamomi*, a large number of these isolates were compared isoenzymically with *P. cinnamomi* reference isolates obtained from M. Coffey (California) and M. Dudzinski (ACT).

P. cinnamomi isolates recovered from mature plant communities in Western Australia have been predominantly of the A2 mating type. Old *et al.* (1984) examined the isoenzyme patterns of two A1 mating type isolates and forty-four A2 mating type isolates from Western Australia. Among the sample of A2 isolates they identified two genotypes, and these are now commonly known as the A2.1 and A2.2 forms. Relative to *P. cactorum* and *P. cambivora*, *P. cinnamomi* was the most isoenzymically diverse species studied by Oudemans and Coffey (1991a). They recognised six isoenzyme types (multi-locus-genotypes) in the A1 mating strain of *P. cinnamomi*, and confirmed the two isoenzyme types in the A2 mating strain reported by Old *et al.* (1984).

Procedure

Following the CAGE procedures described in the previous section, five Western Australian A1 mating type isolates of *P. cinnamomi*, 188 A2 isolates and an isolate of undetermined mating type were assessed for their GPI, IDH, LDH and MDH isoenzyme patterns. Seven reference isolates (five A1 and two A2 mating types) obtained from M. Coffey (California), and three reference isolates (one A1 and two A2) obtained from M. Dudzinski (ACT) were similarly assessed.

Results and Discussion

The four isoenzyme specific stains (GPI, IDH, LDH and MDH) revealed nine isoenzymes (putative loci) in the *P. cinnamomi* isolates studied (Table 2.1). Four loci: GPI-1, LDH-1, MDH-1 and MDH-3, were invariant, and in four others: IDH-1, LDH-2, MDH-2 and MDH-4, a small number of allozyme variants were detected. Relative to these eight loci, IDH-2 was very polymorphic having three alleles and distinguishing four isoenzyme types (Table 2.1).

Table 2.1 Isoenzyme genotypes of reference and Western Australian isolates of *Phytophthora cinnamomi.*

Multi-locus Genotype	Reference Isolate	Source[1]	GPI-1	IDH- 1 2	LDH- 1 2	MDH- 1 2 3 4	Mating Type
CINN 1	P 6379	A	aa	n[2]n bb	aa ab	aa ab aa nn	A1
CINN 2[3]	P 2370	A	aa	nn ab	aa ab	aa bb aa nn	A1
	P 3656	A	aa	nn ab	aa ab	aa bb aa nn	A1
	P 3657	A	aa	nn ab	aa ab	aa bb aa nn	A1
	P 3664	A	aa	nn ab	aa ab	aa bb aa nn	A1
	MD A1 38	B	aa	nn ab	aa ab	aa bb aa nn	A1
		C (n=5)	aa	nn ab	aa ab	aa bb aa nn	A1
CINN 4	P 2110	A	aa	nn bcn	aa ab	aa bb aa nn	A2
	MD A2 420	B	aa	nn bcn	aa ab	aa bb aa nn	A2
		C (n=178)	aa	nn bcn	aa ab	aa bb aa nn	A2
CINN 5	P 3666	A	aa	nn bnc	aa ab	aa bb aa nn	A2
	MD A2 433	B	aa	nn bnc	aa ab	aa bb aa nn	A2
		C (n=5)	aa	nn bnc	aa ab	aa bb aa nn	A2
	N/A	C (n=4)	aa	nn bb	aa ab	aa bb aa nn	A2
	N/A	C (n= 1)	aa	nn bcn	aa nn	aa bb aa nn	?
	N/A	C (n=2)	aa	aa bcn	aa ab	aa bb aa aa	A2

1 A : reference isolates obtained from M Coffey (California)
B : reference isolates obtained from M Dudzinski (ACT)
C : Western Australian isolates recovered from native vegetation, tree crop, and nurseries.

2 n = 'null allele'; where the gene is either switched off or absent in some isoenzyme types.

3 refer to Oudemans and Coffey (1991a) for the isoenzyme classification of P 2370 to P 3664 based on starch gel information.

The nine loci assessed in this study did not differentiate between four of Coffey's A1 standards: P 2370, P 3656, P 3657 and P 3664 (Table 2.1). The A1 isolate provided by Dudzinski (MD A1 38) and five local isolates were isoenzymically indistinguishable from these standards. The P 6379 A1 standard was heterozygous (ab) at the MDH-2 locus and no local isolates matched with it isoenzymically. One hundred and seventy-

eight local A2 isolates were isoenzymically the same as the P 2110 and MD A2 420 reference A2 isolates, and five local A2 isolates were isoenzymically the same as the P 3666 and MD A2 433 references. Interestingly, six local A2 isolates and a local isolate of undetermined mating type did not match exactly with any of the references.

That the isoenzyme patterns of all the WA isolates of *P. cinnamomi* matched those of the standards with a high degree of fidelity indicates that in WA, isolates of *Phytophthora* have been diagnosed to *P. cinnamomi* with a high degree of accuracy. Most of these identifications were based on hyphal morphology.

Relative to the A2 mating type in which only two isoenzyme types have been previously reported (Old *et al.* 1984; Oudemans and Coffey 1991a), the A1 mating type of *P. cinnamomi* is isoenzymically variable, with six isoenzyme types being recognised. The number of Western Australian A1 isolates available for this study was small (n=5), and among these only one isoenzyme type was discriminated with the four specific stains used. These stains matched 183 of the 189 WA A2 isolates tested with one or the other of the two A2 isoenzyme types previously reported. Two new A2 isoenzyme types, and probably a third, were identified in this study indicating that the A2 mating type of *P. cinnamomi* is more variable than has previously been presumed.

2.2 Comparison of the isoenzyme profiles of Western Australian isolates of *Phytophthora megasperma* with those of reference isolates of *P. megasperma* (complex) imported from the UK.

Introduction

Drechsler (1931) described *P. megasperma* Drechs. as an oomycete with unusually large oogonia. Subsequent to this, the species concept has been broadened to include similar *Phytophthora* with smaller oospores, and there has been some confusion about the species concept of *P. megasperma* due to attempts to include the legume pathogens within the originally described species (Hansen and Maxwell, 1991).

Hansen and Maxwell (1991) believed that their interpretation of *P. megasperma*, with *P. sojae*, *P. medicaginis*, and *P. trifolii* removed, fitted comfortably within the original

species description by Drechsler (1931) and the species concept of Tompkins *et al.* (1936). Within the species Hansen and Maxwell (1991) retained three groups:

Broad Host Range	(BHR);
Apple, Cherry	(AC); and
Douglas Fir	(DF)

identifiable by their hosts as well as morphological and chemical characteristics.

Subsequent to Hansen and Maxwell's (1991) treatment of *P. megasperma*, Forster and Coffey (1993) evaluated a worldwide collection of isolates identified as *P. megasperma* using mitochondrial and nuclear DNA polymorphism. The legume pathogens and *P. megasperma* groups (BHR, AC, and DF) identified by Hansen and Maxwell (1991) were assessed in this study, and Forster and Coffey (1993) distinguished these and several other groups as well in their sample of isolates. *P. megasperma,* it seems, may be described as a 'complex' of *Phytophthora* taxa which share key morphological features of their antheridia, oogonia, oospores and sporangia, but which are otherwise sound species. As a consequence of similar studies by Coffey's group in California of Phytophthoras identified as *P. citricola,* and *P. cryptogea/P. drechsleri,* it may be argued that these species are also 'complexes' of *Phytophthora* taxa.

In an isoenzyme survey of Western Australian isolates of *Phytophthora* that had been identified as *P. megasperma* on morphological grounds, Bellgard and Carstairs (1996) distinguished four isoenzyme groups using ten substrate specific stains. Here we compare the isoenzyme profiles of those four isoenzyme groups with *P. megasperma* reference isolates obtained from the UK.

Procedure

Following the CAGE procedures described in the previous section, 77 Western Australian isolates of *P. megasperma* were assessed for their GPI, IDH, LDH, and MDH isoenzyme patterns. Six reference isolates, obtained from C. Brasier and S. Kirk (UK) were similarly assessed.

Results and Discussion

The only Western Australian BHR isolate (see isoenzyme class 'A, B & C a and b' in Table 2.2) was recovered from near the town site of Cataby on the sand plain north of Perth. Five of the eight Western Australian AC isolates (isoenzyme class D b in Table 2.1) identified in the survey were also recovered from the sand plain north of Perth, two were recovered from the Fitzgerald River National Park (FRNP) in the south west of Western Australia, and an isolate was recovered from the town site of Hopetoun near FRNP.

Sixty-eight isolates did not match with any of the standards. Of these, isoenzyme class K was a single isolate recovered from near Cataby, and class L was widely distributed and was recovered from many hosts (Table 2.2). In class L, two isoenzyme types were identified of which there were 62 widely spread isolates (type L a), and the other five isolates (type L b) were all recovered from the sand plain north of Perth.

Table 2.2 Isoenzyme genotypes of reference and Western Australian isolates of *Phytophthora megasperma*.

Isoenzyme Class[1]	Reference Isolate	Hosts	Source[2]	GPI -1	IDH -2	LDH -2	MDH- 2	MDH- 3	Mating Type
A, B, and C a	P 452 (*P. megasperma* BHR)	many	A	bd	ac	cc	dd	dd	H[3]
b		soil	B (n=1)	dd	cc	cc	dd	dd	H
D a	P 471 (*P. megasperma* AC)	Fruit trees	A	dd	cc	cc	cc	dd	H
b		WA natives	B (n=8)	dd	cc	cc	cc	dd	H
F	P. 439 (*P. megasperma* DF)	Douglas Fir, herbaceous weeds	A	cc	dd	cc	dd	cc	H
G	P 450 (*P. trifolii*)	clover	A	bb	cc	aa	bb	bb	H
H	P 484 (*P. medicaginis*)	alfalfa	A	aa	ee	dd	dd	ff	H
I	P 445 (*P. sojae*)	soybean	A	aa	cc	dd	aa	ff	H
K	ND[4]	soil	B (n=1)	ec	ee	bb	ee	cc	H
L a	ND	WA natives	B (n=62)	cc	bb	dd	ee	bb	H
b	ND	WA natives	B (n=5)	bb	bb	dd	ff	aa	H

1 Isoenzyme classes A-I coincide with the *P. megasperma* classes described by Forster and Coffey 1993; and classes K and L were determined by Bellgard and Carstairs 1996.

2 A: reference isolates obtained from C. Brasier and S. Kirk.
B: Western Australian isolates recovered from native vegetation.

3 Homothallic.

4 Not Determined.

That the isoenzyme patterns of only 9 of 77 Western Australian isolates that had been identified by their morphological characters as *P. megasperma* matched with the isoenzyme patterns of *P. megasperma* standards, indicates that we may expect that only a small frequency of local isolates of *Phytophthora* will be diagnosed to recognised taxa within the *P. megasperma* complex using the isoenzyme method of identification.

2.3 Comparison of isoenzyme profiles of Western Australian isolates of *Phytophthora citricola, Phytophthora cryptogea/drechsleri,* and *Phytophthora nicotianae* with reference isolates imported from M. Coffey's Californian collection.

We examined 29 isolates of *P. nicotianae* with CAGE and all matched, with a high degree of fidelity, those reference isolates of *P. nicotianae* obtained for this study from M. Coffey's Californian collection (Table 2.3). However, *circa* 50% of Western Australian isolates of *Phytophthora* which had been diagnosed as being *P. citricola* by their morphology (see Bunny and Shearer 1995), did not match with the isoenzyme profiles of 2 reference isolates we obtained from M. Coffey's Californian collection for this species complex, and 45% of Western Australian cultures of *P. cryptogea/ drechsleri* did not match with 6 reference isolates from Coffey's collection(Table 2.3).

Table 2.3 The number of *P. citricola, P. cryptogea/drechsleri* and *P. nicotianae* isolates used in this study to compare the isoenzyme profiles of Western Australian (and some Eastern Australian) isolates of *Phytophthora* with Coffey's Californian reference isolates, and the frequency (approximate) that did not match with the reference isolates.

Species	No. of W A Isolates Examined	Frequency (approx.) Not Matched with Reference Isolates[1]
Morphologically Distinct		
P. nicotianae	29[2]	0.0
Species Complexes		
P. citricola	12[3]	0.50
P. cryptogea/drechsleri	121	0.45

1. Isolates were deemed to match if there was a high incidence of shared bands i.e.>60%
2. Many of these isolates were obtained from M. Dudzinski (CSIRO, Canberra).
3. These isolates were obtained from F. Bunny (SID of CALM). They represented the isoenzyme variation in *P. citricola* (F. Bunny pers. com.)

Among the respective species complexes, i.e. *P. citricola, P. cryptogea/drechsleri, P. megasperma* (see Service 2.2), the incidence of isolates going unmatched with reference isolates (ex. M. Coffey's Californian collection) was high. This suggests that had isolates of these unmatched Phytophthoras occurred in Coffey's collection, then they were not obtained for this study. Alternatively these may be Phytophthoras that have not as yet been incorporated into the Californian collection.

With CAGE we were able to discriminate among discrete taxonomic units (molecular taxa) within several morphologically recognised taxa (Newhook *et al.* 1978), i.e. *P. megasperma* and other species complexes. This gives some indication of the resolving power of the isoenzyme technique over the morphological.

Recommendations

2.1. In Western Australia there is no recognised centre for housing reference cultures of taxa of *Phytophthora,* and so it is recommended that the VHS service of CALM maintain living isolates of the reference cultures of *Phytophthora* that were obtained for this study from California, the UK and states outside Western Australia.

2.2. It is recommended that the VHS of CALM maintain living reference isolates of all readily available taxa of *Phytophthora,* as determined by their isoenzyme profiles, that have been retrieved from the field in Western Australia.

2.3 When, during routine identification of isolates by the VHS, isolates of *Phytophthora* are encountered which have not as yet been described, i.e. new or unique isoenzyme profiles, it is recommended that:

a. the VHS give these isolates a full morphological examination; and
b. the VHS maintain living reference cultures of these isolates.

