DEPARTMENT OF CONSERVATION AND LAND MANAGEMENT

SCIENCE AND INFORMATION DIVISION

SEMINAR

11 July 1997

Rapid identification of species of Phytophthora
MERIWA Project No M227/M254

presented by S Carstairs and M Stukely

Isoenzyme procedures and protocols for identifying species of *Phytophthora* were developed. The effectiveness of the isoenzyme approach for identifying species of *Phytophthora* was assessed, and it was compared with traditional morphological approaches to diagnosis on the basis of accuracy, time and cost. Except with morphologically distinct species of *Phytophthora*, eg. *Phytophthora cinnamomi* and *Phytophthora nicotianae*, the isoenzyme approach would allow managers to discriminate between Phytophthoras to a level unmatched by current morphological approaches. Morphologically distinct species were identified equally well with both the isoenzyme and morphological approaches, and the morphologically distinct *P. cinnamomi* was more rapidly identified, at less cost to managers, by a "short" (hyphal) morphological method than by the isoenzyme approach. Morphologically indistinct species of *Phytophthora* were more rapidly identified, at less cost, by the isoenzyme method than by morphological methods. A method of directly assaying for *Phytophthora* isoenzymes in infested plant tissue was assessed. Reliability of procedures for assessing the *Phytophthora* status of soil/plant tissue samples collected from the field was progressed, and it was determined that 6-11 field samples, collected from *Phytophthora* infested sites, require assessment to attain high levels of confidence of detecting the *Phytophthora* in those sites and to minimise the likelihood of "false negatives".

Venue: CALM Training Centre, SOHQ, 50 Hayman Road, Como

Time: 3.00pm

Contact: C Farrell, 405 5146

CALM's and the Mining Industry's common goal:

To prevent "disease causing Phytophthoras"[1] being dispersed from infested areas (natural ecosystems) into uninfested areas during operational procedures (see CALM Policy No. 3, 1991).

1. Disease causing Phytophthoras are uniformly destructive to susceptible species, ie. these Phytophthoras kill a high frequency of individuals in susceptible higher plant species in natural ecosystems. By definition some Phytophthoras do not cause disease.

Dieback Review Panel's (Podger *et al.* 1996) comment on this objective:

That situations do occur where uninfested areas may merit attempts to protect them from infestation by *Phytophthora*:

- where areas are of significant size.
- where areas are isolated.
- where animal and other traffic is low.
- where environmental conditions do not often favour the establishment of disease causing Phytophthoras.

The Panel's recommendation:

That significant protectable areas be identified, prioritised and that resources be directed to their protection from disease causing Phytophthoras by rigorous application of hygienic practice.

Relevance of detection and identification of *Phytophthora* to the common goal:

Prescriptions for hygienic practice during operational procedures require that the boundaries of areas which are infested with dieback causing Phytophthoras are set.

These infection boundaries are determined by observation, by trained personnel, of disease symptoms in susceptible species e.g. Banksias and other Proteas, Xanthorrhoeas etc.

Reliability of judgements about the *Phytophthora* status of sites/ ecosystems depend, *inter alia*, on:

