# CONTROL OF *PHYTOPHTHORA* AND *DIPLODINA* CANKER IN WESTERN AUSTRALIA

**ANNUAL REPORT TO THE**

**ENDANGERED SPECIES UNIT,**

**AUSTRALIAN NATURE CONSERVATION AGENCY**

**APRIL 1996**

Property and copyright of this document is vested jointly in the director, Australian Nature Conservation Agency and the Executive Director, WA Department of Conservation and Land Management.

. The Commonwealth disclaims responsibility for the views expressed

Department of Conservation and Land Management
Locked Bag 104, Bentley Delivery Centre, WA 6983

# CONTENTS

## PROJECT 1

## THE CONTROL OF *PHYTOPHTHORA* IN NATIVE PLANT COMMUNITIES


**PART A** - ***Application technologies and phosphonate behaviour in the host*** ......... 1

Summary ......... 1
Introduction ......... 2
1. Assessment of the efficacy of aerial application of phosphonate ......... 2
   1.1 Aerial application trial at South Sister Nature Reserve ......... 4
   1.2 Aerial application trial in the Gull Rock National Park ......... 5
   1.3 Aerial spray trial at Millbrook Reserve ......... 7
   1.4 Aerial application trial at North Dandalup ......... 8
   1.5 Field trial at Eneabba - West (distribution trial) ......... 8
2. Protection of regenerating hosts ......... 10
Cost of aerial application of phosphonate ......... 11
Current work ......... 11
Acknowledgements ......... 12
References ......... 12

**PART B** -***Application of phosphonate on threatened flora populations in the Albany District*** ......... 13

Introduction ......... 13
Summary of threatened flora phosphonate application to March 1996 ......... 13
General Comments ......... 16


## PROJECT 2


**DEVELOPMENT OF A DNA DIAGNOSTIC TEST FOR *PHYTOPHTHORA CINNAMOMI*** ......... 19

Summary ......... 19
SECTION 1: INTRODUCTION ......... 21
Background ......... 21
Objective ......... 23
Experimental Strategy ......... 23

SECTION 2: DEVELOPMENT OF A TEST FOR THE DETECTION OF *PHYTOPHTHORA CINNAMOMI* ..... 25
Background ..... 25
Experimental Results ..... 25
Isolation of a DNA sequence specific for *Phytophthora* ..... 25
Determine the nucleotide sequence of the isolated fragment of DNA ..... 25
Design specific PCR primers for the target site ..... 26
SECTION 3: DEVELOPMENT OF METHODS FOR THE EXTRACTION OF AMPLIFIABLE DNA FROM SOIL ..... 28
Background ..... 28
Experimental Results ..... 28
Extraction of DNA from mycelium ..... 28
Extraction of DNA from mycelium soil mixes ..... 28
Purification of extracted DNA ..... 29
Amplification of DNA from different soils ..... 29
Amplification of zoospore DNA ..... 29
Extraction of DNA from large soil samples ..... 30
SECTION 4: COMPOSITION OF THE PCR REACTION ..... 36
Background ..... 36
Experimental Results ..... 36
SECTION 5: SENSITIVITY OF DETECTION BY PCR ..... 38
Background ..... 38
Experimental Results ..... 38
SECTION 6: DEVELOPMENT OF AN INTERNAL STANDARD FOR THE PCR REACTIONS ..... 40
Background ..... 40
Experimental Results ..... 40
SECTION 7: PROCEDURE FOR ANALYSIS OF SOIL SAMPLES FOR DETECTION OF *P. CINNAMOMI* ..... 41
Procedure for sample preparation and analysis ..... 41
SECTION 8: APPLICATION AND ADOPTION ..... 43
Applications of the detection test ..... 43
Adoption of the detection test ..... 43
Multiple use of PCR technology ..... 44
Modes of technology adoption ..... 45
REFERENCES ..... 46
PUBLICATIONS FROM THIS PROJECT ..... 48

## PROJECT 3

## DEVELOPMENT OF GIS-BASED DECISION-SUPPORT TOOLS AND THE DATABASING OF *PHYTOPHTHORA*-SENSITIVE TAXA


*FINAL REPORT* ........ 49

Objective ........ 49

Introduction ........ 49

Data Systems ........ 49

Results based on action Plan ........ 50

1. Databasing specimen records ........ 50

2. Develop predictive GIS tools ........ 50

    2.1 Development of a predictive model ........ 50

    2.2 Use of remote sensing for monitoring vegetation health in relation to *Phytophthora* dieback ........ 51

3. Transfer specimen records into a relational database ........ 52

*EVALUATION OF CURRENT PROJECT PROPOSALS FOR NEW DIRECTIONS* ........ 52

Predictive modelling ........ 52

Databasing *Phytophthora* susceptible taxa ........ 53


## PROJECT 4


**THE CONTROL AND MANAGEMENT OF *PHYTOPHTHORA MEGASPERMA* IN THE NATIVE PLANT COMMUNITIES OF WESTERN AUSTRALIA** ........ 55

Introduction ........ 55

1. *Phytophthora megasperma* in native plant communities of Western Australia ... 55

    Recommendations ........ 57

2. On the significance of oospores and variability within *P. megasperma* ........ 58

    2.1 An NADP-isocitrate dehydrogenase-2 ab heterozygote of CIT 3 *Phytophthora citricola* arose by oosporogenesis rather than by heterokaryogenesis; and evidence for separate sub-cellular localisations of two NADP specific IDH enzymes in an oomycete ........ 58

        *Introduction* ........ 58

        *Procedure* ........ 60

        *Results* ........ 61

        *Discussion* ........ 62

2.2 Allozymes discriminate between morphologically indistinguishable pathogens recovered in competition experiments between IDH-2 genotypes of the CIT 3 form of *Phytopthora citricola*. .... 63

*Procedure* .... 64

*Results* .... 65

*Conclusions* .... 68

2.3 Measures of outcrossing rates in field populations of homothallic species of *Phytophthora* in the south-west of Western Australia. .... 69

*Introduction* .... 69

*Procedure* .... 70

*Results* .... 71

*Discussion* .... 73

3. Control of *Phytophthora megasperma* in native plant communities of Western Australia. .... 75

3.1 On the relative pathogenicities of taxa within the *P. megasperma* complex and other field occurring *Phytophthora*. .... 75

*Recommendations* .... 77

References .... 78

**PROJECT 5**

**IDENTIFYING, GERMPLASM STORAGE AND IN VITRO PROPAGATION OF *PHYTOPHTHORA* AND CANKER THREATENED TAXA** .... 83

1. Identification of rare and threatened flora at risk from dieback disease .... 83
2. *In vitro* propagation .... 83
3. Cryostorage .... 83
4. Seed collection .... 83
5. Seed storage, viability testing and inventory system .... 84
6. Future collections and proposed developments .... 86

Appendix 1: Accessions List .... 90

**PROJECT 6**

**CONTROL AND MANAGEMENT OF *CRYPTODIAPORTHE MELANOCRASPEDA* CANKER THREATENING *BANKSIA COCCINEA*** .... 97

Summary .... 97

Introduction .... 98

Project Outcomes .... 99

1. To quantify the extent of regeneration in severely degraded stands in the absence of fire .... 99

2. To provide criteria for determining the appropriate stage at which to apply prescribed fire to manage disease within particular stands ... 99

3. To determine whether canker disease develops more slowly in extensive even-aged stands than in mixed age stands ... 99

Work Undertaken ... 100

Outcome (1): Regeneration within disease-affected stands in the absence of fire ... 100

Outcome (2) Criteria for appropriate application of prescribed fire to disease-affected stands ... 100

Outcome (3): Development of disease in extensive even-aged stands ... 103

References ... 103

## PROJECT 1

## THE CONTROL OF *PHYTOPHTHORA* IN NATIVE PLANT COMMUNITIES

## PART A

### *Application technologies and phosphonate behaviour in the host*

B. Komorek, B. Shearer, M. Blumberg, C. Crane and R. Fairman

## SUMMARY

Phosphonate is a very useful fungicide which can successfully be applied on native vegetation to control *Phytophthora cinnamomi* infections. Aerial spraying of the chemical phosphonate is a promising method of application which permits treatment of remote areas cost-effectively. The chemical can be applied to the whole plant canopy effectively.

When applied at low concentration rate (10 %), phosphonate protects plants for twelve to eighteen months but is generally too low to achieve long term protection from infection. Higher concentrations (20 and 40 %) are currently being field tested and appear to ensure longer protection due to much higher initial levels of the active ingredient in plant tissue. Experiments are continuing to determine whether these higher concentrations cause any phytotoxicity to plants.

Phosphonate is distributed in the shoots and roots of the treated plants and the experiment involving *Banksia telmetia* demonstrated that the relative distribution of phosphite ion between shoots and roots changes. The chemical was equally distributed in the shoots and roots in the first harvest but in the subsequent sampling its concentration, although overall significantly lower, was much higher in roots than shoots.

40 % phosphonate applied in our trials is currently the highest concentration commercially available and has not been used to treat plants before.

A new experimental system involving miniplots and the use of an ultra-low volume sprayer has been established. It will allow economical testing of a range of options for foliar application of phosphonate to protect host regenerating in disease centres from infection by *P. cinnamomi*.

## INTRODUCTION

Systemic fungicides such as the phenylamides and phosphonates developed in recent years have provided new opportunities for chemical control of soil-borne *Phytophthora* species that cause diseases of many field and plantation crops. In the past, diseases caused by *Phytophthora* were usually controlled using specific cultural practices, disinfectants or soil fumigation. However, these methods were difficult to apply in field conditions. The chemicals, being non-selective or toxic to plants, could not be used to control the pathogen affecting native vegetation.