Service 3 Variation in species of *Phytophthora* occurring in Western Australia relative to that world-wide.

This service, as required in the schedule of contractual services, is phrased in a form which sets an unattainable goal. "World-wide" variation therefore, is taken to mean the variation found in the extensive collection of Professor M. Coffey in California. The extent to which Coffey's collection reflects true world-wide variability is however unknown. To give an appreciation of how the variation in *Phytophthora* from Western Australia compared with that of world-wide variation we have presented here a case study for *P. cinnamomi.*

Introduction

Podger (1972) argued that *Phytophthora cinnamomi* was introduced into Western Australia during early European settlement, and is being dispersed with soil during earth moving operations. That fewer isoenzyme types were found in the Australian population of *P. cinnamomi*, relative to that in Papua New Guinea (Old *et al.* 1984), further supports Podger's argument that *P. cinnamomi* is a recent introduction to Western Australia. Apparently a subset of the available genotypes of this pathogen has been recently introduced into south-western Australia, and is altering the composition and character of the flora.

In the previous section (Service 2) isoenzyme profiles of Western Australian isolates of *Phytophthora cinnamomi* were matched with standards from California and the Australian Capital Territory. In this section we will be determining a measure of the isoenzyme diversity for the Western Australian population of *P. cinnamomi*, and comparing it with that of populations throughout the world. If it is shown that populations outside Western Australia are genetically much more diverse than the Western Australian population, then we would have provided further evidence in support of Podger's hypothesis that *P. cinnamomi* is a recent introduction to W.A.

Procedure

The Shannon-Weaver measure of diversity, H' (Shannon and Weaver 1949) for the 1996 population of *P.cinnamomi* from Western Australia (n=195) was calculated using the relative frequencies of isolates falling into the isoenzyme classes presented in Section 2 of this report, and Old's *et al.* (1984) results were used to calculate the diversity measure for Western Australian population (n=46) in 1984, the eastern Australian (n=119), and Papua New Guinea (n=18) populations. Oudemans and Coffey's (1991a) results were used to determine the diversity measures for the south-east Asian (n=14) and North American (n=35) populations of *P. cinnamomi.*

Results and Discussion

Papua New Guinea had the most diverse population (H'=0.816) of *P. cinnamomi*, and this was followed by the south-east Asian population (Table 3.1). These were followed by the populations in the eastern states of Australia and North America, both of which were equally diverse. The Western Australian population was the least diverse of the populations examined, with the 1996 study giving a measure of diversity that was about twice that of the 1984 study (Table 3.1).

Table 3.1 Measures of genetic diversity in populations of *P. cinnamomi.*

Location/Region	Year	No. Cultures	H'[1]
Papua New Guinea[2]	1984	18	0.816
China + Indonesia +Taiwan[3]	1991	14	0.508
Eastern States of Australia[2]	1984	119	0.319
North America[3]	1991	35	0.310
Western Australia[4]	1996	194	0.182
Western Australia[2]	1984	46	0.082

1 Genetic diversity (H') was estimated from genotype frequencies by the method of Shannon and Weaver (1949).
2 Genotype frequencies were calculated from the results of Old *et al.* 1984.
3 Genotype frequencies were calculated from the results of Oudemans and Coffey 1991a.
4 Genotype frequencies were calculated from the isoenzyme class numbers for *P. cinnamomi* that were presented in Section 2 of this report.

An interesting outcome from this research was the observed lack of diversity found in the Western Australian population of *P. cinnamomi* relative to that of other populations. Of the two compatibility (mating) types found in *P. cinnamomi,* the A1 type seems to be more variable than the A2 (see Old *et al.* 1984, and Oudemans and

Coffey 1991a). A characteristic of both the 1984 and 1996 studies of the Western Australian population of *P. cinnamomi* was the small representative number of A1 compatibility types examined relative to the number of A2 compatibility types (4% and 2.5% respectively). So the much lower measures of diversity found for the Western Australian population, relative to other populations, may be due to the small representation of the more diverse A1 compatibility type in the samples studied.

At least two hypotheses may account for the relatively low diversity found in the Western Australian population compared to other populations. Firstly, when the subset of *P. cinnamomi* genotypes were introduced into Western Australia from the base population, those genotypes may not have represented the genotypes in the base population proportionally, and some may not have been represented. This being the case, then subsequent to their introduction the relative frequencies of the respective isoenzyme types in the Western Australian population must have remained unchanged until measured in these studies.

Alternatively the isoenzyme types and compatibility types in the base population may have been proportionally represented in the subset of genotypes introduced into Western Australia, and subsequent to their introduction their relative frequencies have changed from those of the base population as a consequence of chance, or because some genotypes were fitter in the Western Australian habitat.

It is worth noting that measures of diversity for the Papua New Guinea and south-east Asian populations were determined from only a small number of cultures, 18 and 14 respectively (Table 3.1). Given that 59 cultures would have to be examined to be 95% confident of detecting genotypes that occur with a frequency of 5% in populations, we might expect that if larger numbers of cultures were used to measure diversity in these populations, then even higher measures may be achieved for the Papua New Guinea and south-east Asian populations. The high measures of diversity observed for these two populations, relative to the others measured, suggest that they may represent the base populations from which the north American and Australian populations were derived.

The relatively low measure of diversity in the Western Australian population of *P. cinnamomi* compared to that of the other populations measured in this study is taken as *prima facie* evidence in support of Podger (1972) who proposed that *P. cinnamomi* was recently introduced into Western Australia. The period in which *P. cinnamomi* was being introduced into Western Australia was probably brief, and this, followed by strict overseas and interstate quarantine laws prohibiting the movement of *P. cinnamomi* infected material into Western Australia, probably accounts for the now quite large differences between the Western Australian population and populations outside the state.

Recommendation

3.1 It is recommended that studies similar to the one above for *P. cinnamomi* be performed for other Phytophthoras found in mining tenements and in the conservation estate of Western Australia to determine whether, like *P. cinnamomi*, they are introduced exotics or not.

Service 4. To evaluate the effectiveness of isoenzymes for identifying species of *Phytophthora.*

Introduction

Erwin (1983) has cited several examples where morphological criteria have produced confusing and doubtful designations of isolates of *Phytophthora* to species. In contrast to the limitations of morphological criteria for assigning isolates of *Phytophthora* to species, Oudemans and Coffey (1991a) found that CAGE could be used to reliably identify isolates of three morphologically distinct species of *Phytophthora* using only three substrate specific stains, and Goodwin *et al.* (1995) used CAGE for rapid identification of allozyme genotypes of *Phytophthora infestans.*

In earlier sections (Service 2.1 and 2.2) of this document it was reported that enzyme profiles of all Western Australian isolates of *P. cinnamomi* and some isolates of *P. megasperma* (complex) showed a high degree of fidelity with imported reference isolates. This observation gave an indication that CAGE identification of isolates to *Phytophthora* species was accurate, and that it may also be efficient. To assess the effectiveness of the isoenzyme method relative to the morphological, the two methods were compared for accuracy of assignment of Western Australian isolates of *Phytophthora* to species or taxa.

Procedure

Fifty isolates of *Phytophthora* (listed in Table 4.1) were selected at random from the Western Australian cultures used to develop the isoenzyme techniques described in Service 1, and used for comparative purposes with reference cultures (see Service 2). These fifty isolates were the subjects of a test comparing the isoenzyme and morphological methods for accuracy of assigning isolates to species. The number of isolates used to assess each of the six species was biased such that the more variable species complexes had higher representation.

Two pure cultures, prepared on corn meal agar (CMA) plates, of each of the fifty isolates were given code numbers, and were subsequently referred to as 'unknowns'. One of the sets of fifty unknowns was subjected to identification to taxa by the isoenzyme method, while the other set was subjected to identification by the morphological method.

The set to be identified by the morphological method was presented to two experienced Vegetation Health Service (VHS) of CALM operators who used hyphal, sporangial, and oogonial characters (see Newhook *et al.* 1978) of the isolates to assign them to species, or species complex in the case of *Phytophthora citricola, P. cryptogea, P. drechsleri* and *P. megasperma.* The operators did not attempt to assign the unknowns to taxa within species complexes on the basis of morphology.

Isoenzyme profiles of the set of fifty unknowns used to assess the accuracy of the isoenzyme method were prepared by the author and one of the aforementioned operators. These profiles were then presented to a third (independent) experienced operator for matching with profiles of standard isolates of taxa of *Phytophthora* identified during research for Services 1, 2 and 3. Where possible, the operator was required to assign the unknowns to taxa within species complexes. Three 'distracters', isolates which had isoenzyme patterns similar to some of the standards but which did not match with any of the unknowns, were included with the standards.

Results

Two classes of *Phytophthora* can be recognised among those species tested in this study. As *P. cinnamomi* and *P. nicotianae* are morphologically distinct species, they were placed into one of the classes (Table 4.2). *P. cinnamomi* may be distinguished from other Phytophthoras by the characteristic coralloid hyphae it produces (Waterhouse 1963), and *P. nicotianae* was the only species to have papillate sporangia (Newhook *et al.* 1978). The other four Phytophthoras represent species complexes, and were placed into a second class (Table 4.2). Both *P. cryptogea* and *P. drechsleri* are heterothallic Phytophthoras, i.e. they require two compatible mating types

Table 4.1 *Phytophthora* isolates used to determine the effectiveness of the isoenzyme method for identifying species of *Phytophthora* 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]
Morphologically distinct					
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
	DCE 114	CINN 4	*Conostephum pendulus*	Gosnells	A2
	DCE 230	CINN 4	*E. marginata*	Gordon Block	A2
P. nicotianae	VHS 3375	?	?	Bunbury District	A2
	VHS 3267	?	*E. ficifolia*	Perth	?
	DCE 17	?	?	?	?
Complexes					
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
P. cryptogea	VHS 3352	E	?	Fitzgerald River National Park	A2
	VHS 3437	E	*B. attenuata*	Jarrahdale	A2
	VHS 3499	E	Soil	Woodarda Track	A2
	VHS 3606	E	*Daviesia* sp.	Fitzgerald River National Park	A2
	VHS 3617	E	*Dryandra* sp.	Fitzgerald River National Park	A2
	MJS 80	E	*P. radiata*	Jarrahwood Plantation	A2
	MJS 105	E	*P. radiata*	Jarrahwood Plantation	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.

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

3 ?: compatibility type not determined; S: sterile; H: homothallic.

Table 4.1 (cont.) *Phytophthora* isolates used to determine the effectiveness of the isoenzyme method for identifying species of *Phytophthora* 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.)	MJS 125	E	*P. radiata*	Jarrahwood Plantation	A2
	DCE 154	E	?	?	?
	DCE 456	E	*B. attenuata*	Cervantes Road	A2
	VHS 3631	?	*Xanthorrhoea preissii*	Bunbury District	?
	DCE 458	?	Water	Encabba	?
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	Fitzgerald River National Park	?
	DCE 457	?	Bait	Cooljarloo	?
	VHS 3360	?	?	?	?
P. megasperma	MJS 216	?	Soil	Baudin Plantation	H
	MJS 217	?	Soil/ *P. radiata*	Baudin Plantation	H
	SEB 250	D(AC)	*Allocasuarina campestris*	Fitzgerald River National Park	H
	SEB 251	D(AC)	*X. platyphylla*	Fitzgerald River National Park	H
	TH 7	D(AC)	*B. prionotes*	North Sand Plain	H
	SEB 218	?	*Isopogon formosus*	Fitzgerald River National Park	H
	SEB 243	?	*B. media*	Fitzgerald River National Park	H
	VHS 3594	?	*B. baxteri*	Fitzgerald River National Park	H
	HSA(DCE441)	?	*B. attenuata*	Minyolo Brook	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 ?: compatibility type not determined; S: sterile; H: homothallic.

to produce oospores, and share most of the morphological characters that are useful for identifying isolates of *Phytophthora* to species. Tucker (1931), cited in Bumbieris (1974), reported in his original description of *P. drechsleri* that it may be distinguished from *P. cryptogea* by its higher optimum growth temperature (30-32.5°C), and its ready development at 35°C. *P. citricola* is a homothallic species and produces semi-papillate sporangia. *P. megasperma* is also homothallic and may be distinguished from *P. citricola* as it produces non-papillate sporangia and larger oogonia.

Table 4.2 Accuracy of assignment of isolates of *Phytophthora* to species or species complex as determined by morphological characters and by isoenzyme patterns.

	Number of Isolates						
		Assigned Correctly on/by					
	Tested	Isoenzyme Pattern	Coralloid Hyphae		1. Incomplete Morphological Data	2. Complete Morphological Data	Morphology (1+2)
			Present	Absent			
Morphologically Distinct Species							
P. cinnamomi	7	7	7	0	2	5	7
P. nicotianae	3	3	0	3	0	3	3
Species Complexes							
P. citricola	9	9	0	9	0	9	9
P. cryptogea	12	12	0	12	10	2	12
P. drechsleri	10	10	0	10	3	1	4
P. megasperma	9	9	0	9	0	8	8
Total	50	50	7	43	15	28	43

Isoenzyme patterns were as effective as classical alpha taxonomic characters for identifying the morphologically distinct species. The three *P. nicotianae* isolates and seven *P. cinnamomi* isolates tested were correctly assigned to species from the isoenzyme patterns they produced (Table 4.2). The three *P. nicotianae* isolates produced all of the structures required to complete the morphological identification, and all were correctly assigned to *P. nicotianae* by this method (Table 4.2). The operators correctly assigned to species the seven isolates of *P. cinnamomi* used in this study, based on the observation of coralloid hyphae (Table 4.2). Although one of the operators was not confident about assigning MJS 171 to *P. cinnamomi* on coralloid hyphae alone, this method was otherwise highly reliable. Two of the seven

P. cinnamomi isolates did not produce all the structures required to complete the fuller morphological identification.

Isoenzyme patterns were more effective than classical alpha taxonomic characters for assigning isolates to the species complexes. By their isoenzyme patterns all of the *P. citricola, P. cryptogea, P. drechsleri* and *P. megasperma* isolates used in this study were correctly assigned to species complex (Table 4.2). The nine *P. citricola* (complex) isolates used in this study produced all the structures required to complete the morphological identification, and all were correctly assigned to *P. citricola* by their morphological characters (Table 4.2). Eight of the nine *P. megasperma* (complex) isolates tested produced all of the structures required to complete the morphological identification, and these were correctly assigned to *P. megasperma* (Table 4.2). The *P. megasperma* (complex) isolate which did not produce all of the structures was misassigned to *P. citricola* (complex) on incomplete morphological data.