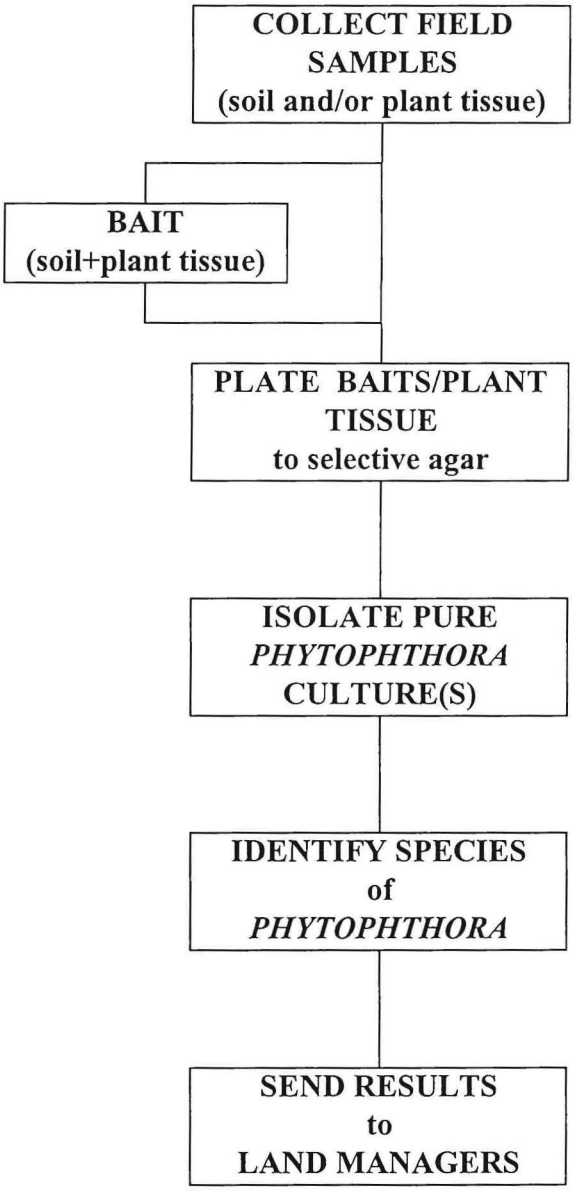
- isolation of dieback causing Phytophthoras from dying plants or soil samples, and their subsequent identification to specific (or biotype) level, from ecosystems suspected to be infested; this requires:

    1. that a sufficiently high number of samples are collected for assessment of the presence of *Phytophthora*, and that these contain a sufficient number of propagules of the pathogen for it to be detected.

    2. that the samples are tested rigorously enough to detect the disease causing Phytophthoras.

- failure to recover Phytophthoras from ecosystems deemed to be free of infection.

TESTING A SITE FOR THE PRESENCE OF *PHYTOPHTHORA*



So what is a sufficiently high number of samples?

The studies:

	Study No. 1	Study No. 2
No. of samples tested:	327	334
No. of sites tested:	45	58
Locality:	SW of WA	FRNP
Collection method:	Kept uniform	Presumed uniform
No. of collectors:	Minimised	Minimal
Time of year:	Winter- spring	Usually spring (1985-95)
Detection service:	VHS of CALM	VHS of CALM

Qn 1. Was the mean number of samples tested from sites that were determined not to be infested with species of *Phytophthora*, the same as that tested from sites that were determined to be infested with Phytophthora?

Qn 2. Was there a positive relationship between the number of samples tested from sites that were determined to be positive for *Phytophthora*, and the number of samples from those sites that were determined to be positive?

Qn 3. From the data for sites determined to be positive for *Phytophthora*, would we be able to derive a minimum number of samples to be tested to achieve 80% or 95% confidence of determining that one of the samples would be positive?

Study 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.***	**Prop^{tn} +ve *Phyt.***	**Site**	**No. samples tested**	**No. +ve for *Phyt.***	**Prop^{tn} +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

Results:

Qn 1. Study No. 1. A two-tailed t-test determined that 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 for *Phytophthora* (mean=8.32; s.d.=4.93).

Qn 2. Study No. 1. There was a weak positive relationship ($r^2=35.5\%$) between the number of samples that were tested from sites that were positive for *Phytophthora*, and the number of samples that were positive from those sites.

Qn 3. Binomial Probabilities

Probability a sample is +'ve for *Phyt.* (P(+'ve)) can be determined by:

$$P(+'ve) = \frac{\text{No. samples +'ve}}{\text{No. samples tested}} = x$$

and, prob a sample is -'ve (P(-'ve)) can be determined by:

$$P(-'ve) = 1-P(+'ve) = 1-x$$

So, for any given number of samples tested for a site, the probability that all the samples will be negative can be determined from:

$P(\text{all -'ve}) = (1-x)^n$ where n is the number of samples tested.

and, the probability that at least one of the samples will be +'ve can be determined from:

$$P(\text{at least 1 +'ve}) = 1-(1-x)^n$$

which is the same as saying 1 minus the probability that all of them will be negative:

$$\text{Confidence (\%)} = 100 * (1-(1-x)^n)$$

Determining the number of samples per site that require to be tested to achieve 95% confidence of detecting a +'ve sample.