The discovery of systemic fungicides, characterised by symplastic mobility, open new possibilities of *Phytophthora* disease control and prompted new research into the effectiveness of such chemicals in controlling some of the *Phytophthora* species present in our forests, heaths and shrublands. Used as part of an integrated management strategy the chemical phosphonate (mono di-potassium phosphonate) promises to be a very useful agent in the control of *Phytophthora cinnamomi* in native plant species.

The fungicide has been field trialed by CALM in various areas in the south-west on plant communities already infested with *Phytophthora cinnamomi*. The trials have shown that one application of phosphonate gives excellent control of the fungus over several years in some *Banksia* species (Shearer, *pers. comm.*).

The chemical can be injected into the trunk using a hydraulic injector. This technique is successful with phosphonate because the $PO_3^-$ ion is transported in the conductive tissues of the tree to the leaves and from leaves to roots. Phosphonate can also be sprayed onto the foliage using a backpack sprayer. Both these methods of phosphonate application have proven to be excellent in controlling infections occurring in small areas where the chemical can be applied manually. However, in order to control the spread of infection effectively over medium to large areas, the chemical must be applied by aircraft. Aerial application of phosphonate permits treatment of remote areas cost-effectively, without disturbance to the treated and neighbouring areas.

Although phosphonate does not eliminate disease, it is a powerful prophylactic fungicide. It is the only short to medium term option to prevent further loss of rare and endangered plant species from dieback through effective protection of healthy plant communities, in areas where the pathogen is present.

## 1. ASSESSMENT OF THE EFFICACY OF AERIAL APPLICATION OF PHOSPHONATE

In order to assess the efficacy of aerial application of phosphonate we set up a number of field experiments involving a range of plant species located in a number of areas in the south-west of Western Australia (Figure 1). These experiments included four trials in which the chemical was applied from the aircraft and four trials using a hand-held ultra-low volume sprayer.

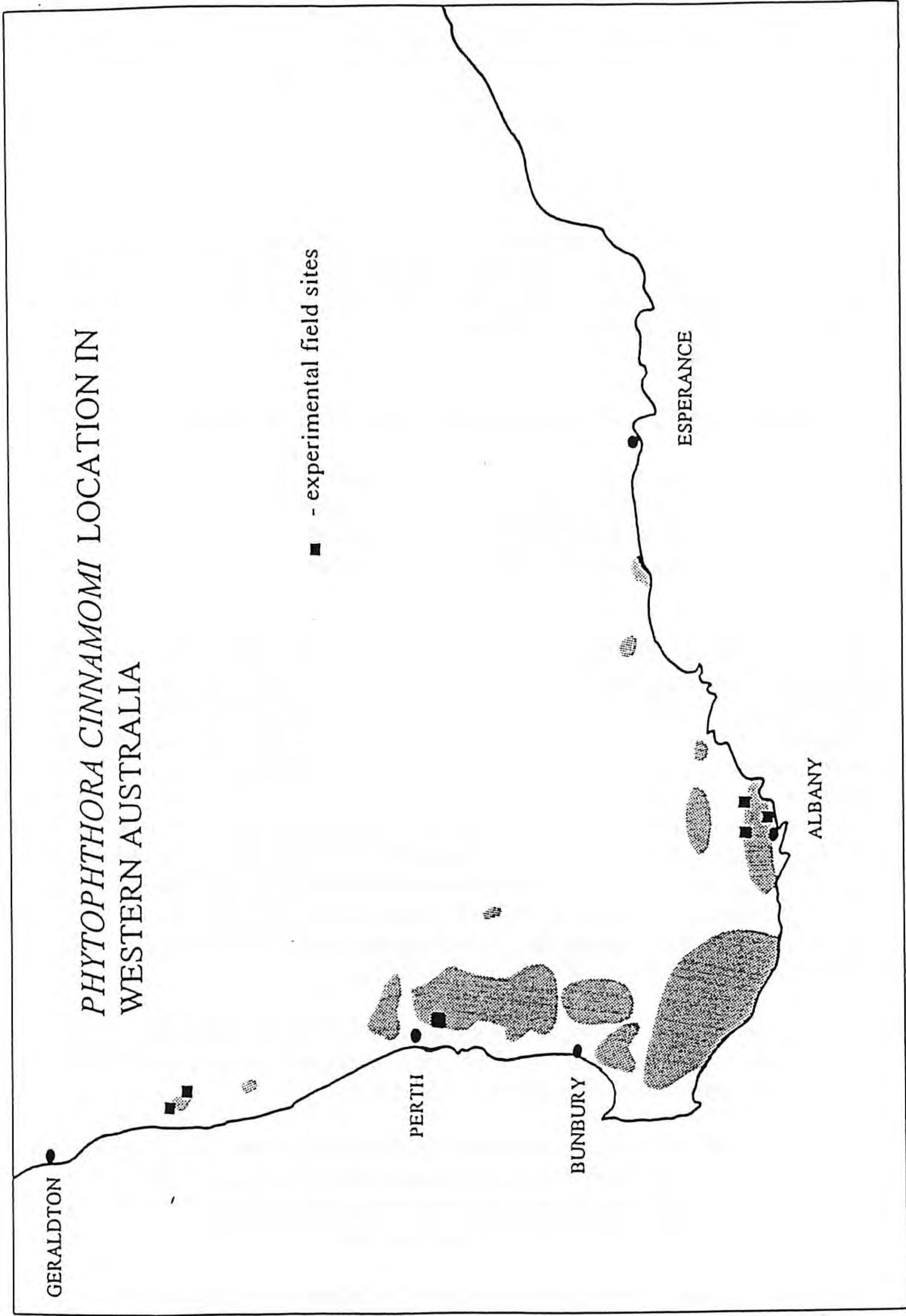



Figure 1. Location of experimental field trials in Western Australia

In the earlier experiments involving the backpack sprayer, phosphonate was applied to runoff (high volume application) and the applied concentration of the chemical that controlled the pathogen ranged from 0.2 to 0.5 %. Aerial application experiments involve low volumes per unit area but high chemical concentration. The concentrations we have been testing are 10, 20 and recently 40 %. 40% is currently the highest concentration commercially available and has not previously been used to treat plants.

The aim of the trials is to provide information on the appropriate rates of application and the duration of protection achieved by utilising phosphonate spraying techniques. Results from these experiments will be used to formulate a management strategy and to determine when re-spraying is required.

## 1.1 Aerial application trial at South Sister Nature Reserve

In early May 1993 we commenced an aerial application trial at South Sister Nature Reserve where there is a population of rare and endangered *Banksia brownii* (feather-leaved banksia). This species of *Banksia* occurs in only a few locations. It is very susceptible to infection from *Phytophthora* and all populations are infected with the pathogen.

The South Sister site (8.8 ha) was sprayed with an 8.5% concentration of Potassium phosphonate, using Synertrol (0.5%) as a wetting agent, in a low volume of 26 l/ha. A follow-up spray was carried out six weeks later. A local contractor (Giles Aviation) undertook the spraying. Twenty *B. brownii* trees were randomly selected and marked. Their health was assessed for any visible signs of phytotoxicity, but none were observed.

The concentration of the active ingredient after the first application was a very low 1.3 $\mu g\ g^{-1}$ (average from 20 trees). It was boosted by the second application to 6.2 $\mu g\ g^{-1}$ however, this decreased to a low level of 0.6 ppm six months after spraying (Komorek *et al*, 1995). Despite the low concentration of the phosphite ion in plant tissues the plants remained healthy for twelve months.

A Year and a half after spraying the concentration of phosphonate residues decreased to 0.17 $\mu g\ g^{-1}$ and two marked trees died. Two years after the application the treated plants had no detectable phosphonate in the plant tissue.

The concentration of the fungicide and the rate per hectare applied to this site was too low to ensure long term protection of the treated plants. The plants were only protected for up to eighteen months. In order to prevent further deaths in the area phosphonate will have to be applied at higher rate and concentration.

The results from the most recent trials indicate that higher concentration of phosphonate can be applied to plants without causing phytotoxicity, therefore after careful assessment of the most recent work it is planned that the area will be resprayed with 40 % phosphonate at 60 l/ha.

## 1.2 Aerial application trial in the Gull Rock National Park

A fully replicated field trial in the Gull Rock area near Albany was established in April 1993 to determine the effectiveness of phosphonate in the control of *Phytophthora cinnamomi* in *Banksia coccinea* (red-flowered banksia). The fungus has had high impact in that area causing wide spread destruction.

In order to assess plant mortality rate, the plots were monitored for six months prior to spraying then treated in early November 1993. A follow-up spraying was carried out in the first week of December 1993.