Two of the twelve *P. cryptogea* (complex) isolates used in this study produced all the structures required to complete the morphological identification, and both were correctly assigned to *P. cryptogea* (complex). The other ten were correctly assigned to *P. cryptogea* (complex) on incomplete morphological data (Table 4.2). Two of the ten *P. drechsleri* (complex) isolates used in this study produced all the structures required for the morphological identification, but only one of them was correctly assigned to *P. drechsleri* (complex). The other isolate was misassigned to *P. cryptogea*. Of the eight *P. drechsleri* (complex) isolates which did not produce all the structures, three were correctly assigned to *P. drechsleri* and the other five were misassigned to *P. cryptogea* (complex).

All of the isolates tested in this experiment were correctly diagnosed to species or species complex by the isoenzyme patterns they produced, and the morphologically distinct species (*P. cinnamomi* and *P. nicotianae*) were also correctly diagnosed by the classical alpha taxonomic method. Among the species complexes, however, misassignment by the classical taxonomic method occurred between the two morphologically very similar heterothallic species (*P. cryptogea* and *P. drechsleri*), and between the two homothallic species (*P. citricola* and *P. megasperma*), and misassignment was usually associated with isolates which did not produce all the structures required for identification. Of those

that produced all the structures 4.8% were misassigned, compared to 31.6% of those isolates which did not produce all the structures required for identification.

When it was required that isolates within the four species complexes be identified down to the lower taxonomic rank of 'taxon' by the isoenzyme patterns they produced, all but one of them were correctly assigned (Table 4.3). The misdiagnosed isolate (one of the *P. drechsleri* isolates) was misassigned to a distracter which had a similar isoenzyme pattern.

Table 4.3 Accuracy of assignment of isolates of *Phytophthora* to taxa within species complexes as determined by morphological characters and by isoenzyme patterns.

Taxa Within Species Complexes[1]	Number of Isolates				
	Tested	Assigned Correctly on/by			
		Isoenzyme Pattern	1. Incomplete Morphological Data	2. Complete Morphological Data	Morphology (1+2)
P. citricola (KAL)	1	1	0	0	0
(CIT 3)	4	4	0	0	0
(JF)	4	4	0	0	0
P. cryptogea(E)	10	10	8	2	10
(Bun)	1	1	0	0	0
(Ene)	1	1	0	0	0
P. drechsleri (WA)	9	8	2	1	3
aff. WA	1	1	0	0	0
P. megasperma (Bau)	2	2	0	0	0
(D)	3	3	0	0	0
(L)	4	4	0	0	0
Total	40	39	10	3	13

1.The key of Newhook *et al.* (1978) does not discriminate between taxa within species complexes. The kinds of taxa recognised here e.g. *P. citricola* (KAL) etc., have been described by others (e.g. Coffey) as "molecular species" in recognition of their taxonomic rank.

The alpha taxonomic method was as sensitive as the isoenzyme method for identifying *P. cryptogea* isolates to taxon (Table 4.3). The classical method however, was less than half as sensitive as the isoenzyme method for identifying *P. drechsleri* isolates, and for the other taxa tested the classical method was not sensitive enough to assign any of the isolates correctly to their respective taxa (Table 4.3).

Discussion

The isoenzyme method of identification used in this study was as accurate as the classical alpha taxonomic method of Newhook *et al.* (1978), as practiced by the VHS, for identifying isolates of *Phytophthora* to *P. cinnamomi* or *P. nicotianae*. These species are morphologically quite distinct and are readily identified by key characters. In its capacity to assign *Phytophthora* isolates to species complexes, the isoenzyme method was superior to the classical morphological method, and in its capacity to assign isolates of species complexes to taxa the isoenzyme method was vastly superior. It was those isolates which did not produce all of the morphological structures required for identification that were most often misdiagnosed by the alpha taxonomic method, and they were usually misassigned to a species complex or taxon with which they shared most characters.

This study did not establish that the isoenzyme method of identifying isolates of *P. cinnamomi* was any more or less effective than the method of identifying *P. cinnamomi* by the coralloid hyphae its isolates produce. Based on this information, there are no grounds for replacing the coralloid hyphae method of identifying *P. cinnamomi* with the isoenzyme method.

The constant association between *P. cinnamomi* and severe dieback in native plant communities in Western Australia has been established (Podger *et al.* 1965; Podger *et al.* 1967; Podger and Batini, 1971; Podger, 1972). However, it has not yet been established that any of the taxa in the species complexes tested in this study are constantly associated with severe dieback. Should it be established that one of them is, then an identification method will be required which distinguishes the disease-causing pathogen from the other taxa. The classical alpha taxonomic method tested in this study was not sensitive enough for this purpose, whereas the isoenzyme method was.

Recommendations

4.1 It is recommended that pathologists and nature managers alike adopt the isoenzyme method for identifying taxa of *Phytophthora* other than *P. cinnamomi*.

4.2 Before deciding whether or not to replace the coralloid hyphae technique of identifying *P. cinnamomi* with the isoenzyme method a comparison needs to be made between the relative efficiencies and opportunity costs of the respective techniques.

Service 5. To compare isoenzyme analysis for identifying species of *Phytophthora* with morphological methods on the basis of accuracy, time and cost.

Introduction

The question as to how the isoenzyme method of identifying species of *Phytophthora,* other than *P. cinnamomi,* compares with morphological methods on the basis of accuracy, has very largely been answered in the previous section (Service 4, on the relative effectiveness of the isoenzyme method). In Service 4 it was determined that identification by isoenzymes was the superior method, and it was subsequently recommended, with no consideration of the relative efficiencies or costs of the respective methods of identification, that the isoenzyme method be adopted in place of the morphological method currently in use by the VHS. It was also determined that the isoenzyme method was as accurate as the coralloid hyphae (Waterhouse, 1963) method of assigning isolates of *Phytophthora* to *P. cinnamomi* or to "*Phytophthora* spp. other than *P. cinnamomi*" as a group.

In this section we will be comparing the relative efficiencies of the methods of identifying species of *Phytophthora*, as well as the cost of diagnosis. The underlying assumptions (null hypotheses) will be:

- that the methods are equally efficient i.e. that the number of days required to assign isolates to species by the isoenzyme method is the same as the morphological methods;

- that the amount of time that operators are actively engaged in assigning isolates to species by the isoenzyme method is the same as the morphological methods; and

- that the isoenzyme method costs no more than the morphological methods for assigning *Phytophthora* isolates to species.

Procedure

The same fifty isolates used in Service 4 (Table 4.1, Service 4) to compare the accuracy of the isoenzyme method with that of the morphological method for assigning isolates of *Phytophthora* to species, were used in this service to compare the efficiencies and costs of the methods of identification.

The efficiency of an identification method may be determined in terms of the number of days it takes from when a field sample is received, until its *Phytophthora* status has been diagnosed e.g. as negative for *Phytophthora*, positive for *P. cinnamomi*, positive for *Phytophthora* spp., or positive for a particular species. Alternatively, efficiency may be determined in terms of the amount of time (min) that operators are actively engaged in diagnosing the *Phytophthora* status of a field sample.

Efficiency in terms of the number of days to diagnosis by morphology.

On the basis of hyphal morphology *P. cinnamomi*, which has coralloid hyphae, may be distinguished from all other species of *Phytophthora* (Waterhouse, 1963). The average number of days (rounded to an integer) required to trap from field soil/plant tissue samples, and identify by their coralloid hyphae on CMA plates, 122 isolates of *P. cinnamomi,* was determined. This time (14 days) was then taken as the average number of days required to diagnose field samples as: negative for *Phytophthora*, positive for *P. cinnamomi,* or positive for *Phytophthora* spp. other than *P. cinnamomi.* This method of diagnosis is hereafter referred to as the "short" morphological method.

To obtain a measure of efficiency in terms of the number of days to diagnosis to particular species of *Phytophthora,* VHS operators recorded the number of days they took to determine the identity (to species) of each of 50 corn meal agar (CMA) plated isolates of *Phytophthora* using hyphal, sporangial and oogonial characters (Newhook *et al.* 1978). This number of days was then combined with the number of days required to perform the short morphological method, and together they gave the number of days required to perform a "fuller" morphological identification.

Efficiency in terms of the amount of time (min) operators were actively engaged in diagnosis by morphology.

The average number of minutes (rounded to an integer) operators were actively engaged in tasks to trap in baits Phytophthoras from 6 field samples, and isolate them on CMA plates, was determined. This amount of time (41 min) was taken to be that required to diagnose field samples by the "short" morphological method.

To obtain a measure of efficiency in terms of the amount of time operators are actively engaged in diagnosis to particular species of *Phytophthora,* VHS operators recorded the amount of time (min) they were actively engaged in identifying, to species, each of the 50 CMA plated isolates of *Phytophthora* using morphological characters and Newhook's *et al.* (1978) key. This time was then combined with the time (min) required to perform the short morphological method, and together they gave the time (min) operators are actively engaged in performing the "fuller" morphological method.

Efficiency of the isoenzyme method.

The operators determined the number of days it took them to identify a second set of 50 CMA plated isolates by the isoenzyme method. This number of days was then combined with the number of days required to perform the short morphological method, and together they gave the number of days required to perform an isoenzyme identification.

The operators also recorded the amount of time (min) they were actively engaged in identifying to species or taxa a second set of 50 CMA plated isolates by the isoenzyme method. This time was then combined with the time (min) required to perform the short morphological method, and together they gave the time (min) operators were actually engaged in performing the isoenzyme method.

Statistical Tests.

One-Way ANOVA was used to compare the efficiency in terms of:

1. days to diagnosis; and
2. time (min) operators are actively engaged in diagnosis;

of the isoenzyme method with that of the fuller morphological method. But first an ANOVA was performed to determine if efficiency was influenced by the 6 species (2 morphologically distinct species and 4 species complexes) used to make up the 50 test isolates used in this study. Before ANOVAs were performed the original data was tested for normality, and transformed as required.

When discrete populations of species were recognised, i.e. subsets of the 6 species that were not significantly different from each other as determined by ANOVA and Tukey (1953) tests [cited in Ott (1993)], the data for these species were pooled into one population of samples. The respective subsets of species were then compared by ANOVA for discreteness, and when found to be different they were considered to be different treatments in subsequent tests.

Case Studies.

Efficiency in terms of days to diagnosis or time operators are actively engaged in diagnosis by the respective methods of identification, may be determined on a 'per-field-sample' basis.

A. We determined the proportion of field samples that were diagnosed as either:

negative for *Phytophthora;*
positive for *P. cinnamomi*;
positive for "*Phytophthora* spp.", or
positive for species other than *P. cinnamomi*;

from the outcomes of over 2000 observations of diagnoses of field samples presented in the VHS 1992-95 triennial report (see the respective proportions in column 2 of Table 5.3). These 2000 observations were heavily biased with field samples from the northern jarrah forest, and long experience has shown that species of *Phytophthora* other than *P. cinnamomi* are seldom retrieved from this region.

B. We also determined the relative proportions of these outcomes of diagnosis for 154 field samples from a small area in the karri forest (see the respective proportions in column 2 of Table 5.4).

For any particular field sample (i.e. per field sample), then, these proportions may be used as probabilities for the diagnosis of the sample. For the various identification methods (see Table 5.1), we determined from these probabilities the number of days it might be expected to take to reach a diagnosis for a field sample, and the amount of time (min) that operators might be expected to be actively engaged in diagnosing the sample.

Table 5.1 The various options (identification methods) available for diagnosing the *Phytophthora* status of field samples.

Outcome of Diagnosis	Identification Method			
	Short Morph	Short Morph + Isoenzyme	Isoenzyme Only	Short Morph + Fuller Morph
-ve for *Phytophthora*	+			
+ve for *P cinnamomi* or +ve for *Phytophthora* spp.	+			
+ve for *P. cinnamomi* or +ve to species other than *P. cinnamomi*[1]		+	+	+

1. *P. cinnamomi* may be identified by the short morphological method, isoenzyme and fuller morphological methods.

Costs.

The respective costs of the methods of identification were calculated by assigning a dollar value to the time operators are actively engaged in diagnosing a sample, and adding to it the cost of materials used to produce that determination.

Results

In terms of the number of days required to reach a diagnosis, the short (hyphal) morphological method of diagnosing field samples as either negative for *Phytophthora,* positive for *P. cinnamomi* or positive for *Phytophthora* spp., was most efficient requiring only 14 days, compared to the isoenzyme method where the means for species ranged from 19.08 for *P. cryptogea* to 20.20 days for *P. drechsleri*, and compared with the fuller morphological method where the mean for species ranged from 27.66 for *P. citricola* to 76.20 for *P. cryptogea* (Table 5.2).

Table 5.2 Days to diagnosis of species of *Phytophthora*, and time (minutes) operators are actively engaged in diagnosis using isoenzymes, the short morphological method and the fuller morphological method.