	Study 1	**Study 2**
No. sites +'ve for *Phyt.*:	28	16
No. samples tested:	233	128
No. samples +'ve for *Phyt.*:	98	87
Prob. a sample is +'ve (x):	0.42	0.68
Prob a sample is -'ve (1-x):	0.58	0.32
Confidence (%):	$100 * (1-(0.58)^n)$	$100 * (1-(0.32)^n)$
When confidence = 95%; n equals:	6 samples	3 samples

Limitations of this study:

1. Inoculum levels, of *Phytophthora cinnamomi* for example, at infested sites may vary from season to season, and from year to year (see Shea 1980, and Shearer and Shea 1987).

2. Floristic composition of the vegetation may also influence inoculum levels (see Shea *et al.* 1978, and Murray *et al.* 1985).

3. In these examples we assumed that inoculum levels at all sites were the same!
   Assuming they were not, 11 samples would require testing per site to achieve circa 85% confidence of detection.

Recommendations:

1. When there is little doubt about the *Phytophthora* status of a site, eg. when high frequencies of individuals of indicator species are dying or have been killed in forested ecosystems, it may be that field samples need not be collected for assessment.

2. When it is required that a site's *Phytophthora* status be verified by testing samples from it, personnel should test no fewer than **6 samples**...from that site.

Estimating the no. "false negatives" Study 1.

No. samples tested	No. sites	No. sites -'ve	No. sites +'ve
Group A			
2	7	5	2
3	8	4	4
5	2	1	1
Totals	**17**	**10**	**7**
Group B			
6	6	2	4
7	3	0	3
8	1	0	1
9	6	2	4
10	3	1	2
12	3	0	3
13	2	1	1
14	2	1	1
15	1	0	1
25	1	0	1
Totals	**28**	**7**[1]	**21**

Group B

Expected proportion of sites -'ve, given 0.95 confidence of detection
= 0.37

Observed proportion of sites -'ve = 7

Probability all 7 negative by chance $= (1-0.95)^7 \quad = 7.8*10^{-10}$

Alternatively it may be that these 7 sites were -'ve because there wasn't sufficient inoculum of *Phytophthora* in the samples for them to be detected.

Expected proportion of sites in Group B not having samples with sufficient inoculum of *Phytophthora*:

$$= \frac{7}{7 + 21} \quad = 0.25$$

Group A.

Expected no. negatives therefore = 17*0.25 = 4.25

Observed no. negatives = 10

and no. false negatives therefore = 10-4.25 = 5.75

$$\% \text{ false negatives} = 100 * \frac{5.75}{17}$$

= 33.8% in sites tested with <6 samples.

Qn 1.

	Study 1	**Study 1A**
No. sites tested:	45	45
No. sites -'ve for *Phyt.*:	17	11 17-6 from Group A
No. sites +'ve for *Phyt.*:	28	34
Mean no. samples tested per -'ve site:	5.53 (4.03)	7.09 (4.21)
Mean no. samples tested per +'ve site:	8.32 (4.93)	7.32 (4.99)
t:	2.07	-0.15
P:	<0.05	0.88

How rigorously should samples be tested?

Part 1:

The Study:

Locality of sites:	SW of WA
Collection method:	Kept uniform
No. of collectors:	Minimised
Time of year:	Winter-spring
Detection service:	VHS of CALM
No. of bait trays assessed:	21
No. of lesioned baits examined:	273

Qn: What number of lesioned baits require examination to achieve 95% confidence of recovering *Phytophthora* from infested bait tray samples?