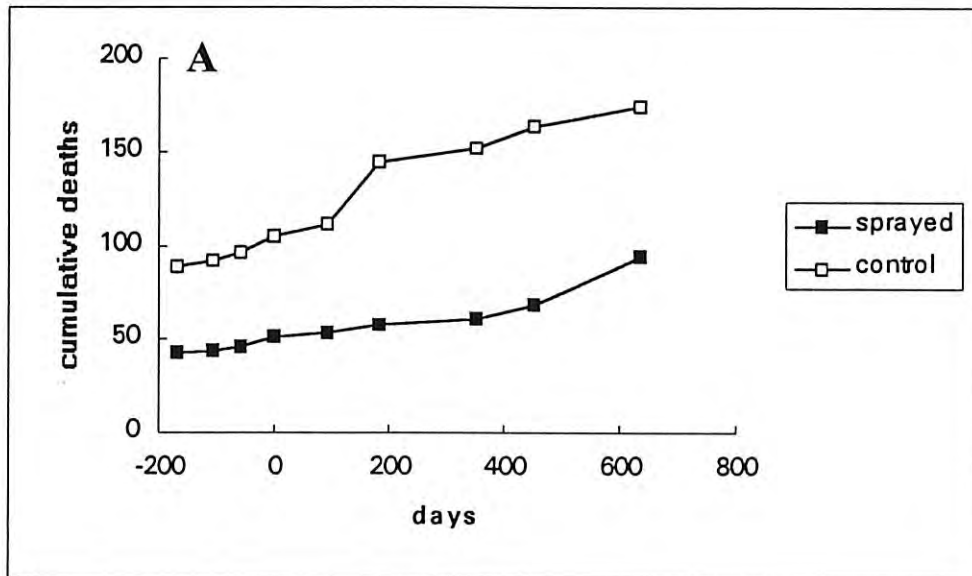
The number of dead plants within each plot was measured to establish the duration of the effectiveness of the fungicide. Plant samples (shoots and roots) were taken for chemical analysis to determine the concentration of the phosphite ion in plant tissue. The plots were sprayed with 10 % phosphonate at 60 l/ha and the second spraying in December was done at the same rate.

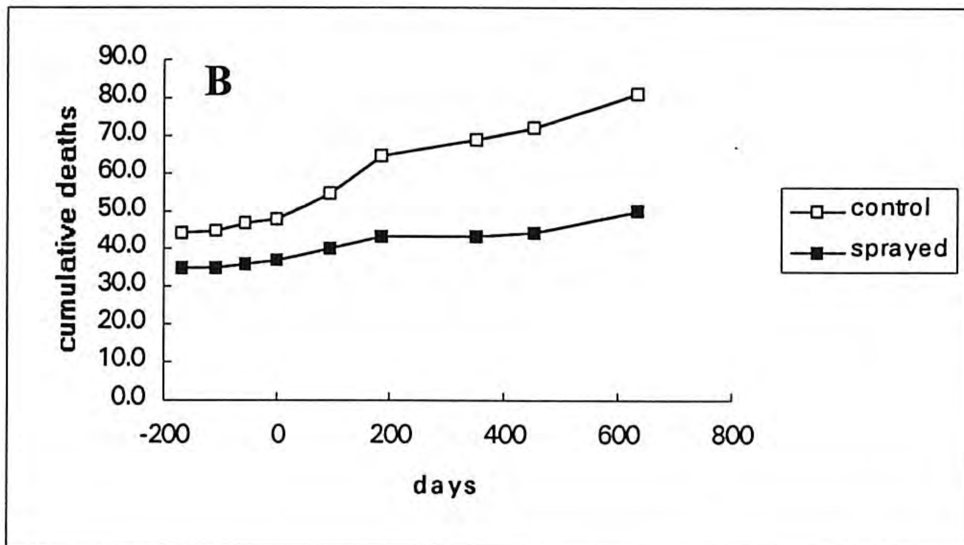
The concentration of the active ingredient ranged from 0.9 to 4.3 $\mu g\ g^{-1}$ after the first spraying and 4.1 to 34.2 $\mu g\ g^{-1}$ after the second application (Komorek *et al*, 1995). One year after the fungicide application the concentration of phosphite ion decreased to between 0.18 and 0.4 $\mu g\ g^{-1}$. Two years after treatment the plants had no detectable phosphonate in the plant tissue.

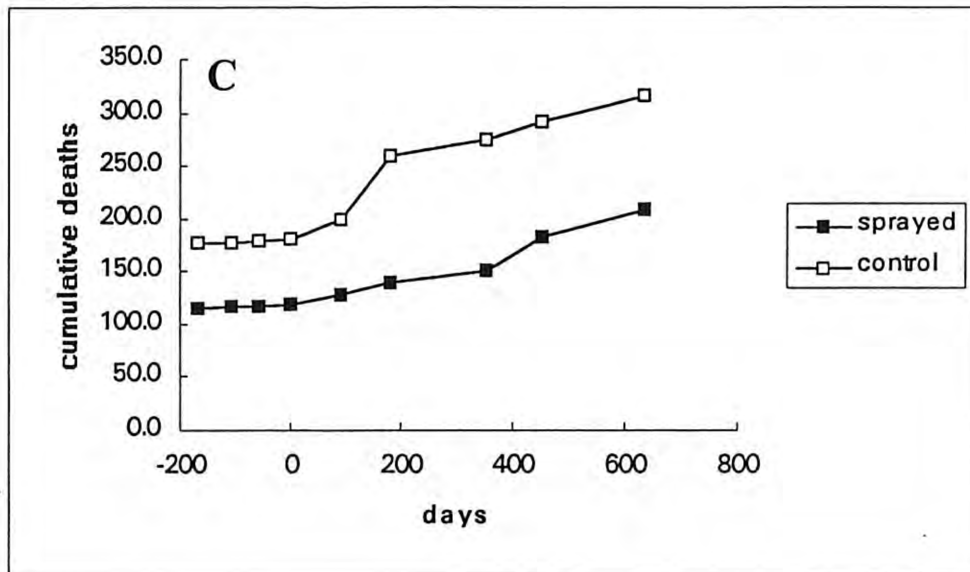
Plant mortality is the ultimate measure of the treatment's effectiveness and in our experiment it has been expressed as cumulative deaths within each plot. Phosphonate, applied aerially, reduced plant deaths in the treated areas (Figure 2) and controlled the disease in all treated plots, in all reps, for twelve to eighteen months. After one year the number of deaths in treated plots started to increase in reps **A** and **C** and after sixteen months the mortality rate, in treated plots and controls, was similar. There was a substantial increase in the number of dead plants in all control plots 90 to 200 days after spraying. That increase coincided with a period of very dry conditions in the 1993/94 summer and early autumn. Drought put additional stress on plants already affected by the fungus.

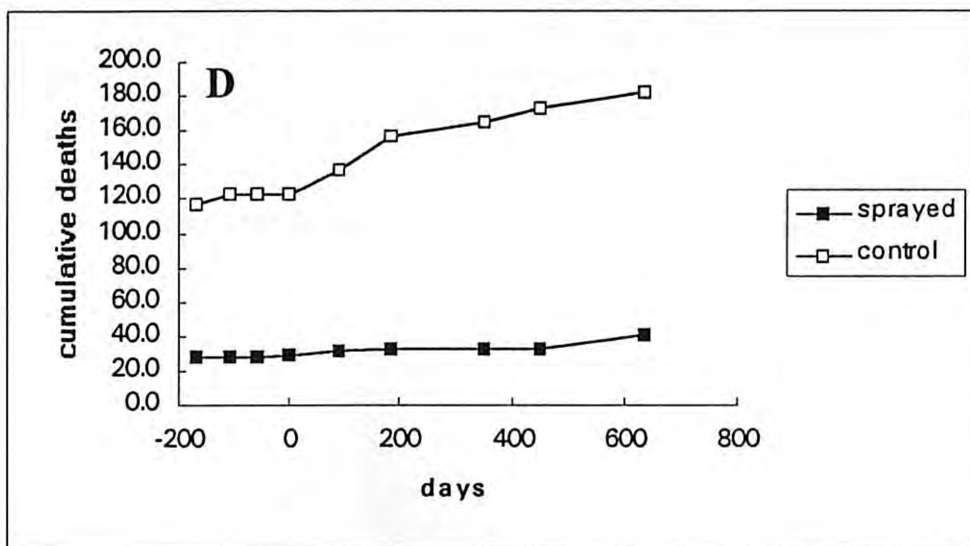
No such increase was recorded in the treated plots in the same period, indicating that the sprayed plants were protected by the chemical and therefore, better able to survive difficult environmental conditions because their roots sustained less damage from the activity of the pathogen.

**Figure 2** Gull Rock aerial application trial - number of dead plants.






## 1.3 Aerial spray trial at Millbrook Reserve

*Phytophthora cinnamomi* has had a high impact on a population of *Banksia brownii* at Millbrook Reserve, 30 km north of Albany. A ground application field trial (using backpack sprayer) carried by CALM several years ago demonstrated that phosphonate protected the plants for up to three years (Shearer, *pers. comm.*).

In November 1993 almost the whole population of *B. brownii* (6 ha) was sprayed aerially, using low volume application. Plants in the Millbrook Reserve and Gull Rock were sprayed at the same time with similar application rates. The concentration of the active ingredient averaged 4.5 $\mu g\ g^{-1}$ after the first application and 22 $\mu g\ g^{-1}$ after the follow-up treatment. Two years after the application, treated plants had no detectable phosphonate in the plant tissue.

In both Gull Rock and Millbrook trials root samples were also collected. The results indicate that phosphonate was distributed in roots as well as shoots.