	Days to Diagnosis (Means)		Time (min) Operators are Engaged in Diagnosis (Means)	
	No. Isolates Tested	Mean (S.D.)	No. Isolates Tested	Mean (S.D.)
Short Morphological Method				
P. cinnamomi or *Phytophthora* spp.	122	14	6	41
Heterothallic species (4 species)				
Isoenzymes	32	19.55 (0.962)	32	61.81 (4.338)
Fuller Morphology	32	62.56 (23.66)	32	83.36 (8.630)
P. cinnamomi				
Isoenzymes	7	19.57 (0.787)	7	59.76 (1.722)
Fuller Morphology	7	47.28 (18.31)	7	85.43 (11.558)
P. cryptogea				
Isoenzymes	12	19.08 (0.793)	12	61.92 (4.130)
Fuller Morphology	12	76.20 (24.351)	12	84.19 (6.695)
P. drechsleri				
Isoenzymes	10	20.20 (1.059)	10	63.67 (5.754)
Fuller Morphology	10	68.50 (18.656)	10	78.92 (7.130)
P. nicotianae				
Isoenzymes	3	19.17 (0.577)	3	59.90 (1.524)
Fuller Morphology	3	35.33 (5.508)	3	91.00 (8.693)
Homothallic species (2 species)				
Isoenzymes	18	19.50 (0.343)	18	59.88 (2.242)
Fuller Morphology	18	32.83 (15.53)	18	70.75 (8.120)
P. citricola				
Isoenzymes	9	19.5 (0.500)	9	60.39 (2.854)
Fuller Morphology	9	27.66 (5.500)	9	68.28 (4.424)
P. megasperma				
Isoenzymes	9	19.5 (0.0)	9	59.38 (1.398)
Fuller Morphology	9	38.00 (20.555)	9	73.22 (10.332)

In terms of the amount of time that operators were actively engaged in obtaining a diagnosis, the short morphological method of diagnosing field samples as either negative for *Phytophthora*, positive for *P. cinnamomi* or positive for *Phytophthora* spp.,

was most efficient, requiring 41 min, compared to the isoenzyme method where the means for species ranged from 59.38 min for *P. megasperma* to 63.67 min for *P. drechsleri*, and compared with the fuller morphological method where the means for species ranged from 68.28 min for *P. citricola* to 91.00 min for *P. nicotianae* (Table 5.2).

Effect of species on treatments.

1. Number of days required to reach a diagnosis by the isoenzyme method.
   When the means of each of the six species of *Phytophthora* were compared it was determined that there was a significant difference ($P_{0.05}$=0.033) among them. Tukey's (1953) test recognised that the mean number of days required to diagnose the *P. drechsleri* isolates by isoenzyme analysis was significantly greater than that required to diagnose the *P. cryptogea* isolates.

2. Number of days to reach diagnosis by the fuller morphological method.
   When the means of each of the six species of *Phytophthora* were compared it was determined that there was a significant difference (P=0.000) among them. Tukey's test recognised that by the fuller morphological method it took significantly more days to reach an identity for *P. cinnamomi* isolates than it did for *P. citricola* isolates, and that it took significantly more days to reach an identity for *P. cryptogea* and *P. drechsleri* isolates than it did for *P. citricola* and *P. megasperma* isolates.

3. Time (min) operators are actively engaged in diagnosis by the isoenzyme method.
   When the means of each of the six species of *Phytophthora* were compared it was determined that there was no significant difference (P=0.13) among them.

4. Time (min) operators are engaged in diagnosis by the fuller morphological method.
   When the means of each of the six species of *Phytophthora* were compared it was determined that there was a significant difference (P=0.000) among them. Tukey's test determined that operators were actively engaged in diagnosing *P.*

cinnamomi isolates for significantly more time than they were in diagnosing *P. citricola* isolates, and they required significantly more time to identify *P. nicotianae* and *P. cryptogea* isolates than to identify *P. citricola* and *P. megasperma* isolates.

Although it took longer (days) to diagnose *P. drechsleri* isolates by the isoenzyme method than it did *P. cryptogea* isolates, from observations 1 and 3 above it was concluded that efficiency in terms of the number of days to diagnosis, and time (min) operators are actively engaged in diagnosis by the isoenzyme method, was not affected by the 6 species making up the 50 subject isolates of *Phytophthora* used in this study, i.e. as far as the isoenzyme method is concerned the 6 species making up the 50 isolates represent one population.

Observations 2 and 4, however, indicated that species did affect both the number of days to diagnosis, and the time (min) operators were actively engaged in diagnosis by the fuller morphological method such that 2 populations, i.e. heterothallic species and homothallic species, were recognised (see Table 5.2). So the fuller morphological data of heterothallic species were pooled, and that for homothallic species were pooled and the two sets compared.

When the mean of the pooled data for the fuller morphological method for heterothallic species was compared with that for homothallic species it was determined that there was a significant difference between them, both in the number of days it took to obtain diagnoses (P=0.000), and in the time operators were actively engaged in diagnosis (P=0.000). Consequently the sample of homothallic isolates was recognised as being discrete from the sample of heterothallic isolates, and these were considered to be separate treatments in subsequent comparisons.

<u>Comparison between the isoenzyme method of identification and morphological methods</u>.

1. <u>Number of days required to reach a diagnosis</u>.
   When the mean number of days required to identify homothallic isolates by the isoenzyme method was compared with that taken by the fuller morphological method, they were found to be significantly different (P=0.000). It took

significantly fewer days to reach a diagnosis for heterothallic isolates by the isoenzyme method (19.55 days, see Table 5.2), than it did for the same isolates by the fuller morphological method (62.56 days). It also took significantly (P=0.000) fewer days to reach a diagnosis for the homothallic isolates by the isoenzyme method (19.5 days, see Table 5.2), than it did for the same isolates by the fuller morphological method (32.83 days).

Irrespective of whether unknown isolates were heterothallic or homothallic we found that by the isoenzyme method of identification, diagnoses for them were reached considerably sooner than by the fuller morphological method. For heterothallic isolates the number of days to diagnosis by the fuller morphological method was 220% higher than for the isoenzyme method, and 68.34% higher for homothallic isolates.

Identification by the isoenzyme method, however, extended the number of days to diagnosis to *P. cinnamomi* or "*Phytophthora* spp." beyond that of the short (hyphal) morphological method by 39.5% for heterothallic and homothallic isolates alike, and identification by the fuller morphological method further extended the number of days by 346.86% for heterothallic isolates and 134.5% for homothallic isolates.

2. Time (min) operators are actively engaged in diagnosis.
   When the mean amount of time (min) that operators were actively engaged by the isoenzyme method in determining identifications for heterothallic isolates was compared with that required to obtain identifications by the fuller morphological method, they were found to be significantly different (P=0.000). Operators were engaged for significantly less time in diagnosing heterothallic isolates by the isoenzyme method (61.81 min) than they were when using the fuller morphological method (83.36 min, see Table 5.2). It also took them significantly (P=0.000) less time to identify homothallic isolates by the isoenzyme method (59.88 min) than it took them to identify the same isolates by the fuller morphological method (70.75 min, see Table 5.2).

Irrespective of whether unknown isolates were heterothallic or homothallic we found that by the isoenzyme method of identification, diagnoses for them engaged operators for considerably less time than the fuller morphological method. Compared to the

isoenzyme method, diagnosis by the fuller morphological method occupied operators for 35.59% more time for heterothallic isolates and 18.15% more time for homothallic isolates.

Identification by the isoenzyme method, however, required that operators be engaged in diagnosis to *P. cinnamomi* or "*Phytophthora* spp." beyond that of the short (hyphal) morphological method by 49.07% for homothallic and heterothallic isolates alike, and identification by the fuller morphological method extended this time by 103.32% for heterothallic isolates and 72.56% for homothallic isolates.

Case studies: efficiency of the respective methods as determined on a per field sample basis.

The following comparisons relate to the outcomes of over 2000 diagnoses carried out by the VHS of CALM in the period 1992-95 on field samples that were taken mainly from the northern jarrah forest, and 154 diagnoses of field samples from the karri forest. These two field sample sets were chosen because between them there was a large difference in both the proportion of samples that were diagnosed as positive for *P. cinnamomi,* and the proportion of samples that were diagnosed as positive for '*Phytophthora* spp.'(see column 2 of Tables 5.3 and 5.4). We were interested in determining if these differences affected the relative efficiencies of the respective methods of identification.

1. In terms of days to diagnosis.

   A. On a "per field sample" basis for the 2000 samples assessed by the VHS, the short morphology method of diagnosis to negative for *Phytophthora* (probability of outcome 0.649), positive for *P. cinnamomi* (0.286) or positive for "*Phytophthora* spp." (0.065) was most efficient in terms of days to diagnosis requiring only 14 days (Table 5.3). If managers were to adopt this approach to diagnosis of field samples that come largely from the northern jarrah forest, then an estimated 6.5% of them would be positive for *Phytophthora,* but the identity of these Phytophthoras would not be determined.

The second most efficient approach to diagnosis for field samples from the northern jarrah forest was the short morphology plus isoenzyme method (14.36 days, see Table 5.3). By this approach the proportion of samples that were negative for *Phytophthora* or positive for *P. cinnamomi* would have been identified by the short morphological method, and Phytophthoras from that proportion of samples having species other than *P. cinnamomi* would have been identified to species by the isoenzyme method (see Table 5.2).

The short morphology plus fuller morphology approach (15.758 days to diagnosis) would be marginally more efficient than if the isoenzyme method (15.942 days) was used exclusively to identify all Phytophthoras, including *P. cinnamomi,* from northern jarrah forest field samples. The fuller morphological approach (29.646 days) would be the least efficient approach to diagnosis (Table 5.3).

B. As might be expected the short morphology method of diagnosis was also the most efficient method of assessing the 154 field samples from the karri forest (14 days, see Table 5.4), however should managers adopt this approach of assessing field samples from areas like the karri forest, then 42.9% of them might be expected to be positive for *Phytophthora,* but the identity of these Phytophthoras would not be determined.

The next most efficient approach to diagnosis for karri forest field samples was the short morphology plus isoenzyme approach (16.36 days, see Table 5.4), and this was followed by the isoenzyme only approach (16.65 days, see Table 5.4), in which case all Phytophthoras, including *P. cinnamomi,* are identified by the isoenzyme method. The short morphology plus fuller morphology approach (24.81 days), and the fuller morphological approach (29.646 days) were the least efficient approaches to diagnosis (Table 5.4).

If it is required that identification of *Phytophthora* species be taken to species complex or taxon level, then for field samples it may be expected that the days to diagnosis by the short morphology plus isoenzyme method would take 2.57% longer than the short

morphology method of diagnosis to either negative for *Phytophthora*, positive for *P. cinnamomi,* or positive for *Phytophthora* spp. for samples from the northern jarrah forest, and 16.84% longer for samples from the karri forest (Table 5.5). The short plus fuller morphological method would take 9.74% longer than the short morphology plus isoenzyme method for northern jarrah forest samples, and 51.17% longer for samples from the karri forest (Table 5.5).

2. In terms of time operators are actively engaged in diagnosis.
   Efficiency may also be assessed in terms of the time in which operators may be expected to be actively engaged in the activity of diagnosing field samples.

   A. On a per field sample basis for the 2000 samples assessed by the VHS, the shorter morphological approach would be the most efficient approach to diagnosis requiring 41 min, and this was followed by the short morphology plus isoenzyme (42.31 min) and short morphology plus fuller morphology (43.16 min) approaches (Table 5.3). The isoenzyme only (48.06 min) and fuller morphological (55.28 min) approaches were least efficient (Table 5.3).

   B. For karri forest samples the shorter morphological approach to diagnosis was once again most efficient (41 min, Table 5.4), and this was followed by the short morphology plus isoenzyme (49.57 min) and isoenzyme only (50.61 min) approaches (Table 5.3). The short morphology plus fuller morphology (54.86 min) and fuller morphology (57.06 min) approaches were least efficient (Table 5.4).

If it is required that identification of *Phytophthora* species be taken to species complex or taxon level, then the time operators may be expected to be actively engaged in diagnosis by the short morphological plus isoenzyme approach would be 3.19% longer than the short morphology method of diagnosis to either negative for *Phytophthora*, positive for *P. cinnamomi* or positive for *Phytophthora* spp. for field samples from the

Table 5.3 Determination of the number of days to diagnosis, and time operators are actively engaged in diagnosis, on a per field sample basis by diagnostic approach for 2000+ samples (predominantly northern jarrah forest) processed by the VHS of CALM in the period 1992-1995.

Field Soil/Plant Tissue Samples Diagnosed as:	Proportion of Samples		Estimate of Days to Diagnosis by:					Estimate of the Time (min) Operators are Actively Engaged				
			Short Morph	Short Morph+ Isoenzyme[1]	Isoenzyme Only	Short Morph+ Fuller Morph[2]	Fuller Morph	Short Morph	Short Morph+ Isoenzyme[1]	Isoenzyme Only	Short Morph+ Fuller Morph[2]	Fuller Morph
Negative for *Phytophthora*	0.649		9.086	9.086	9.086	9.086	9.086	26.609	26.609	26.609	26.609	26.609
Positive for *P. cinnamomi*	0.286		4.004	4.004	5.586	4.004	11.105	11.726	11.726	17.479	11.726	23.841
Phytophthora spp.	0.065		0.91					2.665				
P. nicotianae		0.001		0.020	0.020	0.062	0.062		0.061	0.061	0.083	0.083
P. cryptogea		0.015		0.293	0.293	0.938	0.938		0.917	0.917	1.250	1.250
P. drechsleri		0.002		0.039	0.039	0.125	0.125		0.122	0.122	0.167	0.167
P. citricola		0.022		0.430	0.430	0.722	0.722		1.344	1.344	1.553	1.556
P. megasperma		0.025		0.488	0.488	0.821	0.821		1.528	1.528	1.769	1.769
Totals	1.0	0.065	14.0	14.360	15.942	15.758	29.646	41.0	42.307	48.060	43.16	55.275

1 Samples that are negative for *Phytophthora* and positive for *P. cinnamomi* are identified by the short morphology method, and species other than *P. cinnamomi* are identified by the isoenzyme method.

2 Samples that are negative for *Phytophthora* and positive for *P. cinnamomi* are identified by the short morphology method, and species other than *P. cinnamomi* are identified by the fuller morphological method.