Results:

Sample No.	No. of Lesioned Baits Tested	No. of Baits -'ve for *Phyt.*	No. of Baits +'ve for *Phyt.*
1. *Phyt.* A.	10	4	1
Phyt. B.			5
2. *Phyt.* A.	8	0	1
Phyt. B.			7
3. *Phyt.* A.	8	5	1
Phyt. B.			2
4. *Phyt.* A.	12	0	2
Phyt. B.			10
5. *Phyt.* A.	10	1	4
Phyt. B.			5
6. 1 *Phyt.* only	9	5	4
7. 1 *Phyt.* only	8	7	1
8. 1 *Phyt.* only	9	0	9
9. 1 *Phyt.* only	24	20	4
10. 1 *Phyt.* only	29	6	23
11. 1 *Phyt.* only	13	9	4
12. 1 *Phyt.* only	16	10	6
13. 1 *Phyt.* only	15	9	6
14. 1 *Phyt.* only	17	9	8
15. 1 *Phyt.* only	13	9	4
16. 1 *Phyt.* only	14	1	13
17. 1 *Phyt.* only	10	9	1
18. 1 *Phyt.* only	12	11	1
19. 1 *Phyt.* only	11	8	3
20. 1 *Phyt.* only	6	5	1
21. 1 *Phyt.* only	9	4	5
Totals	**273**	**144**	**129**

Determining the number of lesioned baits per baiting dish that require to be examined to achieve 95% confidence of detecting a +'ve lesion.

No. of bait dishes +'ve for *Phyt.*:	21
No. of lesioned baits examined:	273
No. lesioned baits +'ve for *Phyt.*:	129
Prob. a lesioned bait is +'ve (x):	0.47
Prob. a lesioned bait is -'ve (1-x):	0.53
Confidence (%):	$100*(1-(0.53)^n)$
When confidence = 95%; n equals:	5 lesioned baits

Limitations of this study:

1. This experiment has not been repeated.

2. We assumed that all species of *Phytophthora* behaved the same.

3. We assumed that inoculum levels were the same in all of the bait dishes!
   Assuming they were not, 28 lesioned baits would require testing per bait dish to achieve *circa* 95% confidence of detection.

Recommendation:

For each baiting dish the entire lesions of 28 baits (where available), or at least 5 lesioned baits, should be plated onto selective antibiotic agar.

Part 2:

The Study: 5 day baiting vs 5 *and* 10 day baiting.

Locality of sites:	SW of WA
Collection method:	Kept uniform
No. of collectors:	Minimised
Time of year:	Winter-spring
Detection service:	VHS of CALM
No. of samples assessed:	27

Step 1: Contents of a sample divided between 2 plastic bait dishes.

Step 2: *Circa* 300 mL of distilled water was added to each dish.

Step 3: 30-40 pinnae of WA blue lupin and the same no. of *Eucalyptus sieberi* cotyledons was added to each dish.

Step 4: 4 and 5 days later 8-12 lesioned baits from each dish were transferred to selective antibiotic agar plates. All other baits were discarded.

Fresh baits were added, and 8-12 of these were plated onto plates after 5 days exposure ie. 10 day baits.

Step 5: 16-40 hrs after harvest, the plated lesioned baits were examined microscopically for colonies of *Phytophthora*.

Qn 1: Did the 2nd harvest of baits substantially increase (where a substantial increase would be 7.5% or more) the number of samples determined to be positive for 6 species of *Phytophthora*?

Qn 2: Were the species of *Phytophthora* recovered in the 2nd harvest of baits (ie. 10 day baits) the same as those recovered in the 1st harvest (ie. 4-5 day baits)?

Results:

Qn 1: Recovery of species...

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			% increase in recovery due to 2nd harvest
	same species of Phyt. was recovered on both harvestings	+ve for Phyt. 1st harvest, & -ve for that Phyt. 2nd harvest	-ve 1st harvest & +ve for same Phyt. 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**

A Wilcoxon Signed Rank Test for paired differences between expected and observed increase in recoveries due to a 2nd (day 10) harvest of baits, was used to test the hypothesis that **the second harvest increased recovery of species of Phytophthora by 7.5% or more**.

In this case, for the one-tailed test where α=0.10 and n=6, T_o=18 (where T_o is the expected or critical value).