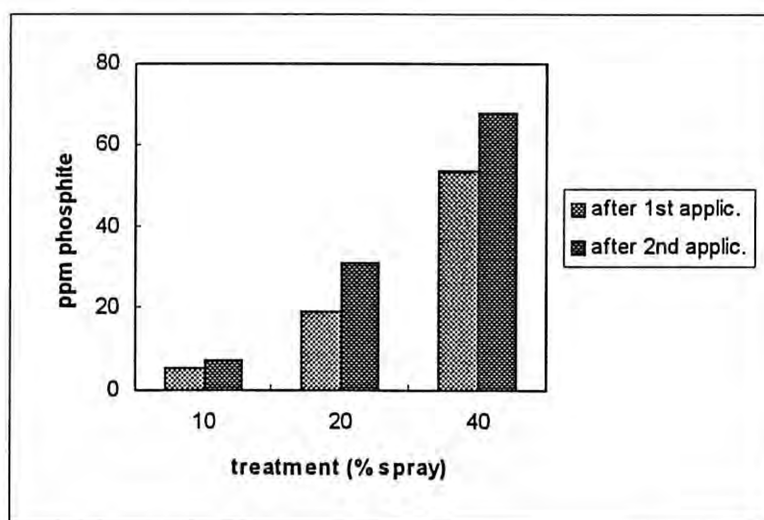
## 1.4 Aerial application trial at North Dandalup

In October 1994 we established a new phosphonate aerial application trial involving three plant species: *Banksia attenuata, Banksia ilicifolia and Banksia menziesii.* Plants were sprayed twice with three concentrations (10, 20 and 40 %) at 60 l/ha of phosphonate, using 2% Synertrol as a wetting agent.

The first spraying was done in early November and the second spraying four weeks later. The second treatment increased the concentration of the active ingredient significantly at all spray concentration rates.

The following figure (Figure 3) illustrates the concentration of phosphite ion in leaf tissue after the first and follow-up spraying. 40 % treatment is likely to ensure long term protection due to the high initial phosphonate concentration in plant tissue. This treatment did not result in any burning of leaf margins but in order to establish whether the high concentration does definitely not cause any phytotoxicity we will also determine the effect of phosphonate on flowering and seed setting. Seed from the sprayed and control plots were collected and will be counted and germinated.

**Figure 3** Phosphite concentration ($\mu g\ g^{-1}$) in leaf tissue of *Banksia* plants.




## 1.5 Field trial at Eneabba - West (distribution trial)

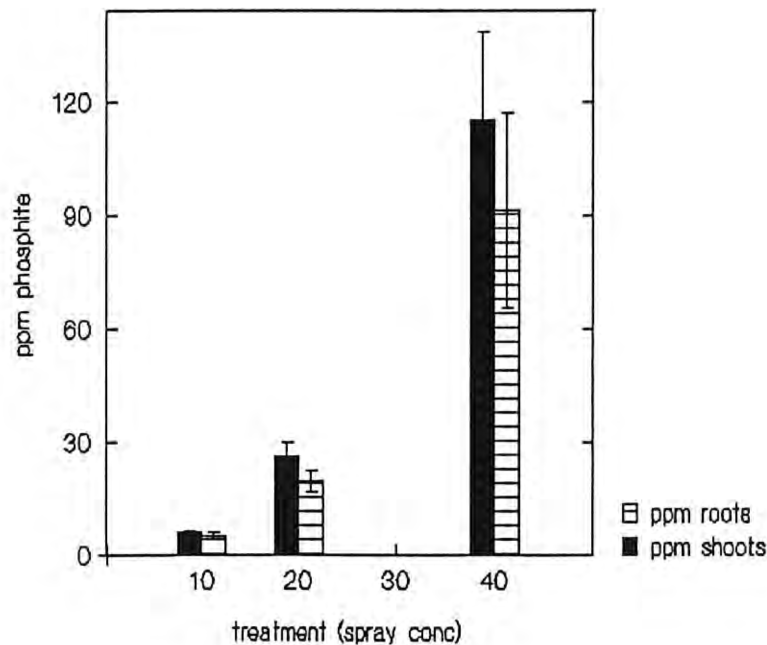
A new phosphonate application was established in November 1994. Small plots (4 m *1 m) containing regenerating seedlings (post fire) were sprayed twice with three different concentrations of phosphonate (10, 20 and 40 %) using hand-held ultra-low

volume sprayer. Each plot was sprayed at the rate equivalent of 15 l/ha and the spray was mixed with 2 % Synertrol. The number of droplets per unit area and droplet size were similar to droplet number achieved by aerial application at North Dandalup as determined by water sensitive papers.

The use of an ultra-low volume sprayer and small plots is a cost effective method of doing experiments and providing results that can be directly extrapolated to larger, aerial application trials. This particular method is especially useful when studying the relative distribution of phosphonate in the roots and shoots.

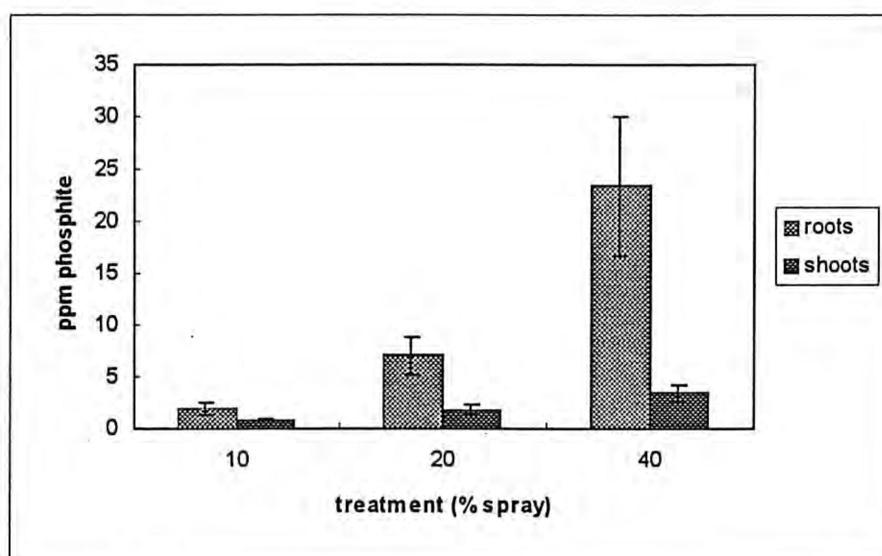
Leaf and root samples from the first (spring) harvest (after two applications) were analysed and the data is presented in Figure 4. The average concentration of phosphite in the leaf tissue was 5.9, 26.3 and 115.4 $\mu g\ g^{-1}$, root tissue 5.2, 19.6 and 91.3 for 10, 20 and 40 % treatment respectively (Komorek *et al*, 1995). The concentration of phosphite ion in the first harvest was slightly higher in shoots than in roots at all spray concentrations, but the difference was not statistically significant ($P=0.01$).

**Figure 4** Phosphite concentration ($\mu g\ g^{-1}$) in leaf and root tissue of *Banksia telmetia* plants after treatment.




Six months later the concentration of phosphonate decreased significantly in all treatments but was significantly higher in roots than shoots (Figure 5). This drop in overall plant tissue phosphonate concentration coincided with a large increase in the mass of each individual plant. Being a two year old post fire regeneration, the plants' above-ground dry weight increased by 400 % in all treatments and the controls, causing dilution of phosphonate and therefore lowering its concentration.

**Figure 5** Phosphite concentration ($\mu g\ g^{-1}$) in leaf and root tissue of *Banksia telmetia* plants six months after the treatment.




The results from this trial indicate that the concentration of phosphonate in young plants decreases substantially over a relatively short time because of a dilution factor due to the high growth rates.

The relative distribution of the active ingredient between shoots and roots changes during the growing season but the mechanism that is responsible for these changes and the ultimate fate of the phosphite ion are unknown and must be studied in detail.

## 2. PROTECTION OF REGENERATING HOSTS

An experimental system has been established that will allow economical testing of a range of options for foliar application of phosphonate to protect the host, regenerating in disease centres, from infection by *P. cinnamomi*. The system uses a combination of miniplots, a hand held micro-drop applicator to simulate aircraft application and controlled introduction of inoculum.

To test the system, plots have been established in naturally regenerating *Banksia coccinea* in a disease centre in Gull Rock National Park and naturally regenerating *B. telmatiaea* in a disease centre near Eneabba. After pre-treatment monitoring in October 1994-August 1995, the regenerating Banksias were sprayed once or twice with 20 and 40% phosphonate or not sprayed at all. Mortality has continued to increase in the unsprayed plots, but not in the sprayed plots.

Variation in host and inoculum distribution causes considerable variation in mortality in disease centres, which necessitates monitoring of treated areas over a number of years. In plots established in a disease centre in *Banksia* woodland on the Swan Coastal Plain, host and inoculum distribution is being controlled by planting seedlings and introducing inoculum on a systematic grid arrangement. These plots will be planted this autumn and inoculated in winter to test various spray concentration and surfactant combinations.

## COST OF AERIAL APPLICATION OF PHOSPHONATE

Phosphonate is sold in Australia under various brand names and available in 20 or 40 percent strength with 40 % being available only recently. The cost of the chemical has dropped by about half in the last four years and currently the price is around $2.85/litre of 40 % strength if purchased in bulk. 40 % phosphonate is actually cheaper than 20 % so in situations where it is necessary to apply fungicide containing only 20 % of the active ingredient, it is more cost effective to purchase the concentrated solution and dilute it to the desired strength. In Western Australia the only registered concentrated (40 %) phosphonate is sold as FOS-4-PINE by Robert Linton Pty Ltd.