Table 5.4 Determination of the number of days to diagnosis, and time operators are actively engaged in diagnosis, on a per field sample basis by diagnostic approach for 154 samples taken from a small area in the karri forest.

Field Soil/Plant Tissue Samples Diagnosed as:	Proportion of Samples		Estimate of Days to Diagnosis by:					Estimate of the Time (min) Operators are Actively Engaged				
			Short Morph	Short Morph+ Isoenzyme[1]	Isoenzyme Only	Short Morph+ Fuller Morph[2]	Fuller Morph	Short Morph	Short Morph+ Isoenzyme[1]	Isoenzyme Only	Short Morph+ Fuller Morph[2]	Fuller Morph
Negative for *Phytophthora*	0.519		7.266	7.266	7.266	7.266	7.266	21.279	21.279	21.279	21.276	21.276
Positive for *P. cinnamomi*	0.052		0.728	0.728	1.016	0.728	3.253	2.132	2.132	3.178	2.132	4.335
Phytophthora spp.	0.429		6.006					17.589				
P. nicotianae		0.000		0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000
P. cryptogea		0.011		0.215	0.215	0.688	0.688		0.672	0.672	0.917	0.917
P. drechsleri		0.082		1.606	1.606	5.130	5.130		5.011	5.011	6.836	6.836
P. citricola		0.335		6.543	6.543	10.998	10.998		20.474	20.474	23.701	23.701
P. megasperma		0.000		0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000
Totals	1.0	0.428	14..0	16.358	16.646	24.81	27.335	41.0	49.568	50.614	54.862	57.065

1 Samples that are negative for *Phytophthora* and positive for *P. cinnamomi* are identified by the short morphology method, and species other than *P. cinnamomi* are identified by the isoenzyme method.

2 Samples that are negative for *Phytophthora* and positive for *P. cinnamomi* are identified by the short morphology method, and species other than *P. cinnamomi* are identified by the fuller morphological method.

northern jarrah forest (Table 5.5), and 20.9% longer for field samples from the karri forest. For field samples from the northern jarrah forest operators would be engaged in diagnosis by the short morphological plus fuller morphological method for 2.02% longer than the short morphology plus isoenzyme method, and for karri forest samples for 10.68% longer (Table 5.5).

Table 5.5 Percent that alternative diagnostic approaches increase the days to diagnosis and the time operators are actively engaged relative to the short morphological and short plus isoenzyme approaches, on a per field sample basis.

	% Increase per Sample on Short Morphology Method	% Increase per Sample on Short + Isoenzymes Method
Days to Diagnosis		
Short + Isoenzymes		
1. Northern jarrah forest[1]	2.57	
2. Karri forest[2]	16.84	
Short + Fuller Morphology		
1. Northern jarrah forest	12.56	9.74
2. Karri forest	77.21	51.17
Isoenzymes Only		
1. Northern jarrah forest	13.87	11.02
2. Karri forest	18.90	1.76
Fuller Morphology Only		
1. Northern jarrah forest	111.76	106.45
2. Karri forest	95.25	67.10
Time Operators are Engaged in Diagnosis		
Short + Isoenzymes		
1. Northern jarrah forest	3.19	
2. Karri forest	20.90	
Short + Fuller Morphology		
1. Northern jarrah forest	5.27	2.02
2. Karri forest	33.81	10.68
Isoenzymes Only		
1. Northern jarrah forest	17.22	13.60
2. Karri forest	23.45	2.11
Fuller Morphology Only		
1. Northern jarrah forest	34.82	30.65
2. Karri forest	39.18	15.12

1. See Table 5.3
2. See Table 5.4

Costs

The material costs of identifying isolates of *Phytophthora* by the isoenzyme method ($5.54) was more expensive than the short morphological ($1.87) or full morphological ($2.85) approaches (Table 5.6).

Table 5.6 The cost ($) of materials to identify *Phytophthora* spp. by each of three diagnostic approaches: Short morphology to *P. cinnamomi* or to *Phytophthora* spp. (genus only); comprehensive 'fuller' morphology to species; and isoenzymes to species and electromorphs.

Materials	+/- *Phytophthora* spp. (Short Morphology)	Full Morph. Approach	Isoenzyme Approach
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	0.54
CAGE Plate			0.21
Stains and Buffers			1.54
Liquid nitrogen			0.70
Incidentals			0.65
Totals per Diagnosis	1.87	2.85	5.54

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

When labour and material costs are considered, the short morphological approach ($14.42) would be most cost effective irrespective of where the samples were taken from. For the northern jarrah forest and the karri forest samples, the short morphological plus isoenzyme approach was the next most efficient method ($15.07 and $18.62 respectively, see Table 5.7).

Diagnosis of isolates of *Phytophthora,* other than *P. cinnamomi,* to species, species complexes, or taxa by the short morphological plus isoenzyme approach increased costs for northern jarrah forest samples by 4.51% compared to the short morphological method alone, and increased karri forest samples by 29.13%. Diagnosis by the short morphological plus fuller morphological approach increased costs by 5.06% for the northern jarrah forest samples, and 32.38% for karri forest samples.

Table 5.7 Computation of total costs to diagnosis of a soil/plant tissue sample by the various diagnostic approaches.

Diagnostic Approach	Operation Time (mins)		$ cost per minute		Admin. Loading		Materials	Total
Short Morphological								
1. Northern jarrah forest[1]	41	x	0.232	x	1.32	+	1.87	14.42
2. Karri forest[2]	41	x	0.232	x	1.32	+	1.87	14.42
Short Morphological + Isoenzymes								
1. Northern jarrah forest	42.31	x	0.232	x	1.32	+	2.11	15.07
2. Karri forest	49.57	x	0.232	x	1.32	+	3.44	18.62
Isoenzymes Only								
1. Northern jarrah forest	48.06	x	0.232	x	1.32	+	3.15	17.79
2. Karri forest	50.61	x	0.232	x	1.32	+	3.64	19.14
Short + Fuller Morphological								
1. Northern jarrah forest	43.16	x	0.232	x	1.32	+	1.93	15.15
2. Karri forest	54.86	x	0.232	x	1.32	+	2.29	19.09
Fuller Morphology Only								
1. Northern jarrah forest	55.27	x	0.232	x	1.32	+	2.21	19.14
2. Karri forest	57.06	x	0.232	x	1.32	+	2.60	20.07

1. See Table 5.3
2. See Table 5.4

Discussion

Irrespective of whether unknown isolates were heterothallic or homothallic, their identity was diagnosed sooner by the isoenzyme method than by the fuller morphological method, and the isoenzyme method engaged operators in diagnosis for considerably less time.

Because they have not been proven otherwise, all *Phytophthora* species are considered to be a threat to Western Australian plant communities, and the same management approach is adopted for all. Managers therefore may not require that diagnosis be

taken beyond presence or absence of *P. cinnamomi* or "*Phytophthora* spp." (collectively) in their samples. This being the case, the shorter hyphal morphology approach to diagnosing field samples would be the most efficient method, both in terms of the number of days it would take to assess samples and the time (min) operators are actively engaged in diagnosis, and it is also the most cost-effective option irrespective of where the samples come from. Notwithstanding this, the shorter morphological method may not be the most reliable approach for distinguishing some Phytophthoras from Pythiums.

Research for management purposes therefore may require that diagnosis be taken to specific or taxon level. Chevis and Stukely (1982), for example, associated pine mortalities in the Donnybrook Sunkland plantations with species of *Phytophthora* other than *P. cinnamomi,* and plant deaths in native vegetation on the Northern Sand Plains and in the Fitzgerald River National Park have more often been associated with species of *Phytophthora* other than *P. cinnamomi* than with *P. cinnamomi* itself.

For taxa in the species complexes of *Phytophthora* the fuller morphological approach to diagnosis was not as accurate as the isoenzyme method for diagnosis to taxa (see Service 4), particularly when distinguishing between species in the homothallic complexes (e.g. *P. citricola* and *P. megasperma*), or between species in the heterothallic complexes (e.g. *P. cryptogea* and *P. drechsleri*). Consequently it was recommended that managers adopt the isoenzyme method for identifying species of *Phytophthora,* other than *P. cinnamomi,* in preference to the fuller morphological method (see Section 4).

The above recommendation was made with no consideration for either the efficiency of, or costs incurred by, the respective methods. In this section we found that when determining the identity of isolates of *Phytophthora* both efficiency, i.e. in the number of days required to reach a diagnosis and the amount of time operators are actively engaged in diagnosis, and the cost of diagnosing field samples were dependent upon:

1. the expected outcome of diagnosis; and
2. the diagnostic approach used.

If the short morphology plus isoenzyme approach is used, it may be expected that field samples from the northern jarrah forest would require 14.36 days to diagnosis (Table 5.3), engage operators for 42.31 min (Table 5.3), and cost $15.07 (Table 5.7), compared to 16.36 days (Table 5.4), 49.57 min (Table 5.4), and $18.62 (Table 5.7) for samples from the karri forest (Table 5.4). Field samples from the karri forest are expected to take longer to diagnose, to engage operators in diagnosis for more time, and therefore cost more to diagnose because the probability that they will be positive for a species of *Phytophthora* other than *P. cinnamomi* is high (0.429, see Table 5.4) relative to samples from the northern jarrah forest (0.065, see Table 5.3). Diagnosis of these samples is more likely to involve isoenzyme analysis which affects the outcomes of efficiency and cost.

For field samples from the northern jarrah forest there was little difference in the cost per sample of adopting the isoenzyme method ($15.07 per sample) for diagnosing species other than *P. cinnamomi,* relative to the fuller morphological approach ($15.15 per sample), and greatest benefits of adopting the isoenzyme method over the fuller morphological approach included: considerably more accurate diagnosis, and increased efficiency in that, on average, samples may be expected to be diagnosed 9.74% faster from the time of receipt.

For field samples from the karri forest there was also little difference in the cost per sample of adopting the short morphological plus isoenzyme method ($18.62 per sample), relative to the short morphological plus fuller morphological approach ($19.09 per sample). However if the short morphological plus isoenzyme approach is adopted in preference to the short morphological plus fuller morphological approach, then substantial benefits may be expected in that samples would be diagnosed 51.17% faster and engage operators for 10.68% less time.

The influence that the expected outcome of diagnosis has on efficiency and cost is of particular relevance to mining companies operating in the sand plains north of Perth. Hart *et al.* (1991) found that relative to the proportion of field sites in the Northern Sand Plain that were positive for *P. cinnamomi* (0.12) the proportion of sites that were positive for other species of *Phytophthora* was very high (0.55). These respective proportions are comparable to those found for the 154 field samples collected from the

karri forest. The efficiency and cost of diagnosing samples from the Northern Sand Plain by the respective diagnostic approaches, therefore, is more likely to approach that found for karri forest samples than for northern jarrah forest samples, in which case the short morphological and isoenzyme approach to diagnosis was substantially more efficient than the short morphological plus fuller morphological approach.

Recommendations

5.1 Mining companies wishing to have field samples from their tenements in the Northern Sand Plain diagnosed for species of *Phytophthora* are advised to use the short morphological plus isoenzyme approach because it will afford them greater accuracy and efficiency at no extra cost.

5.2 Where long experience has shown that the proportions of the isolates of the various species of *Phytophthora* were dominated by species other than *P. cinnamomi*, e.g. Northern Sand Plains and Fitzgerald River National Park, all isolates other than *P. cinnamomi* should be directly routed to isoenzyme analysis.

Service 6. Test new procedures for directly assaying bait tissues for *Phytophthora* by isoenzyme analysis, and compare time and cost with the present method.

Introduction

A standard baiting procedure for *Phytophthora*, and the one employed by the VHS of CALM, involves plating onto a selective agar plate lesioned *Lupinus angustifolius* and *Eucalyptus sieberi* baits that have been exposed to soil/plant tissue samples suspected of being infested with the fungus. It usually takes 14 days to determine whether a sample is positive or negative for *Phytophthora* (see Services 5 and 7.2) by this method. Goodwin *et al.* (1995) found that pieces of potato (size not given) cut from *Phytophthora infestans* infected tubers usually produced enough sporangia after 1 to 2 days of incubation to give sufficient Glucose-6-phosphate isomerase (GPI) signal on CAGE plates to discriminate between *P. infestans* genotypes. They subsequently suggested that the method of directly assaying infected potato tubers by CAGE may be a useful diagnostic tool in field situations where rapid determination of *P. infestans* genotypes could aid disease management strategies.

The objectives of this research were to:

- determine whether or not the direct assay of *L. angustifolius* and *E. sieberi* bait tissue for *Phytophthora* isoenzymes is possible; and if it is possible, to:
- determine whether or not it would be an efficient and cost-effective alternative to the 'plate and wait' technique currently employed by the VHS of CALM.

Procedure

Into each of 4 plastic baiting dishes was added *circa* 300 g of *Phytophthora cinnamomi* infested soil. Into each of 2 of the dishes was added 300 mL of distilled water, and into each of the other 2 dishes was added 300 mL of distilled water to which 15 mg of Hymexazol™, 30 mg of Ampicillin™, and 0.3 mL of Nystatin™ had been added. Hymexazol™ inhibits Pythiums, and the other additives are antibacterial. Twenty *E. sieberi* cotyledons and ten *L. angustifolius* radicles were then floated on the liquid in each dish.

After three days of exposure to the *P. cinnamomi*-infested soil, four *L. angustifolius* and four *E. sieberi* baits were removed from each dish and transferred to V8 juice and pea broth in 1 oz McCartney bottles. The baits were incubated for two days, then all of the material (bait tissue plus pathogens from the soil) was assayed by the CAGE method (see Section 1) for the Glucose-6-Phosphate Isomerase (GPI), Isocitrate dehydrogenase (IDH), Lactate dehydrogenase (LDH), and Malate dehydrogenase (MDH) isoenzymes of *P. cinnamomi.*

Results

Direct assay by CAGE of *P. cinnamomi*-infested *L. angustifolius* or *E. sieberi* baits from dishes to which distilled water only was added, and dishes which included the additives Hymexazol etc., gave intense signal and excellent resolution of GPI, IDH, LDH, and MDH isoenzymes (data not presented). These isoenzymes, from the assayed baits, produced by the four test stains matched those of a reference isolate of *P. cinnamomi* from M. Coffey's Californian collection. Faint bands, additional to the *P. cinnamomi* isoenzyme, were also visible in some of the samples. These may be attributed to contamination of the *P. cinnamomi* isoenzymes with those from the bait tissue or from another organism (e.g. other fungi or bacteria).