In testing the hypothesis T_+=18 (where T_+ is the observed value), and since this was the same as the critical value (ie. T_o=18), it was concluded that this sample provided sufficient evidence to support the hypothesis ie. the 2nd harvest of baits substantially increased (7.5% or more) the recovery of species of *Phytophthora* from these samples.

Qn 2: Recovery of species of *Phytophthora*...

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**

When species of *Phytophthora* recovered from samples in the 2nd harvest were compared with those in the 1st, it was found that on 21 occasions they were the same; and on 9 occasions (or 30% of the time) the species were not the same!

This result might be expected given that the 2nd harvest of baits substantially increase the recovery rates of most species recovered in these samples.

Notably: on 1 occasion *P. cinnamomi* was recovered in the 2nd harvest of baits, while only *Phytophthora citricola* was recovered in the 1st. If not for the 2nd harvest of baits, this sample would have been classified as negative for *P. cinnamomi*.

Limitations:

1. These tests have not been repeated.

2. Technicians would be required to invest an additional *circa* 22 min to process a 2nd harvest of baits. This is equivalent to an increase of *circa* 76% of operator time invested in each sample.

3. The 2nd harvest would increase by almost 100% the consumables used when processing samples.

Recommendation:

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.

Part 3: Single baiting vs double baiting samples.

Palzer (1976) for *P. cinnamomi* and Bunny (pers com.) for *P. citricola*, found that some field samples that were 1st determined to be negative for Phytophthora, were subsequently determined to be positive when subjected to a 2nd baiting process ie. they were "double baited". In his experiments Palzer baited samples for 2 days with the root radicles of *Lupinus angustifolius*. Double baiting increased the number of samples determined to be positive by 26 to 142%.

The Study:

Locality of sites:	SW of WA
Collection method:	Kept uniform
No. of collectors:	Minimised
Time of year:	Winter-spring
Detection service:	VHS of CALM
No. of samples assessed:	117

Qn: For samples found to be negative for *Phytophthora* after a 1st 10 day baiting program, were a substantial (ie. increased recovery by 7.5% or more) number of them subsequently found to be positive for Phytophthoras when baited a 2nd time for 10 days?

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 *Phyt.* 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**

For a Wilcoxon Signed Rank one-tailed test where α=0.10 and n=7, T_o=23 (where T_o is the expected or critical value).

In testing the hypothesis T_+=22 (where T_+ is the observed value), and since this was less than the critical value (ie. T_o=23), it was concluded that this sample did not provide sufficient evidence to support the hypothesis ie. the 2nd (or double) baiting process did not substantially increase (by 7.5% or more) the recovery of species of *Phytophthora* from these samples.

What about Palzer?

Limitations:

1. these tests have not been repeated.

2. Double baiting would effectively double the duration taken to assess samples, and would increase by *circa* 65% (or 41 min) the time operators would be required to spend on a sample.

Recommendation:

Proceed with the single baiting process.

Recapping:

For **Study 1**: An ideal scenario.

45 sites from which 6 samples were taken from each ie. 270 samples.

75% of sites ie. 34 of them, would have been determined to be positive for *Phytophthora*.

Expect 3.5 samples/ site to be positive ie. 34* 3.5, or 119 samples.

At 5 day harvest of baits, expect 3 baits to be positive/ bait dish, or 3*119 = 357 isolates of *Phytophthora*.

At 10 day harvest also expect 357 isolates of *Phytophthora*.

Total 714 isolates of *Phytophthora*.

In this study 5% os isolates recovered were *P. cinnamomi*, therefore expect to recover 36 isolates of *P. cinnamomi*, and expect 678 isolates of other species of Phytophthora

ie. 3* *P. citricola*

2* *P. cryptogea*

1* *P. drechsleri*

2 Situations:

1. where long experience has shown that *P. cinnamomi* is by far the most abundant *Phytophthora* recovered from samples eg. northern jarrah forest: forest ecosystems, and

2. where long experience has shown that species other than *P. cinnamomi* are most abundant eg. coastal sand plain where the annual rainfall is between *circa* 500-700 mm, wetlands, and unsealed pavements.