At present the cost of the concentrated (40 %) chemical per hectare is $171 for single spraying when applied at 60 l/ha and $342 if a follow-up treatment is applied. The cost of aircraft hire is around $300-500/hour. The time that is required to spray any particular area can vary considerably depending mainly on the following factors:

- size, shape and location of the treated area
- distance from the air strip as frequent chemical and fuel reloading is required
- maximum load of the aircraft
- type of aircraft's spraying system/equipment

As an example, the time required to spray South Sister Nature Reserve (~9 ha) including loading was around 1.5 hours.

## CURRENT WORK

- Chemical analysis of plant material from all the aerial and ground applications is continuing. The analysis involves shoot and root material from eight field trials. This will enable us to determine the longevity of the chemical in plants and to establish the length of protection achieved by various phosphonate application rates. It will ensure the selection of the most efficacious rate of application as well as tte timing of subsequent treatments.
- We determined that the application of concentrated phosphonate does not affect the growth of plants. We are going to commence a germination experiment to determine the effect of the fungicide on seed number and viability.

- As 40 % phosphonate applied at 60 l/ha appears not to cause phytotoxicity to treated plants we will determine the maximum concentration of the chemical in tissue that is tolerated which does not result in leaf burning.
- We will field test a number of plant species of different ages from various locations. This information is necessary in formulating prescriptions of phosphonate application as the chemical will usually be applied in areas of diverse species and age composition.
- We will also determine whether phosphonate is stored in plant tissue (roots ?) as polyphosphonate and acts as a source of the phophite ion.
- Earlier established aerial application experiments in the Albany area (sprayed with 10 % phosphonate) will be re-treated with 40 % phosphonate in April this year.
- The longevity of the effect of fungicide application will be tested by stem inoculation of selected trials that have no detectable phosphonate in the plant tissue.

**ACKNOWLEDGMENTS**

We wish to thank the staff of Western Australian Chemistry Centre, Agricultural Chemistry Laboratory: Dr Neil Rothnie for his aid and advice in this project and to Wayne Best for his assistance in the quantitative determinations of phosphonate in plant samples.

## REFERENCES

Komorek, B.M. (1995). The control of *Phytophthora* in native plant communities. *Annual report to the Australian Nature Conservation Agency* - March 1995.

**PROJECT 1 (cont.)**

# THE CONTROL OF *PHYTOPHTHORA* IN NATIVE PLANT COMMUNITIES

## PART B

## *Application of Phosphonate on threatened flora populations in the Albany District*

K. Gillen, M. Grant and E. Hickman

## INTRODUCTION

In 1993 a program was implemented in CALM's Albany District for the application of phosphonate to populations of threatened flora, critically affected by *Phytophthora*. Based on the research outlined in Part A, appropriate concentrations and application procedures were determined. The program for each population generally involved two foliar sprays within a 6 week period.

## SUMMARY OF THREATENED FLORA PHOSPHONATE APPLICATION TO MARCH 1996

The relevant species and actions of the Phosphonate Program to date are as follows:

**1993 Program**

| Species | Location | Comments |
|---|---|---|
| *Andersonia* sp. Two Peoples Bay | Boulder Hill | Set up two 20mx20m plots, one control (unsprayed), 52 plants and other sprayed with 0.3% Phosphonate, 57 plants. Sprayed 23.6.93 and 30.6.93. |
| *Banksia brownii* | Cheyne Road | Sprayed with 0.3% Phosphonate on 23.6.93 and 30.6.93, 35 plants. |

**1994 Program**

| Species | Location | Comments |
|---|---|---|
| *Banksia brownii* | Vancouver Penninsula | Sprayed with 0.3% Phosphonate on 10.1.94 and 1.3.94, ~310 plants. |
| | Hassell Hwy N.P. | Sprayed with 0.3% Phosphonate on 11.1.94 and 10.3.94, 84 plants. |
| | Hassell Beach Rd, Waychinicup N.P. | Sprayed with 0.3% Phosphonate on 16.2.94 and 10.3.94, ~58 plants. |

| | | |
|---|---|---|
| | Mt Hassell, S.R.N.P. | Sprayed with 0.3% Phosphonate on 12.1.94 and 3.3.94, ~375 plants. |
| *Isopogon uncinatus* | Vancouver Penninsula | Sprayed with 0.3% Phosphonate on 10.1.94 and 1.3.94, 9 plants. |
| | Mutton Bird | Sprayed with 0.3% Phosphonate on 14.2.94 and 1.3.94, 96 plants. |
| *Darwinia wittwerorum* | Talyuberlup | Set up 4mx4m plot. Sprayed with 0.3% Phosphonate on 14.6.94 and 5.7.94. |
| *Darwinia oxylepis* | Mondurup Pass | Sprayed with 0.3% Phosphonate on 14.6.94 and 5.7.94. |

**1995 Program**

| Species | Location | Comments |
|---|---|---|
| *Banksia brownii* | Coyanarup | Survey for presence of dieback Summer '96. |
| | Mt Success | Survey for presence of dieback Summer '96. |
| | Mondurup | Surveyed for presence of dieback June '95. No *B.brownii* located. |
| | Moongoongoonderup | Surveyed for presence of dieback April '95. *Phytophthora cinnamomi* present. Further survey required. |
| | Yungermere | To be sprayed. |
| | Ellen Peak | 2 populations sprayed August '95, 2nd spray required. |
| | Cheyne Road | Sprayed June '95, 2nd spray required. |
| | Vancouver Penninsula | To be sprayed. |
| | Hassell Hwy N.P. | Sprayed June '95, 2nd spray required. |
| | Hassell Beach Rd, Waychinicup N.P. | Sprayed June '95, 2nd spray required. |
| | Mt Hassell, S.R.N.P. | Reviewed last years spray June '95. Ground spray legistically difficult, consider aerial spray. |
| *Isopogon uncinatus* | Vancouver Penninsula | Review last years spray and respray. |
| | Mutton Bird | No *Phytophthora cinnamomi* present, no need to spray. |
| *Banksia verticillata* | Waychinicup N.P. | Surveyed for disease presence April '95, decline due to aerial canker |

| | | |
|---|---|---|
| | | (*Zythiostroma*). |
| *Darwinia oxylepis* | Talyuberlup | Review results of last years spray program. |
| *Darwinia wittwerorum* | Mondurup Pass | Review results of last years spray program. |
| *Dryandra ionthocarpa* | Kamballup | Samples taken and analysed for presence of dieback. No *Phytophthora cinnamonii* present so spraying not required, more survey needed, particularly western population. |
| *Dryandra montana* | Bluff Knoll | Survey for presence of dieback. |
| *Andersonia axilliflora* | Ellen Peak | Priority 2 species surveyed and considered impractical to spray. Further survey of other populations needed. |
| *Andersonia sp.* Two Peoples Bay | Boulder Hill | Reviewed effect of Phosphonate spray of 20mx20m monitoring plot in June '95. Found Phosphonate concentration used was in effective to stem decline due to dieback. 4 new monitoring plots set up with control (unsprayed) + 3 conc. (0.5%, 1.0%, 2.0%). |
| | Goodga River | 3 new monitoring plots set up of 4 cells (5mx5m) with control (unsprayed) + 3 conc. (0.5%, 1.0%, 2.0%). |
| | Moyle Road | 10mx10m decline monitoring plot set up. |
| | Reservoir Hill (new) | 10mx10m decline monitoring plot set up. |
| *Lambertia orbifolia* | Narrikup | D.R.F. in Southern Forest Region included in Albany District Phosphonate Program because of its proximity. Sprayed once 5.5.95, second spray 15.6.95. New plants found first spray 15.6.95, second spray 2.8.95. |

| | | |
|---|---|---|
| *Andersonia* Mt Lindsay | Mt Lindsay | D.R.F. in Southern Forest Region included in Albany District Phosphonate Program because of its proximity. Surveyed for presence of dieback.Possible larger populations found therefore not such a priority. |

**Update of 1996 Program**

| Species | Location | Comments |
|---|---|---|
| *Adenanthos cunninghamii* | All | On calcareous soils so unlikely to have *Phytophthora cinnamomi* associated with it. However samples still necessary. |
| *Adenanthos dobagii* | Quion Head | Surveyed for dieback Summer '94. Not present on site. |
| *Adenanthos ellipticus* | East Mt Barren | Surveyed for dieback August '94 and June '95. No *Phytophthora* recovered from dead plants but *Phytophthora megasperma* known to be present down slope of Hamersley Drive. Further survey needed. |
| | Thumb Peak | Surveyed for dieback January '94. Population very healthy (no dead plants) therefore assume no dieback present. |
| | West Mt Barren | Survey for presence of dieback. |
| *Adenanthos pungens* | Hamilla Hill | Surveyed for dieback June '94. Requires further sampling. |

## GENERAL COMMENTS

***Banksia brownii*** is in the second year of the Phosphonate program. The focus for this species is on the populations at greatest risk. The Ellen Peak, the Cheyne Road, the Hassell Hwy and the Hassell Beach Road populations have all received their first spray of 0.3% Phosphonate solution. The Ellen Peak population has been extended from one population of approx. 300 plants to 3 populations of a total of approx. 550 plants all regenerating from the fire in 1991. Due to the close proximity of the Ellen Peak population of *B.brownii* to a population of *Lambertia fairallii* it is also included in the spray program. The Vancouver Peninsula population is still to be sprayed and the Mt Hassell population was visited, however this population is consider beyond the scope of a ground spray, at this time, due to the logistical difficulties involved. This situation is being reviewed in March 1996.