Bait samples from the dishes to which only water was added gave the same intensity and resolution of staining for *P. cinnamomi* isoenzymes as samples from dishes to which Hymexazol™, Ampicillin™, and Nystatin™ had been added. The additives (Hymexazol, etc.) did not reduce the number of isoenzyme bands additional to those of *P. cinnamomi* (results not presented), and the additives did not appear to inhibit infection of the baits by the zoospores of *P. cinnamomi.*

Discussion

P. cinnamomi-infected *L. angustifolius* and *E. sieberi* baits produced the same intense and highly resolved banding patterns when directly assayed for GPI, IDH, LDH and MDH isoenzymes by CAGE, as those patterns produced by pure cultures of *P. cinnamomi.*

Field soil/plant tissue samples contain a variety of plant pathogens (fungi and bacteria) which infect *L. angustifolius* and *E. sieberi* baits and produce lesions similar to those of Phytophthoras. It is well known that fungicides and antibiotics, such as those tested here (Hymexazol™, etc.), will not effectively inhibit all of them. Although 65% of field samples may be expected to be negative for *Phytophthora* (Service 5), in most cases the baits used to test these negative samples will develop lesions similar to those produced by Phytophthoras. In Service 7.2 it was established that as many as 28 baits require testing to be 95% confident of recovering *Phytophthora* if it is present.

In order to maintain a high level of confidence of detecting Phytophthoras therefore, either 28 baits need to be examined on selective agar plates, or 28 baits need to be directly assayed by CAGE for GPI, IDH, LDH, and MDH. In cost of materials alone, detection by the plating technique would cost $2.53, which is considerably less than the $16.69 it would cost by the isoenzyme method. Operators would be actively engaged for more time (48.06 vs 41.0 min, see Service 5) using the isoenzyme method.

However, relative to the number of days it would take to diagnose field samples as negative or positive for *Phytophthora* by the 'plating and waiting' method (14 days, see Service 5), assessment by directly assaying baits from them for *Phytophthora* isoenzymes would be faster (*circa* 10 days).

Finally, the direct assay technique involves the destruction of the bait containing the *Phytophthora* isolate. An interesting isolate, as identified by its isoenzymes, may not be subsequently retrieved from the field sample from which it was originally detected.

Recommendations

6.1 Managers are advised not to adopt the direct assay of baits by CAGE technique for detecting species of *Phytophthora* in soil/plant tissue samples.

Service 7. To research modifications to *Phytophthora* baiting techniques, and compare by cost and time the efficiency of retrieving *Phytophthora* by traditional and modified methods.

7.1 Determination of the number of *Eucalyptus sieberi* and *Lupinus angustifolius* baits that require testing to be 95% confident of retrieving *Phytophthora* from soil/plant tissue samples.

Introduction

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. The 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 baiting procedure used here was recommended to us by F.D. Podger (pers. com.), and is a modified version of that practised by the VHS :

1. *Circa* 300 g of soil/plant tissue sample was distributed into each of two plastic baiting dishes (replicates).
2. *Circa* 300 mL of distilled water was added to the baiting dishes.
3. Thirty to forty W.A. blue lupin baits and *circa* twenty *Eucalyptus sieberi* cotyledons were then added to the baiting dishes.
4. On the third to fifth day following baiting a number of baits with lesions (column 2 of Table 7.1: 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).

The proportion of lesioned baits (from a baiting dish) that were positive for *Phytophthora* was used to determine the percent confidence of detecting *Phytophthora* (given the number of baits tested) from binomial probabilities, and was also used to determine the number of baits that require testing to be 95% confident of detecting the fungus.

Results

In samples containing two *Phytophthora* species, one species produced far fewer colonies than the other (column 3 of Table 7.1).

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 7.1), 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 7.1) 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 7.1) 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

When screening baits for *Phytophthora,* these results indicate that a large number (n=28) of baits having lesions need to be tested 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 differences in the size of the baits used (within lupin, within Eucalypt, and between lupin and Eucalypt) and variation in lesion sizes, these analyses should be taken to be indicative and not definitive.

Recommendations

7.1 40+ lupin and Eucalypt baits should be added to each baiting tray.

7.2 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

examined for baits having lesions on day 3, and each day thereafter to day 10.

7.3 For each baiting dish the entire lesions of 28 or more baits (where available), should be plated on to selective agar (i.e. 2 selective plates with 14 baits per plate).

Table 7.1. 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 number of baits requiring plating to be 95% confident of retrieving the fungus (column 5).

Sample No.		No. of Baits Plated	% Colonies Per Bait	% Confidence of Detection	No. Baits for 95% Confidence
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	99.99	1
9		24	16.6	98.3	17
10		29	79	99.99	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	99.99	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

7.2 Recovery of species of *Phytophthora* from baits harvested on two occasions (five or six days apart) from the same soil samples.

Introduction

A standard procedure for recovering *Phytophthora* from soil/plant tissue samples is to 'bait' the samples with young, rapidly expanding live plant material 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 Service 7.1 it was reported that large numbers of baits (up to 28) having lesions require testing to attain a 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 F.D. Podger (pers. com.) and is a modified version of that practised by the VHS of CALM:

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

2. *Circa* 300 mL 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 (numbers as above in item 3) 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 was treated as in steps 4 and 5 above.

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

Results and Discussion

Six biological species* of *Phytophthora* were recovered from the 27 samples tested in this study (Table 7.2). From column four of Table 7.2 it is seen that for each species, except the two biological species of *P. cryptogea*, there were soil samples which contained Phytophthoras, but which were not identified as such in the first harvest of baits. The percent increase in recovery of the various species of *Phytophthora* ranged from 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 7.2).

Table 7.2. Recovery of species of *Phytophthora* from 27 soil samples from which baits were harvested on day 4 or 5 and again on day 10.

Species	No. samples from which the same species of Phyt. was recovered at both harvests	No. samples +ve for a Phyt. 1st harvest, and -ve for that Phyt. 2nd harvest	No. samples -ve 1st harvest, and +ve for 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

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

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 to the first harvest. So for the one-tailed test where $\alpha=0.10$ and $n=6$, $T_o=18$. In testing the null hypothesis $T^+=18$, which is the same as the critical value (T_o). Since $T_o=18=T^+=18$, it is 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 7.3), it was found that on 21 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 substantially the recovery rates of most species found in these soil samples.

Table 7.3. 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

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 28 baits from any one harvest may require testing to attain the 95% confidence level of detecting species of *Phytophthora* in that harvest (see Section 7.1). 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 advantages for baits at different times during the period a sample is being baited. Repeating the experiment and assessing 28 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* 22 min 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. In addition to this the cost of consumables would increase by almost 100%.

Recommendations

7.4 Management should identify the species of *Phytophthora* recovered from baits exposed to field samples on day 1 to day 5, and those from a second set of baits exposed to the same field samples on day 6 to day 10.

7.5 Management should adopt a 15 day programme for double harvesting baits from samples (Table 7.4). This programme maximises the use of down-time on weekends, and according to it 13 to 15 days are required from when a sample is received until it has been given a complete assessment.

Table 7.4. Fifteen day double harvest programme for recovering species of *Phytophthora* from soil / plant tissue samples in two batches, A and B.

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	Receive samples A1 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	Receive samples B1 Bait B1 samples
8	Sat		
9	Sun		
10	Mon	Second (final) harvest of baits for Batch A.	
11	Tues		First harvest of baits for Batch B.
12	Wed	Examine baits harvested day 10	First harvest of baits ...

7.3 Effect of a second baiting on recovery of species of *Phytophthora* from soil.

Introduction

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 of *Phytophthora* (where a substantial increase would be 7.5% or more) 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 F.D. Podger (pers. com.), and has been described previously in Section 7.2. 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 isoenzymically.

Results and Discussion

Seven biological species of *Phytophthora* were recovered from the 347 soil samples tested (Table 7.5). The rate of recovery of these species in the first baiting ranged from 0.29% for *P. citricola* 1 to 9.22% for *P. citricola* 2. Recoveries of the two biological species of *P. cryptogea* were both low (0.58, 0.86%). 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 7.5).

Table 7.5. 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)	100.0
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

From column three of Table 7.5 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 did not increase the number of soil samples found to be positive for *P. cryptogea* 1 and *P. cryptogea* 2, both of which were recovered in low frequencies in the first baiting. It did however increase by 1 the number of samples found to be positive for *P. cinnamomi* and *P. citricola* 1; and up to 5 for *P. citricola* 2 and *P. drechsleri,* both of which were recovered in high frequencies in the first baiting (Column 1, Table 7.5).

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 of species of *Phytophthora* from soil samples relative to the first baiting (where a substantial increase would be 7.5% or more). 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 baiting.

Double baiting soil samples identified more of them as being positive for *Phytophthora,* albeit an insubstantial amount, however the benefit of reducing the frequency of 'false negatives' 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). Double-baited samples would require 28 days assessment before they would be deemed to be negative. In addition to the delay to diagnosis, double baiting would increase by *circa* 65% the time (41 minutes for single baiting compared to *circa* 68 minutes for double baiting) operators are required to spend on a sample.

Because the important issue is to know whether or not the source of the sample is positive for *Phytophthora*, it may be that increasing the intensity of sampling at the source is a more efficient approach to assessment than increasing the amount of resources invested in diagnosing individual samples. The issue of sampling intensity at the source is taken up in Service 8.

As might be expected, species that were recovered in highest frequencies in the first baiting seemed to be recovered in highest frequencies in the second baiting as well (Table 7.5). When the rates of recovering the various species of *Phytophthora* in the second baiting (i.e. the values in column 3 of Table 7.5) were regressed against the rates of recovering them in the first baiting, it was found that there was a positive (r^2 = 0.508) and significant (P = 0.044) linear 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. The expression:

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

describes this linear relationship, such that the expected number (y) of samples that would be positive on rebaiting those samples that were negative for *Phytophthora* in the first baiting, may be derived from the observed number of samples (x) that were positive for *Phytophthora* in the first baiting.

The predictive value of expression 1, however, is not expected to extend to situations with *circa* 10% or more of samples positive for a species of *Phytophthora* in the first baiting, this being about the upper limit in our study. To increase the utility of the predictor, sample sets with more than 20% of samples positive for Phytophthoras would need to 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:

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

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.

Double baiting reduced the number of samples that were falsely identified as negative for *Phytophthora*. From this it may be inferred that when samples are single baited, cases would arise when sites are deemed to be free of *Phytophthora* when they are in fact not free of *Phytophthora*. While double baiting would reduce the number of *Phytophthora* infested sites that are deemed to be *Phytophthora* free, this would only be by an insubstantial amount, and the added cost (of double baiting) to management would be quite substantial (*circa* a 65% increase in costs per sample). So the next step for management is to determine which approach, i.e. double baiting samples from sites, or single baiting a larger number of samples from sites, most effectively reduces the number of sites that are deemed to be *Phytophthora* free when *Phytophthora* is actually present, and which approach is most cost effective. The issue of the number of times a site should be sampled is taken up in the next section.

Recommendations

7.6 Determine which of the two approaches, i.e. double baiting samples from sites, or single baiting a larger number of samples from sites, most effectively reduces the number of sites that are deemed to be negative for *Phytophthora* when they are in fact infested, and which is most cost effective.

7.4 Effect of pH on the competitive infective abilities of *P. cinnamomi* and CIT 3 *P. citricola* for *Eucalyptus sieberi* baits.

Introduction

Zentmyer and Marshall (1959) (cited in Zentmyer 1980) found that the production of sporangia by *P. cinnamomi* was not reduced in non-sterile soil extract in which the pH had been artificially reduced from pH 7.4 to 4.5. Chee and Newhook (1965) (cited in Zentmyer 1980) reported good sporangial production in *P. cinnamomi* over a pH range from 4.0 to 7.0 in non-sterile soil extract adjusted with HCl and NaOH. At pH 3 only one of seventeen isolates tested by Chee and Newhook (1965) (cited in Zentmyer 1980) produced sporangia, and at pH 8.0, six of them formed sporangia. Pegg (1977) (cited in Zentmyer 1980) found that at pH 3.8 sporangial production by *P. cinnamomi* was considerably reduced.

A small number (5) of field samples from the south-west of Western Australia were infested with two species of *Phytophthora*. In all of these samples it was found that one of the species was recovered in much higher frequency than the other (see Service 7.1). The common species was recovered 4 times (on average) more often than the uncommon species. In Service 7.2 it is reported that *P. citricola* was recovered in the first harvest of lesioned *E. sieberi* cotyledon baits tested after five days exposure to a soil sample, and that *P. cinnamomi* was recovered in a sample of baits tested after ten days exposure to the same soil sample. Using hyphal morphology to distinguish between colonies of *Phytophthora palmivora* and *P. parasitica,* Zitko and Timmer (1994) compared the abilities of these Phytophthoras in mixed culture to infect fibrous roots of citrus. In combination with *P. parasitica* the proportion of roots from which *P. palmivora* was retrieved was usually near that 'expected' (that being the proportion of roots infected when the fungus was tested on its own), while fewer roots than expected yielded *P. parasitica*. Zitko and Timmer (1994) compared the competitive abilities of these pathogens under a range of screenhouse conditions, but pH was not one of the conditions tested for.