Part 4: Factors affecting the recovery of species of *Phytophthora*: pH

Qn: Do species of *Phytophthora* infest *E. sieberi* cotyledons equally well under a range of pH conditions?

The Study:

P. cinnamomi
CIT 3 *P. citricola*

4 bait dishes per treatment; 4 treatments pH 4, 4.5, 5, and 5.5.

2 cotyledons of E. sieberi infested with *P. cinnamomi*.

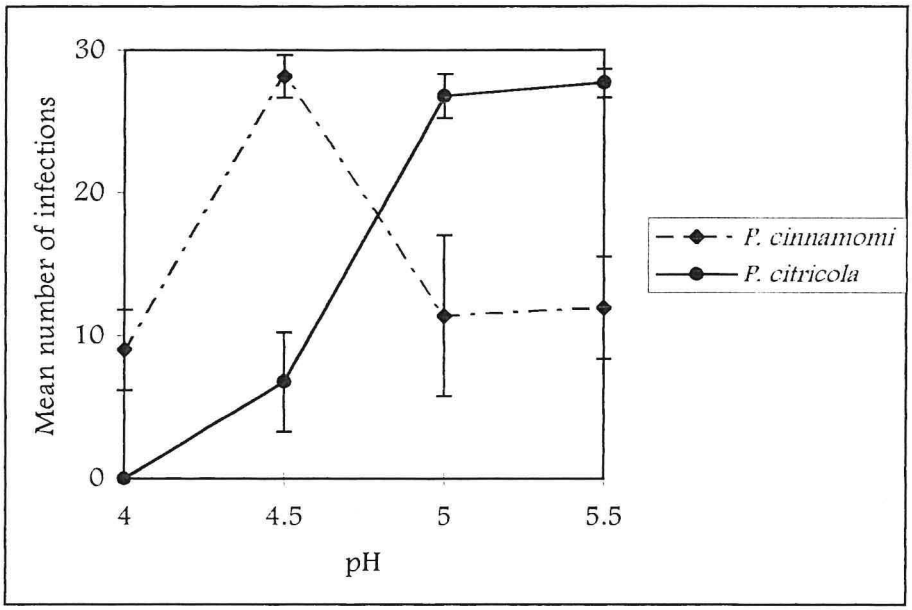
2 cotyledons of E. sieberi infested with CIT 3 *P. citricola.*

Non-sterile soil extract, 30 *E. sieberi* cotyledons.

Constant temperature for 48 hrs.

Harvest cotyledons onto selective antibiotic agar, and

Examine 48 hrs later



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.

Limitations:

This experiment has not been repeated.

Recommendations:

1. Repeat experiments like this comparing competitive abilities of a range of Phytophthoras, and Pythiums, under a range of pH.

2. To optimise recovery of *P. cinnamomi* from samples when in combination with *P. citricola*, buffer sample trays to pH 4.5.

Why CAGE Electrophoresis?

CRCTPP Queensland's response to the use of CAGE:

1. CALM had a CAGE facility at SID Como.

2. a. Oudemans and Coffey (1991) found that CAGE was superior to starch gel electrophoresis for resolving some isoenzymes of 3 species of *Phytophthora*, and subsequently concluded that CAGE had potential as a rapid diagnostic method.

   b. Subsequently Goodwin *et al.* (1995) got an article into Am. Path. Soc. Plant Disease Journal under "Spotlight on Diagnosis"- titled:

   > Use of Cellulose - Acetate Electrophoresis for Rapid Identification of Allozyme Genotypes of *Phytophthora infestans*.

   and concluded:

   i. As a component of and integrated disease management strategy, CAGE could be very useful to potato growers; and

   ii The useful life of this particular application may only be a few years, ie. as more sensitive DNA techniques become available.

3. Stages:
   i. Developing the protocols.

   ii. Determining the isoenzymic variation in morphological species of *Phytophthora*.

   iii. Comparing the traditional morphological method of identification with an isoenzyme (CAGE) method.

Stage 1: Developing the protocols for Cellulose Acetate Gel Electrophoresis (CAGE).