***Andersonia* sp. Two Peoples Bay** Boulder Hill population had two 20m x 20m monitoring plots set up in 1993, one plot sprayed with 0.3% Phosphonate solution and

the other plot unsprayed as a control. The results from these plots were reviewed in June of this year and it was found there was no difference in the decline rates of plants from *Phytophthora* infection between the sprayed and the unsprayed plot. It was decided that a new set of monitoring plots should be set up. This time 6 monitoring plots were set up consisting of 4 cells (20m x 20m) with a control (unsprayed) and 3 different concentrations of Phosphonate solution (0.5%, 1.0%, 2.0%). These monitoring plots have received there first spray and are awaiting their second spray. At the Goodga River population 3 similar monitoring plots were set up also each consisting of 4 cells, however this time the cells are only 5m x 5m as the density of plants at this population is much higher. This population was burnt in 1989 and 1991. The cells consisted of a control (unsprayed) and 3 different concentrations of Phosphonate solution (0.5%, 1.0%, 2.0%). The Moyle Road population discovered in January this year and the Reservoir Hill population discovered in June this year are not as yet included in the Phosphonate spraying program. However it was decided to set up a 10m x 10m monitoring plot at each site, in order to record the decline in plant numbers, due to dieback, and to estimate regeneration following a possible fire event.

***Lambertia orbifolia*** is a Declared Rare Flora species from the Southern Forest Region with 2 populations near Narrikup. Since the Southern Forest Region does not have a Phosphonate program and this population is in close proximity to the Albany District it has been included in the Albany District Phosphonate Program. The first spray for these plants was conducted in May of this year. The second spray was undertaken in June of this year. At this time a number of new plants were located also suffering the effects of dieback so received their first spray. These new plants received their second spray in August of this year.

Of the other species covered by the Phosphonate program it was suggested that *Andersonia* sp. Mt Lindsay needs to be reviewed by the Environmental Protection Officer, CALM, Albany and Mountain Ecologist, CALM, Albany, who is doing a survey of Mt. Lindsay to determine its status in terms of actual population size and also susceptibility to, and presence of *Phytophthora*. *Adenanthos ellipticus* at East Mt. Barren was reviewed in June of this year. Again no *Phytophthora* was recovered, even though there are a lot of plant deaths on the lower slopes. It was suggested that Spring might yield a dieback recovery. Mark True, the Ranger in this area of the Fitzgerald River National Park, will sample recent plant deaths for analysis.

## PROJECT 2

## DEVELOPMENT OF A DNA DIAGNOSTIC TEST FOR DETECTION OF *PHYTOPHTHORA CINNAMOMI* IN SOIL

P.A. O'Brien

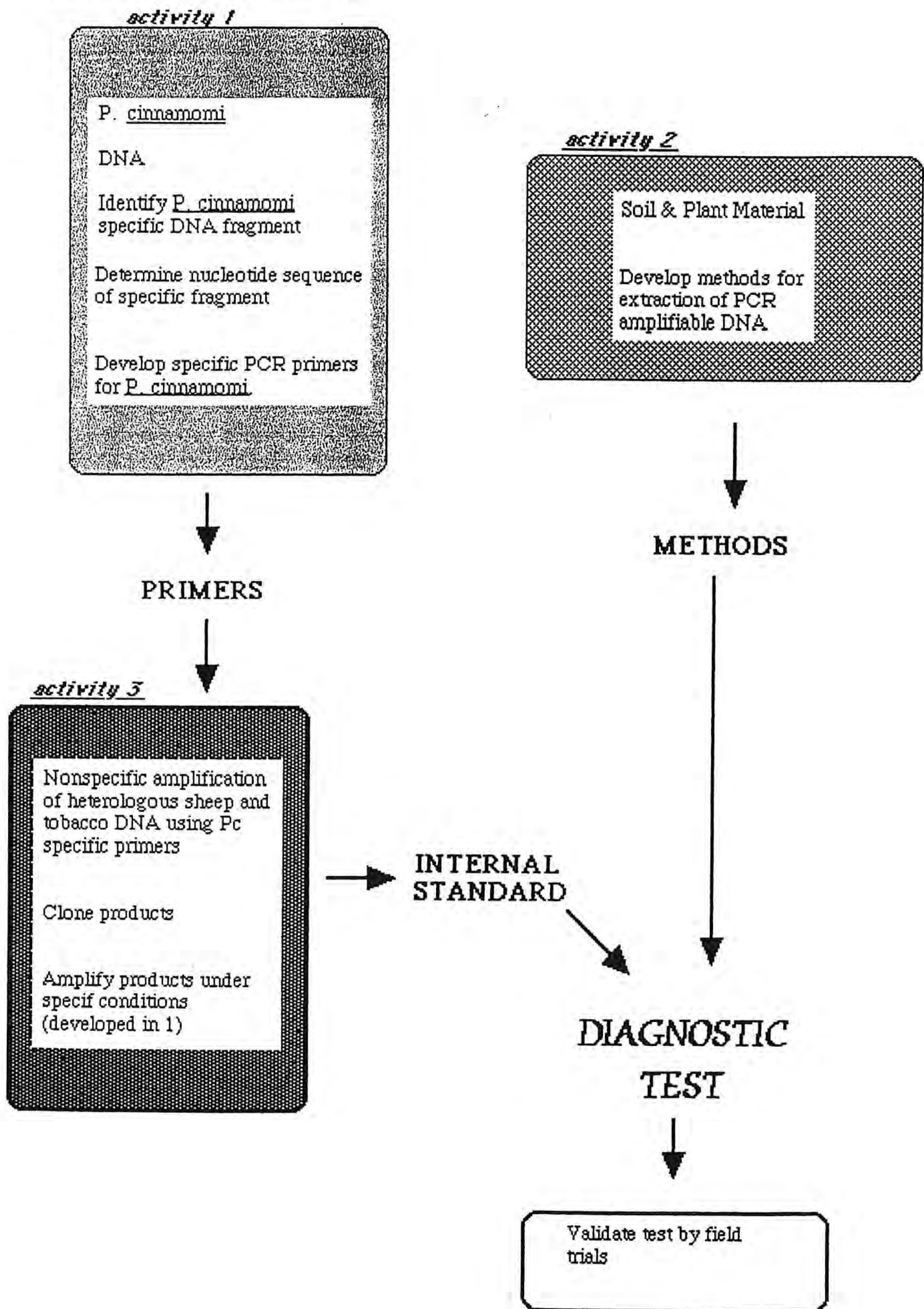

## SUMMARY

Current methods for identification of *Phytophthora* species in soil or plant material are time consuming, insensitive and frequently misleading. In view of the importance of *Phytophthora* as a pathogen of a wide range of plant species there is a need for the development of faster and more accurate methods. The methods developed should also be simple to carry out. In this project methods for the detection of *Phytophthora cinnamomi* in soil or plant material using polymerase chain reaction (PCR) technology were investigated. The investigation used *P. cinnamomi* as a model system to develop the techniques. The method is based on detection of *P. cinnamomi* specific DNA sequences by PCR.

A DNA sequence specific to *P. cinnamomi* was identified and cloned. This sequence was used to develop a PCR reaction which recognised only DNA from *P. cinnamomi.* Initial attempts to amplify DNA extracted from soil were unsuccessful due to the presence of inhibitors in the extract. This inhibition was eventually overcome by modification of the extraction and amplification buffers. The final procedure for analysis of DNA extracted from one gram samples of soil involves boiling the soil, followed by centrifugation to remove soil particles, concentration of the DNA using glassmilk, amplification and electrophoresis to detect the product. The time required for analysis is several hours and the procedure is 10 to 100 times more sensitive than the currently used methods. It is also amenable to simultaneous processing of multiple samples.

Attempts to increase the sensitivity by extracting DNA from soil samples larger than 1g were unsuccesssful as there was too high a level of inhibitor groups in the extract. The approach of first separating the mycelium from the soil using biomagnetic beads and then extracting DNA from the mycelium was also unsuccessful.

Because of the simplicity of the methods the system would be suitable for use in routine diagnostic procedures involving the analysis of multiple samples. On a cost basis it compares favourably to current methods. Aspects which remain to be developed, and possible modes of adoption of this technology are discussed.


OVERVIEW OF PROJECT
activity 1
P. cinnamomi
DNA
Identify P. cinnamomi specific DNA fragment
Determine nucleotide sequence of specific fragment
Develop specific PCR primers for P. cinnamomi
PRIMERS
activity 2
Soil & Plant Material
Develop methods for extraction of PCR amplifiable DNA
METHODS
activity 3
Nonspecific amplification of heterologous sheep and tobacco DNA using Pc specific primers
Clone products
Amplify products under specif conditions (developed in 1)
INTERNAL STANDARD
DIAGNOSTIC TEST
Validate test by field trials

## SECTION 1: INTRODUCTION

### Background

Plant pathogenic *Phytophthora* species are responsible for significant economic losses across horticultural, ornamental and pasture crops in Australia (Cahill, 1993). It is estimated that direct losses due to *Phytophthora* diseases amounted to $223 million across these industries in 1991/92. *Phytophthora* diseases are responsible for causing catastrophic widespread damage to native vegetation in the forests and other ecosystems of Australia, and in particular Western Australia, and as a result many species of plants are at risk of extinction. This has a significant impact on the continuing viability of the tourist, wildflower, and timber industries in Western Australia, and seriously diminishes the recreational value of the Western Australian forests. In addition the loss of trees from large areas by disease has the effect of changing catchment flow including raising the water table leading to increased salinity of farmland and potable water supplies. In 1993, 84% of diseases caused by *Phytophthora* were considered able to cause greater damage, or unable to be controlled (Cahill, 1993). *Phytophthora cinnamomi* is the most widespread species and the most damaging to native vegetation. Other species which are widespread and cause considerable damage include, *P. infestans* (potatoes), *P. nicotianae* (tomato, ornamentals), *P. cactorum* (pome and stonefruits), *P. megasperma, P citricola*, and *P. parasitica* (ornamentals and native spp.) (Cahill, 1993).