Using allozymes to discriminate between two morphologically indistinguishable CIT 3 *P. citricola* cultures Carstairs and Newcombe (1996) compared the abilities of these

pathogens in mixed culture to infect *Eucalyptus sieberi* cotyledons. In combination at pH 5.7, both Phytophthoras infected *E. sieberi* baits in near expected proportions, although significantly fewer baits were infected by each of the pathogens than when they were treated singly. At pH 5.7 Carstairs and Newcombe (1996) found that in mixed culture both Phytophthoras were equally able to access the Eucalypt baits offered, but that following infection selection favoured the isolation of one of them.

The baiting technique employed by the VHS, and indeed by many laboratories interested in determining the *Phytophthora* status of samples, involves adding to 1 part soil/plant tissue sample *circa* 2 parts distilled water. Young, rapidly expanding plant tissue (e.g. *Eucalyptus sieberi* cotyledons) is then added as a bait for *Phytophthora* zoospores. The final pH of this system, in which the Phytophthoras are required to produce sporangia, will be determined by the properties of the soil/plant tissue sample. If it can be shown that pH affects the competitive abilities of species of *Phytophthora* to infect baits, it may be possible to buffer systems to optimise infection of baits by particular species/taxa of *Phytophthora*. Our objective in this study was to measure the competitive abilities of *P. cinnamomi* and *P. citricola* in combination under a regime of differing pH conditions.

Procedure

To assess the competitive abilities of *P. cinnamomi* and CIT 3 *P. citricola* to infect a host, we followed the procedure of Carstairs and Newcombe (1996). Inoculum is stimulated to produce sporangia and zoospores and the zoospores are trapped with *Eucalyptus sieberi* cotyledon baits. The Phytophthoras thus recovered are then identified by hyphal morphology. *P. cinnamomi* typically produces colonies with coralloid hyphae (Waterhouse, 1963), where as CIT 3 *P. citricola* produces hyphae which are not coralloid.

Ten *E. sieberi* cotyledons were surface sterilised with 70% ethanol and placed adaxial surface down onto the surface of each of six selective agar plates. To three of these plates 2 mm square inoculum plugs of an A2 mating type *P. cinnamomi* isolate were placed alongside the Eucalypt cotyledons, and to the other three plates inoculum plugs of a CIT 3 *P. citricola* isolate were placed alongside the cotyledons. The plates were

sealed and allowed to incubate at 23° C in the dark for 48 hr by which time the Phytophthoras had grown into the cotyledons.

Soil extracts were prepared by combining 400 g of garden soil and 2.5 L of distilled water in a 3 L conical flask. These were allowed to stand in a constant temperature room at 24° C in the light for 24 hrs before being filtered. The extracts were then diluted 1:1 with distilled water and the final pH was determined. Two batches of soil extract were prepared and on each occasion the final pH was 5.7. Citric acid was used to buffer 1 L aliquots of this non-sterile soil extract to pH 5.5, 5.0, 4.5, and 4.0.

On the bottom of each of three plastic baiting dishes and 8 cm apart were attached two *E. sieberi* cotyledons that had been infected with the *P. cinnamomi* isolate. To each dish was added 250 mL of the crude soil extract and 30 fresh *E. sieberi* cotyledons which were floated adaxial surface down. These dishes were incubated in a constant temperature room at 24° C with constant light. After 48 hrs ten cotyledons were removed from each dish and plated onto selective agar plates. After 48 hrs each cotyledon was assessed for *P. cinnamomi* colonies growing into the agar. From the *Phytophthora* colonies growing out of five Eucalypt baits, half-centimetre-square inoculum plugs were excised and transferred to McCartney bottles containing 4 mL of V8/Pea broth. These were incubated at 23°C in the dark for 48 hrs, then the mycelium was harvested and assessed isoenzymically by the CAGE method for GPI, IDH, LDH and MDH (Hebert and Beaton 1993). Three dishes to which *E. sieberi* cotyledons infected with CIT 3 *P. citricola* were added were treated in the same manner.

To each of four dishes both *P. cinnamomi* and CIT 3 *P. citricola* infected *E. sieberi* cotyledons were added. On the bottom and toward one end of the dish was attached a *P. cinnamomi* and a CIT 3 infected cotyledon and 8 cm from these and toward the other end of the dish was attached a second pair of cotyledons, one infected with *P. cinnamomi* and the other infected with CIT 3 *P. citricola.* Non-sterile soil extract at pH 4.0, and 30 *E. sieberi* cotyledons which had been surface sterilised with 70% ethanol were added to each dish and the dishes were incubated as above. Similar sets of four dishes were set up with non-sterile soil extract at pH 4.5, 5.0 and 5.5. All of the *E. sieberi* cotyledon baits from each of the four dishes for the four pH treatments

were then plated onto selective agar plates and the *Phytophthora* colonies were allowed to grow out for 24 hrs. Presence or absence of the two species of *Phytophthora* growing out of each Eucalypt cotyledon was then recorded.

As a control to test for the presence of Phytophthoras in the soil extract, to each of three 17x11x5 cm deep plastic containers 250 mL of crude soil extract was added, along with thirty surface-sterilised *E. sieberi* cotyledons, each being floated adaxial surface down. The dishes were incubated as above, and on the fifth day ten cotyledons were harvested from each of the three dishes and plated onto selective agar plates. After 48 hrs the cotyledons were assessed for the presence of Phytophthoras.

Results

No Phytophthoras were recovered from the three control bait dishes to which no inoculum was added, so there was no evidence that the soil extract was contaminated with *P. cinnamomi*, CIT 3 *P. citricola* or any other *Phytophthora*. Baits which had been exposed to either *P. cinnamomi* or CIT 3 *P. citricola* inoculum showed symptoms of infection by *Phytophthora* (which included the loss of anthocyanin pigment from the abaxial surface of the cotyledons). All of the baits plated from these dishes proved positive for *Phytophthora,* and those colonies tested by their isoenzymes proved to be positive for either *P. cinnamomi* or CIT 3 *P. citricola* (data not presented). There was no evidence of the *P. cinnamomi* inoculated dishes being contaminated with CIT 3 *P. citricola* inoculum or *vice versa*.

When tested in combination with *P. cinnamomi* at pH 4.0, CIT 3 *P. citricola* was not recovered from any of the plated baits (Figure 7.1). The number of baits from which CIT 3 *P. citricola* was recovered increased as pH increased such that at pH 5.0 and 5.5 it was recovered in near expected numbers i.e. 30 (Figure 7.1), as predicted by the single species result described above.

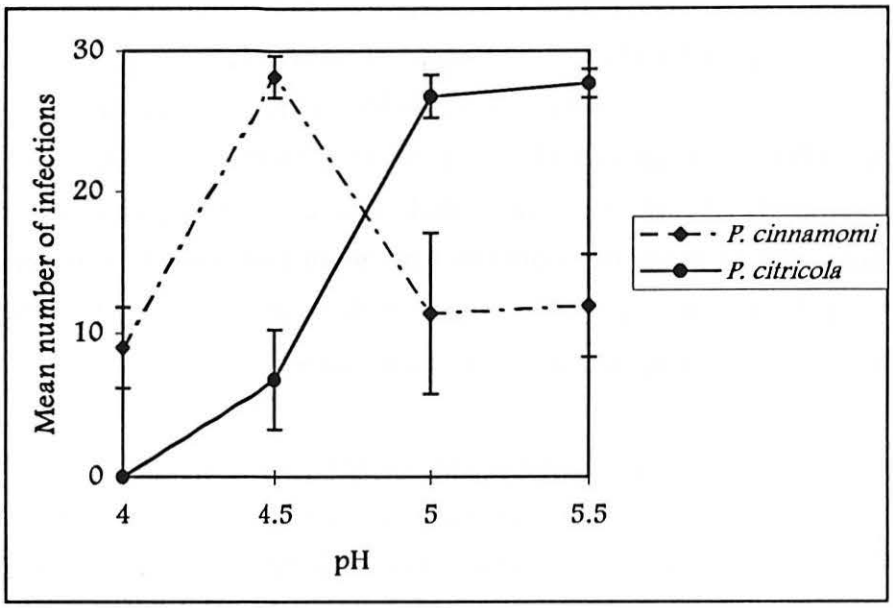

Figure 7.1 Effect of pH on the number of *Eucalyptus sieberi* cotyledons from which *Phytophthora cinnamomi* and CIT 3 *P. citricola* were recovered when these two fungi were tested in combination.

When tested in combination with CIT 3 *P. citricola* at pH 4.0, *P. cinnamomi* was recovered from nine (on average) of thirty baits (Figure 7.1). The number of baits from which *P. cinnamomi* was recovered increased to near the expected number of 30 (mean number infected was 28.14) at pH 4.5, while recoveries of CIT 3 *P. citricola* were still very low (mean number infected was 6.72). The level of recovery of *P. cinnamomi* from baits at pH 5.0 and 5.5 then returned to about the same as that recorded for pH 4.0 (Figure 7.1).

Discussion

Below pH 5.0, and in combination with *P. cinnamomi,* recovery of CIT 3 *P. citricola* from *E. sieberi* baits was considerably reduced such that at pH 4.0 no CIT 3 *P. citricola* was recovered. Although recovery of *P. cinnamomi* from baits was considerably reduced at pH 4.0, it was not suppressed altogether and *P. cinnamomi* seemed to out-compete CIT 3 *P. citricola* for baits. At pH 5.0 and 5.5 CIT 3 *P. citricola* out-competed *P. cinnamomi* for baits.

Relative to CIT 3 *P. citricola, P. cinnamomi* seems better able to access and infect hosts at low pH, while at higher pH CIT 3 *P. citricola* seems to be better able to access and infect hosts. It may be that at pH 4.5 and below, only *P. cinnamomi* was recovered from the baits because in that species the process of sporangial production continued, whereas in CIT 3 *P. citricola* it was disrupted. Conversely it may be that at higher pH CIT 3 *P. citricola* was recovered from the baits more frequently because once having infected the baits, CIT 3 *P. citricola* is antagonistic to *P. cinnamomi* thereby reducing the number of baits proving positive for *P. cinnamomi.*

Carstairs and Newcombe (1996) demonstrated that at pH 5.7 two CIT 3 *P. citricola* cultures were equally able to access and infect *E. sieberi* cotyledons, whereas we found that at pH 5.5 CIT 3 P. citricola out-competed *P. cinnamomi* for *E. sieberi* baits. At pH 5.5 and 5.0 only 2 baits would need to be assessed to achieve 95% confidence of recovering CIT 3 *P. citricola* compared with 6 baits for *P. cinnamomi* at the same pH. In mixed *P. cinnamomi* and CIT 3 *P. citricola* culture, these pH conditions promoted recovery of CIT 3 *P. citricola.* If it is desirable to optimise recovery of *P. cinnamomi,* when in mixed culture with CIT 3 *P. citricola,* then the lower pH of 4.5 would give the best results. At this pH *P. cinnamomi* was recovered at near expected proportions, and only 2 baits would need to be assessed to achieve 95% confidence of recovering the pathogen. In contrast 12 baits would need to be assessed at this pH to be 95% confident of recovering CIT 3 *P. citricola.*

Only one of many variables was altered in this study. Another variable that may be altered to promote the recovery of a particular *Phytophthora* is temperature, and another is light. Additives to the water component of the system, such as Hymexazol, may also promote the recovery of particular Phytophthoras under some situations.

Recommendations

7.7 It is recommended that similar competition experiments be conducted to compare with *P. cinnamomi* the relative abilities of Pythiums and key Phytophthoras in the *P. megasperma* and *P. cryptogea* complexes to infect baits at different pH.

7.8 To optimise the chance of recovering *P. cinnamomi* from field samples, when in combination with other species of *Phytophthora* and Pythiums, managers are recommended to buffer their sample solutions to about pH 4.5.

Service 8. Test ways of establishing the intensity of field sampling necessary to achieve given levels of certainty of detecting *Phytophthora* if present.

Introduction

Warm moist soil conditions are associated with high levels of *P. cinnamomi* inoculum in soils (Shearer and Tippett, 1989). Shea *et al.* (1980) reported greatest numbers of sporangia of *P. cinnamomi* being formed in autumn and spring in a jarrah forest site, and Shearer and Shea (1987) reported that seasonal changes in inoculum levels of *P. cinnamomi* in jarrah forest sites varied from year to year depending on the timing of opening and closing rains. Floristic composition of the vegetation can also affect inoculum levels of *P. cinnamomi* (Shea *et al.* 1978; Murray *et al.* 1985).

So, inoculum levels of Phytophthoras at diseased sites are probably not static. Because verification of the presence of *Phytophthora*, by isolation from samples and identification, is an integral part of monitoring disease caused by these fungi in Western Australia, it would be valuable to know how many soil/plant tissue samples from a site need to be tested so as to determine the site's *Phytophthora* status with some degree of certainty. Intuitively it is to be expected that if large numbers of samples are taken from sites which are infested with *Phytophthora* and tested for the pathogen, then the likelihood that the sites will be verified as positive for *Phytophthora* would be greater than if fewer samples were tested.

Our objective, therefore, was to determine if testing larger numbers of samples from sites, i.e. more intensive sampling, is associated with more frequent positive detection of *Phytophthora.*

Procedure

Three hundred and twenty seven soil samples, of *circa* 1 kg each, from 45 sites from the south of Western Australia were assessed for their *Phytophthora* status by the baiting method as employed by the VHS of CALM. The number of samples assessed per site ranged from 2 to 25.

A two-tailed t-test was then used to determine whether or not the mean number of samples taken from sites that proved negative for *Phytophthora* was the same as that taken from sites that proved positive.

Results

Of the 45 sites examined for the presence of *Phytophthora*, 17 were diagnosed as negative for *Phytophthora*, and 28 were diagnosed as positive (Table 8.1). The mean number of samples tested from sites that were negative for *Phytophthora* (mean = 5.53; S.D. = 4.03) was significantly ($t_{obs}=2.07 > t_{0.05}=1.684$ for 43 d.f.) less than the mean number of samples that were tested from sites that proved positive (mean = 8.32; S.D. = 4.93). This indicated that relative to sites which were diagnosed as *Phytophthora* free, sites which were positive for *Phytophthora* had significantly more samples tested from them.