Objective:
to identify simple, and therefore cost effective, specific activity stains which when used routinely would discriminate between taxa of *Phytophthora*.

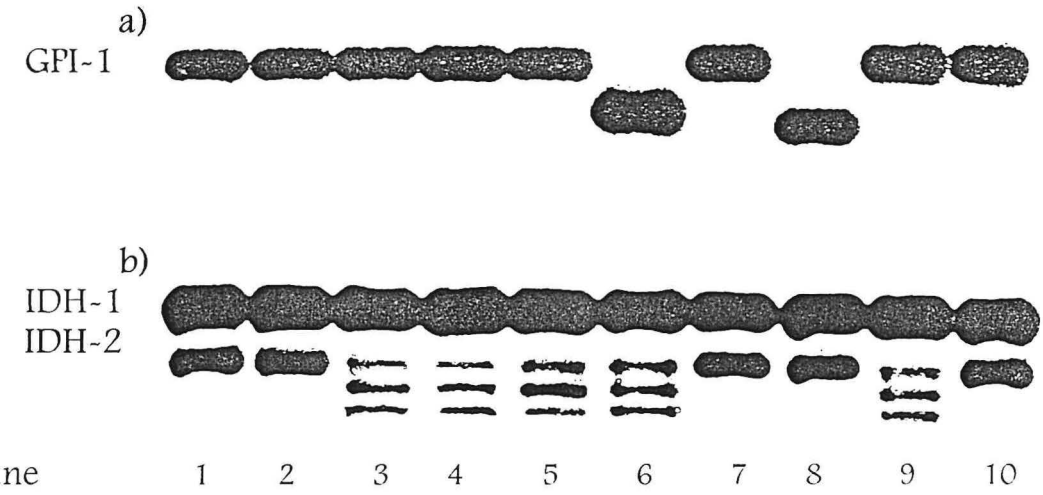
The Study:
Representative isolates of *Phytophthora* that had been determined on morphology to be:

P. cinnamomi
P. citricola
P. cryptogea
P. drechsleri
P. megasperma
P. nicotianae

were used to develop the protocols.

Results:

Examples of isoenzymes and allozymes developed on CAGE plates. 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.



Genotypic interpretation of electrophoretic phenotypes of a) GPI-1 and b) IDH-1 and 2.

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

Objective: to determine isoenzyme profiles for species of *Phytophthora*.

These profiles may then be incorporated into a library of profiles, with which the profiles of unknowns may be compared for rapid identification.

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

Recommendation:

Use the isoenzyme (banding) patterns derived from CAGE with GPI, IDH, LDH and MDH to distinguish between taxa of *Phytophthora.*

Stage 2: Determining the isoenzymic variation in morphological species of Western Australian isoaltes of *Phytophthora*.

Based on morphological determinations, isolates used in these studies were placed into their respective species.

An example of a study using P. megasperma:

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

Isoenzyme Class[1]	Reference Isolate	Hosts	Source[2]	GP I-1	IDH -2	LDH-2	MDH-2 3	Mating Type
A, B, and C	P 452 (*P. megasperma* BHR)	many	A	bd	ac	cc	dd dd	H[3]
a								
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.

Erwin and Ribiero's (1996) comment of the taxonomy of biotypes of *P. megasperma*: **Be conservative!**

Summary of isoenzymic variation.

	Researchers	No. Cultures Tested	No. Biotypes[1]
Morphologically distinct species			
P. cinnamomi	This study	194	1
P. nicotianae	This study	29	1
Morphologically indistinct species			
P. citricola	Bunny & Shearer	132	4
	This study	12 (ex B&S)	ditto
P. cryptogeal drechsleri	This study	121	10
P. megasperma	Bellgard & Carstairs	77	5[2]
Total		**553**[3]	**21**

1. Biotypes = isoenzyme classes identified.
2. Two of these biotypes shared many isoenzymes in common, and may be insipient taxa.
3. A high proportion of *P. nicotianae* isolates originate from Australian states other than Western Australia (M. Dudzinski pers. com.).

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

Example using *P. cinnamomi*:

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.