A range of management practices are used for the control of *Phytophthora* species, which include rotation, fumigation, pasteurisation, filtration of water supplies, resistant cultivars, quarantine and the use of fungicides. A key process to these practices is the isolation and diagnosis of the pathogen. Current methods of diagnosis are time consuming and frequently give inaccurate results in that they fail to detect the pathogen (Marks and Kassaby, 1974; Tsao and Guy, 1977) (Hardy unpublished). On old impacted jarrah forest sites, baiting will often give negative results and *P. cinnamomi* is only recovered by collecting soils and growing a susceptible species such as *Banksia grandis* for between 3-6 months in the soil before the pathogen can be recovered (MacDougal & Hardy, in prep.). Finally, plating of infected plant material onto *Phytophthora* selective agar can also give incorrect negative results and it is not until the plant material is baited often in conjunction with the wetting and drying procedure that *P. citricola* and *P. cinnamomi* can be isolated (Bunny *et al.*, 1996) (Hardy, unpublished). Therefore, diagnosis by conventional baiting or plating techniques and confirmation of the presence or absence of *Phytophthora* species can be time consuming and often incorrect.

At present the use of fungicides to control *Phytophthora* diseases is often prophylactic and predominantly systemic fungicides based on phosphorus acid and metalaxyl are used. Metalaxyl resistance is commonly reported for *Phytophthora*, whilst there have been no reports so far of resistance to phosphonate (Cahill, 1993). The increasing use of fungicides raises concerns about their effects on the environmental, in particular contamination of water supplies by leaching into groundwater supplies or by run-off in above ground water supplies. Fungicide application may also lead to decreased

acceptability of horticultural produce due to contamination by residual levels of the fungicide. In addition, recent work on the use of phosphonate to control *P. cinnamomi* in jarrah forest understorey plant species, indicates that flowering and seed set can be reduced at recommended rates of application (Bennallick, Hardy & Shearer, in press). This raises concerns regarding the long term use of phosphonate. In view of these concerns, management practices will play an increasing role in control of *Phytophthora* diseases. Management practices would greatly benefit from the availability of a rapid diagnostic method which overcomes the problems outlined above for the detection of *Phytophthora* spp. in soil or infected plant material. The availability of such a method would allow the more timely and effective application of management practices, and the more selective use of chemical control at a time when it is likely to be of maximum benefit.

As an alternative to the plating or baiting procedure, the pathogen can be detected by detection of DNA sequences specific to the pathogen using PCR (Polymerase Chain Reaction) technology. In this procedure, DNA is extracted from the sample, (soil or plant material), mixed with DNA precursors, oligonucleotide primers which bind to sites flanking the species specific sequence (the target site), and DNA polymerase (Fig 1.1). The DNA is first denatured by using a high temperature, the temperature is then lowered to allow the binding of the oligonucleotide primers to their binding sites, and finally the temperature is changed again to allow DNA polymerase to synthesize two new strands of DNA. This is a typical PCR cycle in which the number of DNA strands corresponding to the target site is doubled (Saiki *et al.*, 1988). The cycle is repeated 20-30 times resulting in an exponential accumulation in the number of copies of the target site. The products of the amplification can be detected by electrophoresis on an agarose gel. The specificity of the reaction is determined by the binding of the oligonucleotide primers to the target, so that despite the presence of DNA from other species in the sample, only the target site is amplified. If the pathogen is not present in the sample no product will be observed.

The advantages of PCR as a diagnostic test are

- The procedure is technically simple: All of the reagents are mixed in a tube and placed in a thermocycler which automatically goes through the temperature changes.

- The test is not affected by environmental conditions or stage of the life cycle. Unlike protein markers which may or may not be expressed depending on environmental conditions or stage of the life cycle, the target DNA site is always present.

- The process is rapid. The entire process from sample to result takes about 5-7hr, compared to 1-2 weeks for the plating or baiting procedures. This time can be reduced considerably (to about 1 hr) by using a nonelectrophoretic method for detection and quantitation of the amplification products.

- The process is very specific. The test is specific not only at the species level, but also at the pathotype, or mating type level.

- The test is very sensitive. The sensitivity depends on the target sequence and the chemical composition of the sample, but all studies using PCR for detection of organisms in environmental samples have shown that it is more sensitive than microbiological or immunological detection methods (Steffan and Atlas, 1991).

- Multiple pathogens can be analysed simultaneously. Primers which amplify target sequences from different pathogen species can be added to the amplification simultaneously. Reactions for the detection of multiple species have been developed (Ersek *et al.*, 1994).

- DNA extracts can be stored in the freezer for a number of years, and used for further analysis during this time. Thus significant information can be generated as new tests are developed without having to repeat sample collection.

- Multiple samples can be processed simultaneously. DNA extraction techniques can be performed on 12-24 samples simultaneously.

- New tests can be developed easily by isolating specific DNA sequences. This process may take a few months. The procedures for extraction and analysis of the DNA do not have to be redeveloped each time a new test is required.

Because of these advantages PCR detection tests compare very favourably to immunological detection tests, which although simpler to carry out, are less sensitive and less specific (Schots *et al.*, 1994).

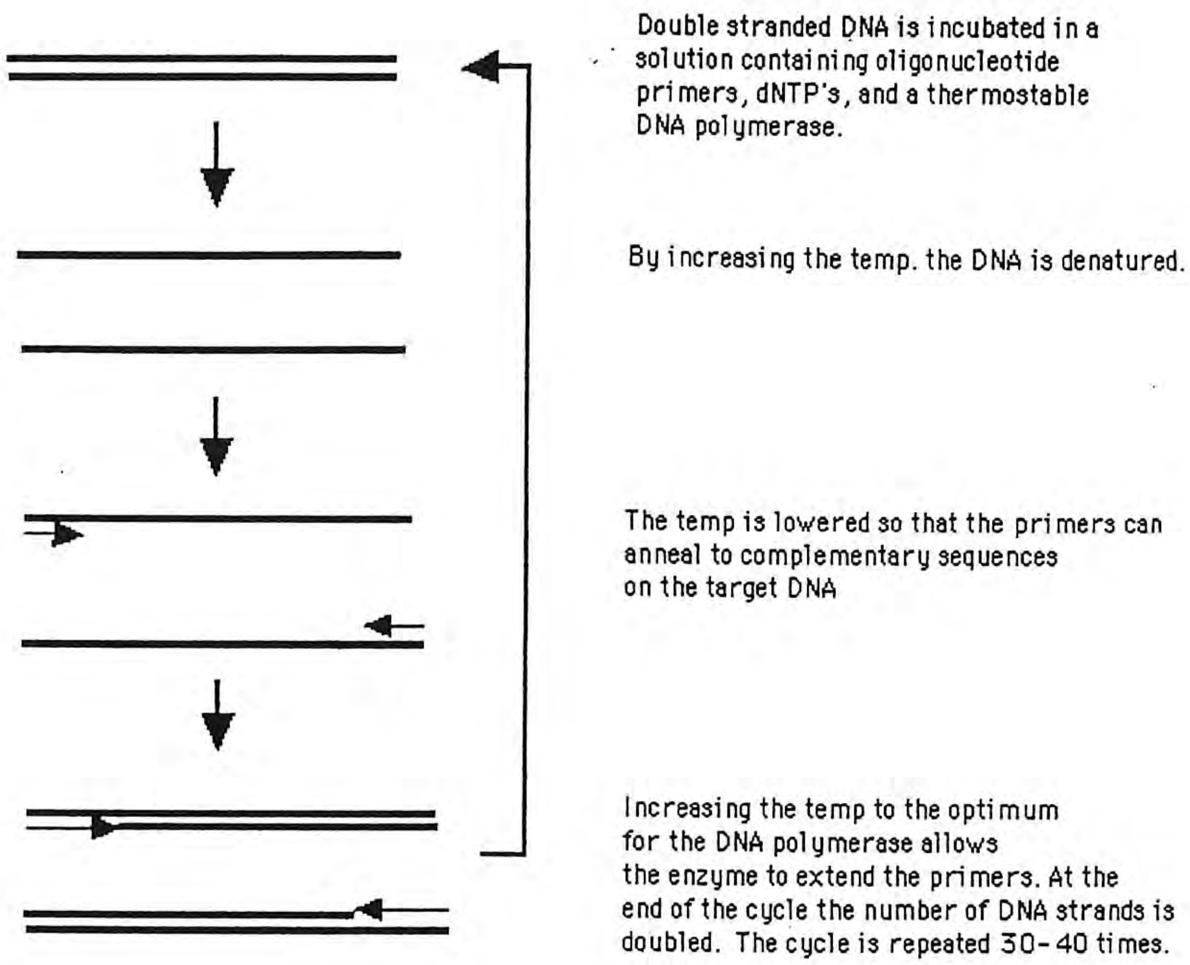
**Objective**

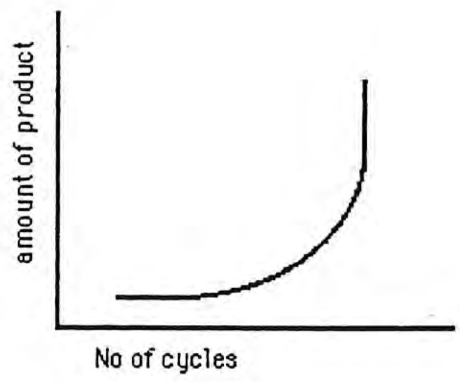
To develop diagnostic PCR procedures for the detection of species of *Phytophthora* in soil and plant material.