There was a positive relationship, though not a strong one ($r^2=35.5\%$), between the number of samples that were tested from sites which were positive for *Phytophthora*, and the frequency of samples that were positive from those sites.

For those sites which proved positive for *Phytophthora*, the mean frequency of samples per site which tested positive was 0.455 (n=28, and S.D.=0.256), and from about 80% of the sites that were positive for *Phytophthora* one might expect to yield a frequency of positive samples equal to or greater than 0.240. Eleven samples would need testing to be 95% confident of detecting a positive sample from a site in which 0.24 of the samples were positive. So in order to be 95% confident of detecting 80% of sites that were positive for *Phytophthora*, eleven samples would need to be tested per site. Twenty-two samples would need to be tested per site to be 95% confident of detecting 90% of sites that are positive for *Phytophthora*, and 74 samples per site would need to be tested to be 95% confident of detecting 95% of sites that are positive.

Table 8.1 The number of samples from 45 sites that were tested for *Phytophthora*, and the number and proportion of these that proved to be positive for *Phytophthora*.

Sites negative for *Phytophthora*				Sites positive for *Phytophthora*			
Site	No. samples tested	No. +ve for *Phyt.*	Proportion +ve *Phyt.*	Site	No. samples tested	No. +ve for *Phyt.*	Proportion +ve *Phyt.*
1	2	0	0	18	2	1	0.50
2	2	0	0	19	3	1	0.33
3	2	0	0	20	9	1	0.11
4	2	0	0	21	9	1	0.11
5	2	0	0	22	12	1	0.08
6	3	0	0	23	15	1	0.07
7	3	0	0	24	2	1	0.50
8	3	0	0	25	3	1	0.33
9	3	0	0	26	3	1	0.33
10	5	0	0	27	6	2	0.33
11	6	0	0	28	9	2	0.22
12	6	0	0	29	7	2	0.28
13	9	0	0	30	6	3	0.50
14	9	0	0	31	7	3	0.43
15	10	0	0	32	13	3	0.23
16	13	0	0	33	14	3	0.21
17	14	0	0	34	3	3	1.0
				35	5	3	0.60
				36	6	4	0.67
				37	12	4	0.33
				38	6	5	0.83
				39	12	6	0.50
				40	8	6	0.75
				41	9	6	0.67
				42	7	6	0.86
				43	10	6	0.60
				44	10	8	0.80
				45	25	14	0.56

These are 'worst case' determinations. Less restrictive assumptions, i.e. using $p=^{98}/_{233}$ over all 45 sites where p is equal to the number of samples that were positive for *Phytophthora* (98) divided by the number of samples tested (233), would yield a sampling intensity of six samples needing testing to be 95% confident of declaring a site *Phytophthora* free.

Seven of the seventeen sites in which *Phytophthora* was not detected were sampled six or more times. As only two of the 17 (11.8%) sites that were negative for *Phytophthora* were sampled more than eleven times, we can be 80% confident that only 11.8% of the 17 sites which did not prove positive for *Phytophthora* were *Phytophthora* free. None of them were sampled as many as 22 times. Of those sites that were positive for *Phytophthora* seven (25%) were sampled more than eleven times, and only one of them was sampled more than 22 times.

Discussion

No level of sampling will provide 100% assurance that a site is free of *Phytophthora*. Sites which proved positive for *Phytophthora* had a significantly higher number of samples tested than those sites which proved negative, and the more often positive sites were sampled the greater the number of samples that proved to be positive for *Phytophthora*. That there is a relationship between intensity of sampling and the likelihood that *Phytophthora* sites will be confirmed positive, has been established.

In the best case by assessing 6 samples from sites, sites may be determined to be free of *Phytophthora* with 95% confidence. In the worst case researchers would be required to assess 74 samples from sites for *Phytophthora* to achieve 95% confidence of identifying 95% of sites which are infested with *Phytophthora*. Conversely, sites which prove to be negative for *Phytophthora* after being sampled 74 times, may be assumed to be *Phytophthora* free with 95% confidence.

The level of sampling required to assess the *Phytophthora* status of a site will vary from site to site as is indicated by the wide range in frequency of positive samples found in our sample of 28 sites confirmed positive for *Phytophthora* (0.07 to 1.0, and mean=0.455). Not only are there likely to be differences in *Phytophthora* recovery rates between sites due to physical and vegetation differences (Shea *et al.* 1978; Murray *et al.* 1985), but at any one site temporal differences can also be expected differences(Shea *et al.* 1980; Shearer and Shea 1987). The levels of sampling intensity determined in this study, to achieve certain confidence levels of establishing the *Phytophthora* status of sites, should therefore only be considered to be first approximations. This is especially so since all the sites tested for this study were sampled at one time, in late winter and early spring when inoculum levels of

Phytophthora might be expected to be higher than in summer for example (Shea *et al.* 1980; Shearer and Shea 1987). Testing the same sites in different seasons is therefore likely to result in quite different sampling intensities being required for the same levels of confidence.

When a site's *Phytophthora* status is being confirmed by assessing field samples by baiting in the laboratory, reasonable levels of confidence about the results may only be achieved by testing quite large numbers of samples, i.e. 6-11. The concomitant laboratory costs of analysing these samples, at *circa* $40.00 per sample, is therefore also going to be large. Given that a considerable cost has also been incurred by putting the Interpreters (dieback assessors) in the field, the laboratory costs of assessing the field samples are probably not imposing substantial additional costs to management given that a degree of confidence about the *Phytophthora* status of the site will flow from it. So when Interpreters have little doubt about the *Phytophthora* status of a site, they need not collect field samples from it for assessment. However, in cases where there is some doubt, then the site should be sampled intensely.

Recommendations

8.1 When Interpreters have little doubt about the *Phytophthora* status of a site, they need not collect field samples from it for assessment.

8.2 When it is required that a site's *Phytophthora* status be verified by testing samples from it, managers should test no fewer than 6 and as many as 11 samples from that site.

Service 9. To train staff at the Vegetation Health Service (VHS) of CALM in the use of isoenzyme analysis for the identification of species of *Phytophthora*.

Introduction

The research reported in this document has shown that the isoenzyme method of identifying unknown isolates of *Phytophthora* other than *P. cinnamomi* is more accurate, efficient and cost effective than the morphological method currently in use by the VHS of CALM. Our objective therefore, was to train CALM staff in the method of *Phytophthora* identification by isoenzyme assessment, such that this service may be performed on a regular basis.

Procedure

Two CALM technicians from the VHS attended individual training sessions in the use of the isoenzyme laboratory and isoenzyme protocols for one day per week for 3 weeks, and then for half a day per week over the 3 succeeding weeks during the third quarter.

Results and Discussion

Both staff members now have sufficient experience to work in an isoenzyme laboratory and carry out the various CAGE protocols required to produce isoenzyme patterns necessary for identification of local species of *Phytophthora*. In the course of their using the isoenzyme method for routine identification of isolates, the VHS staff have recently diagnosed one Western Australian isolate as being *Phytophthora boehmeriae*, which may be the first record of this species in W.A. (J Webster and N North pers. com.).

Recommendations

9.1 It is now necessary for the VHS staff to work together in the isoenzyme laboratory to form a cohesive team. Several more half-day sessions may be required to achieve this goal.

9.2 The isoenzyme protocols are varied (see Service 1) and require that laboratory staff perform them on a regular basis in order to maintain familiarity and efficiency.

9.3 As it has been recommended that VHS staff maintain living reference isolates of all readily available taxa of *Phytophthora* retrieved from the field in Western Australia (see Section 2.2), it is now recommended that the VHS staff establish their own reference library of isoenzyme banding patterns for those reference isolates.

LIST OF RECOMMENDATIONS

The recommendations made in the preceding Services are listed here for convenience:

1.1 Plant pathology services which detect and identify Phytophthoras should adopt as standard procedure the practice of accumulating, on CMA, unidentified pure cultures of species of *Phytophthora* other than *Phytophthora cinnamomi* through the week. Inoculum plugs of the cultures should then be added to McCartney bottles of Pea/V8 juice broth on Friday afternoons, and the mycelium grown from them should be harvested on the following Monday mornings (*circa* 64 hrs later) in readiness for CAGE. In so doing the services will be maximising the use of down-time on weekends.

1.2 In daily record sheets note the date, Sample Well Plate number, and well position of each isolate of *Phytophthora* tested by Cellulose Acetate Gel Electrophoresis (CAGE).

1.3 It is recommended that the isoenzyme (banding) patterns derived from CAGE with GPI, IDH, LDH and MDH stains be used to distinguish between taxa of *Phytophthora.*

2.1. As there is no recognised centre in Western Australia for housing reference cultures of taxa of *Phytophthora,* it is recommended that the Vegetation Health Service (VHS) of CALM maintain living isolates of the reference cultures of *Phytophthora* that were obtained for this study from California, the UK and states outside Western Australia.

2.2. The VHS of CALM should maintain living reference isolates of all readily available taxa of *Phytophthora,* as determined by their isoenzyme profiles, that have been retrieved from the field in Western Australia.

2.3 When, during routine identification of isolates by the VHS, isolates of *Phytophthora* are encountered which have not as yet been described, i.e. new or unique isoenzyme profiles, it is recommended that:

a. the VHS give these isolates a full morphological examination; and
b. the VHS maintain living reference cultures of these isolates.

3.1 Studies comparing measures of diversity between Western Australian and non-Western Australian populations of *Phytophthora,* similar to that for *P. cinnamomi,* should be conducted for other Phytophthoras found in mining tenements and in the conservation estate of Western Australia.

4.1 Pathologists and nature managers alike should adopt the isoenzyme method for identifying taxa of *Phytophthora* other than *P. cinnamomi.*

4.2 Before deciding whether or not to replace the coralloid hyphae technique of identifying *P. cinnamomi* with the isoenzyme method a comparison needs to be made between the relative efficiencies and opportunity costs of the respective techniques.

5.1 Mining companies wishing to have field samples from their tenements in the Northern Sand Plain diagnosed for species of *Phytophthora* are advised to use the short morphological plus isoenzyme approach because it will afford them greater accuracy and efficiency at no extra cost.

5.2 Where long experience has shown that the proportions of the isolates of the various species of *Phytophthora* were dominated by species other than *P. cinnamomi*, e.g. Northern Sand Plains and Fitzgerald River National Park, all isolates other than *P. cinnamomi* should be directly routed to isoenzyme analysis.

6.1 Managers are advised not to adopt the direct assay of baits by CAGE technique for detecting species of *Phytophthora* in soil/plant tissue samples.

7.1 At least 40 lupin and Eucalypt baits should be added to each baiting tray.

7.2 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 examined for baits having lesions on day 3, and each day thereafter to day 10.

7.3 For each baiting dish the entire lesions of 28 or more baits (where available), should be plated on to selective agar (i.e. 2 selective plates with 14 baits per plate).

7.4 Management should identify the species of *Phytophthora* recovered from baits exposed to field samples on day 1 to day 5, and those from a second set of baits exposed to the same field samples on day 6 to day 10.

7.5 Management should adopt a fifteen-day programme for double harvesting baits from samples (Table 7.4). This programme maximises the use of down-time on weekends, and requires thirteen to fifteen days between receipt of samples and completion of assessment.

7.6 Determine which of the two approaches, i.e. double baiting samples from sites, or single baiting a larger number of samples from sites, most effectively reduces the number of sites that are deemed to be negative for *Phytophthora* when they are in fact infested ("false negatives"), and which is most cost effective.

7.7 Competition experiments similar to that described in Service 7.4 should be conducted to compare with *P. cinnamomi* the relative abilities of Pythiums and key Phytophthoras in the *P. megasperma* and *P. cryptogea* complexes to infect baits at different pH.

7.8 To optimise the chance of recovering *P. cinnamomi* from field samples, when in combination with other species of *Phytophthora* and Pythiums, managers are recommended to buffer their sample solutions to about pH 4.5.

8.1 When Interpreters have little doubt about the *Phytophthora* status of a site, they need not collect field samples from it for assessment.

8.2 When it is required that a site's *Phytophthora* status be verified by testing samples from it, managers should test no fewer than 6 and as many as 11 samples from that site.

9.1 It is now necessary for the VHS staff to work together in the isoenzyme laboratory to form a cohesive team. Several more half-day sessions may be required to achieve this goal.

9.2 The isoenzyme protocols are varied (see Service 1) and require that laboratory staff perform them on a regular basis in order to maintain familiarity and efficiency.

9.3 As it has been recommended that VHS staff maintain living reference isolates of all readily available taxa of *Phytophthora* retrieved from the field in Western Australia (see Section 2.2), it is now recommended that the VHS staff establish their own reference library of isoenzyme banding patterns for those reference isolates.

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Appendix 1 Chemical / Compound List

Chemical Compound	Concⁿ (mg/mL)	Supplier	Product No.
DL-Aspartic Acid		Sigma	A-9006
Bacto Agar		Difco	0140-01
Calcium Carbonate			
Citric Acid		Ajax	5009
D-Fructose-6-Phosphate	10	Sigma	F-3627
D-glucose		Sigma	G-7528
Glucose-6-Phosphate-Dehydrogenase		Boehringer Mannheim	165 875
DL-Glutamic Acid		Sigma	G-1626
Glycine		BDH	10119
L-Histadine		Sigma	H-8000
DL-Isocitric Acid	75	Sigma	I-1252
DL-Lactic Acid	75	Sigma	L-1500
Magnesium Chloride Hexahydrate	10	BDH	10149
DL-Malic Acid	75	Sigma	M-6773
2-Mercaptoethanol		Sigma	M-6250
MTT	10	Sigma	M-2128
NAD	5	Sigma	N-7381
NADP	5	Sigma	N-3886
Peptone (N-2-Soy)		Sigma	P-1265
PMS	5	Sigma	P-9625
Trizma Base (Tris)		Sigma	T-1378