Limitations:

Comparing measures of diversity determined from CAGE data, with that determined from starch gel electrophoresis data.

Recall Oudemans and Coffey (1991): "CAGE was superior to starch gel electrophoresis for resolving some isoenzymes"!

Recommendations:

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. 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.

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.

Stage 3: Comparison of identification methods on the basis of accuracy, time, and cost.

The Study:

Double blind experiment.
50 isolates for isoenzyme identification.
Same 50 isolates, but with different identification numbers for morphological identification.
Reference library of isoenzymes for isoenzyme identification.
Key of Newhook *et al.* (1978) for morphological identification.

1. Accuracy:

a. Accuracy to morphological species.

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	Isoz. Pattern	Coralloid Hyphae		Incomplete Morph. Data	Complete Morph. Data	Morph. (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**

b. Accuracy to biotype (taxa) within morphological species.

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			
		Isoz. Pattern	Incomplete Morph. Data	Complete Morph. Data	Morph. (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.

2. Time taken to determine a diagnosis:

Time can be:

a. the duration (in days) from when a sample is received by a diagnostic service until they have completed their assessment and reported the identification; or

b. the amount of time diagnosticians are actively engaged with diagnosing the *Phytophthora* status of a sample.

All results were analysed by ANOVA of raw data.

Duration and operator time

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 Morph. 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)

Outcomes:

1. Duration/ Rapidity

a. With the isoenzyme method of identification, diagnosis of heterothallic isolates was reached considerably sooner, relative to the fuller morphological method.

b. Diagnosis of heterothallics was 220% (43 days) longer by the fuller morphological method than the isoenzyme method and homothallics was 68.34% (13.33 days) longer by the fuller morphological method than isoenzymic.

c. Isoenzymes extended the number of days to diagnosis of *P. cinnamomi* by 39.5% (5.57 days) beyond that taken by the short or hyphal morphological method.

2. Time operators were engaged in diagnosis.

a. Operators were engaged by the isoenzyme method of identification for considerably less time than by the fuller morphological method.

b. Heterothallics: 35.59% (21.55 min) longer.
Homothallics: 18.15% (10.87 min) longer.

c. Compared to the short/ hyphal method, isoenzymes were 49.07% (20 min) longer.

3. Cost.

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 Morph.)	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.

Study 1. 2000 northern jarrah forest samples.

		Frequency
Sample diagnosis:	-'ve for *Phytophthora*	0.65
	+'ve for *P. cinnamomi*	0.29
	+'ve for other *Phytophthora*	0.06

Study 2. 154 field samples from the karri forest.

		Frequency
Sample diagnosis:	-'ve for *Phytophthora*	0.57
	+'ve for *Phytophthora*	0.43

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	41	x	0.232	x	2.8	+	1.87	28.50
.....2. Karri forest	41	x	0.232	x	2.8	+	1.87	28.50
Short Morphological + Isoenzymes								
1. Northern jarrah forest	42.31	x	0.232	x	2.8	+	2.11	29.59
.....2. Karri forest	49.57	x	0.232	x	2.8	+	3.44	35.64
Isoenzymes Only								
1. Northern jarrah forest	48.06	x	0.232	x	2.8	+	3.15	34.37
.....2. Karri forest	50.61	x	0.232	x	2.8	+	3.64	36.52
Short + Fuller Morphological								
1. Northern jarrah forest	43.16	x	0.232	x	2.8	+	1.93	29.97
.....2. Karri forest	54.86	x	0.232	x	2.8	+	2.29	37.93
Fuller Morphology Only								
1. Northern jarrah forest	55.27	x	0.232	x	2.8	+	2.21	38.11
.....2. Karri forest	57.06	x	0.232	x	2.8	+	2.60	39.67

Outcomes:

a. Relative to short/ hyphal ID approach, the short ID + isoenzymes increased costs for the northern jarrah forest samples by 4.51% ($0.65). Which is negligible.

b. Short + isoenzyme approach increased the cost for karri samples by 29.13% ($4.20). This was a substantial increase.

Recommendations:

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.

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.

n.b. Direct Assay!