It is anticipated that the operation of a routine diagnostic process for detection of *Phytophthora* will involve the analysis of a large number of samples from different locations, and from a large number of different plant species. In terms of chemical composition every sample can be considered to be unique. Thus in terms of the development of a routine diagnostic procedure, this is the challenge that has to be overcome.

**Experimental Strategy**

The experimental approach is; (i) to identify a DNA sequence unique to *P. cinnamomi* and use this to develop a specific PCR reaction; (ii) develop techniques for the extraction of amplifiable DNA from soil and plant material; (iii) develop procedures for the detection of false negative reactions and quantification of the PCR reaction.




With increasing numbers of cycles we get an exponential increase in product.

After 30-40 cycles we get a billion fold increase and the product can easily be seen after gel electrophoresis.

By designing the primers appropiately the reaction can be made to be very specific. A one base pair mismatch between the primer and target can prevent binding.

Figure 1.1: The PCR Cycle

## SECTION 2: DEVELOPMENT OF A TEST FOR THE DETECTION OF *PHYTOPHTHORA CINNAMOMI*

### Background

The first stage in developing a test for detection of *P. cinnamomi* is the isolation of a DNA sequence specific for *P. cinnamomi* (target sequence). This can be achieved using a variation of the PCR reaction called RAPD-PCR[1] (Duncan *et al.*, 1993). In this procedure a single oligonucleotide primer of arbitrary sequence is used to amplify multiple loci within the target organism. Many of the sequences amplified are derived from polymorphic repetitive DNA and are frequently species specific.

The fragment of DNA containing the target sequence is cloned and the nucleotide sequence determined. Based on this sequence PCR primers are designed. These need to be tested to determine if specificity is retained.

### Experimental Results

#### *Isolation of a DNA sequence specific for Phytophthora cinnamomi*

DNA extracted from isolates of *P. cinnamomi* and other *Phytophthora* species, was used for RAPD-PCR analysis. A number of products specific to *P. cinnamomi* were observed on the gel. To confirm that these were present only in *P. cinnamomi*, the DNA was blotted onto a membrane and probed with radioactive DNA from *P. cinnamomi*. From this we could identify bands that were specific to *P. cinnamomi*.

Several of these fragments were extracted from the gel and because the yield of DNA was very low, it was amplified by PCR prior to cloning into an *E. coli* plasmid vector. Analysis revealed that most of the clones contained a fragment other than the desired fragment. The conclusion was that each of the bands recovered from the gel was a complex mixture of more than one sequence, and that the wrong sequence was preferentially amplified. This problem was overcome by omission of the amplification step after extraction from the gel. The DNA was cloned into the bacterial plasmid pUC18. The specificity of the cloned fragments for *P. cinnamomi* was confirmed by hybridisation to DNA from different fungal species.

#### *Determine the nucleotide sequence of the isolated fragment of DNA*

The nucleotide sequence of the cloned fragments was determined by dideoxy nucleotide sequencing. Analysis of the sequence shows that the cloned fragment is a repetitive element.

---

[1] Random Amplification of Polymorphic DNA by the Polymerase Chain Reaction.

*Design specific PCR primers for the target site*

Oligonucleotide primers were designed to the left and right ends of the insert in pMDab3-2,. Three primers were designed for each end of the insert. The binding sites of the primers overlap.

(A) Primers designed for pMDab3-2.

All combinations of these primers amplified DNA from *P. cinnamomi*. Optimum amplification was obtained with 2mMMgC12 in the reaction (photo p2).

The LPC1/RPC1, LPC2/RPC2 and LPC2/RPC1 combinations amplified sequences present in *Pythium* and *Rhizoctonia* as well as sequences in other species of *Phytophthora* (D27 32 & 28). The LPC2/RPC3 combination was however found to be specific for *P. cinnamomi* (Fig 2.1).

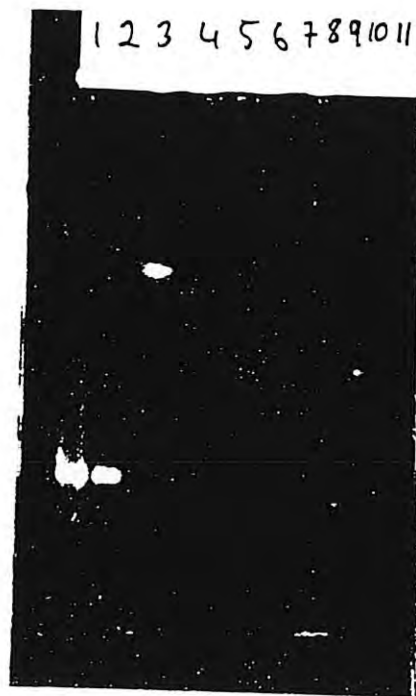



Figure 2.1: *Specificity of PCR primers for P. cinnamomi.*
DNA was extracted from mycelium of different species of Phytophthora and used for amplification with the primers LPC2/RPC3. The products were electrpophoresed on a 5% polyacrylamide gel which was stained and photographed under UV light. Lanes (**1**) *P. cinnamomi*; (**2**) *P. cinnamomi*; (**3**) *P. drechsleri*; (**4**) *P. citricola*; (**5**) *P. citophthora*; (**6**) *P. megasperma* var *megasperma*; (**7**) *P. megasperma* var *sojae*; (**8**) *P. nicotianae*; (9) *P. cryptogea*; (**1 0**) *R. solani*; (**1 1**) *Pythium*

# SECTION 3: DEVELOPMENT OF METHODS FOR THE EXTRACTION OF AMPLIFIABLE DNA FROM SOIL

## Background

The use of PCR technology for identification of fungal pathogens requires the ability to isolate DNA in a form in which it can be amplified. However, phenolic and other compounds, e.g., polysaccharides present in soil, or in many plants species inhibit amplification (Young *et al.*, 1993). Techniques have been developed for the extraction of PCR amplifiable DNA from soil or plant material but they are technically complex, time consuming, expensive, and unsuitable for use in routine diagnostic procedures which may involve analysis of large numbers of samples (Jacobsen and Rasmussen, 1992; More *et al.*, 1994; Rochelle and Olsen, 1991; Selenska and Klingmuller, 1992; Tsai and Olsen, 1992). What is needed is a simple procedure which results in the extraction of PCR amplifiable DNA from soil or plant material. In this the development of simple procedures for the extraction of PCR amplifiable fungal DNA from soil are described. These procedures would be suitable for routine diagnostic use.

## Experimental Results

### *Extraction of DNA from mycelium*

Initially conditions for extraction of DNA from mycelium of *P. cinnamomi* were established. For extraction of DNA the mycelium was suspended in 10mMTris.HCl pH8.0 and boiled for various times. Extracts were concentrated by geneclean, and analysed by electrophoresis on an agarose gel to check the integrity of the DNA. The results show (Fig 3.1) that after 10 min boiling significant quantities of high molecular weight DNA were released from the mycelium. Increasing the time of boiling to 40 min resulted in a marginal increase in the amount of DNA released, but significantly increased the colouration of the extract.

### *Extraction of DNA from mycelium soil mixes*

Extracts prepared by boiling mycelium alone did not inhibit amplification of *P. cinnamomi* DNA. At longer times of boiling there may be a slight inhibitory effect as judged by the intensity of the bands (Fig. 3.2). In contrast, extracts prepared by boiling mycelium in the presence of soil (1g mycelium / g soil) resulted in the total inhibition of amplification.

*Purification of extracted DNA*

In an attempt to remove the inhibitors which may be small molecular weight phenols (Young *et al.*, 1993), the soil extract was chromatographed through Sephadex G-50 columns . Fractions containing DNA were identified by spotting aliquots onto ethidium bromide agarose plates and viewing under UV light. The addition of aliquots of these fractions to PCR reactions did not inhibit amplification (Fig 3.3). In an attempt to increase the sensitivity of the reaction, the DNA in the G-50 fractions was concentrated by geneclean. However, with this treatment inhibition returned.

A number of DNA purification systems were evaluated for their ability to separate the inhibitors from the DNA. The only successful methods appeared to be the addition of the ion exchange resin AG-1X2 to the extraction buffer, or centrifugation through cDNA spun columns containing Sephacryl S-300 gel. However, as with the Sephadex G-50 purified DNA, inhibition returned when the DNA was concentrated using geneclean.

Attempts to improve the quality of the DNA preparation by varying the composition of the extraction buffer were made. The addition of NaCl to the buffer had no effect. The signal obtained with 200mM NaCl was as strong as that obtained with no NaCl in the buffer. Addition of PVP or PVPP to the extraction buffer resulted in significant removal of the inhibitory activity (Fig 3.4). A slight improvement in the amplifiability of the DNA was obtained after repeated cycles of freezing and thawing. The presence of low levels of spermidine in the extraction buffer at 6.25x10-7M resulted in the appearance of an amplification product whereas no product was observed without spermidine. The presence of both spermidine and PVPP in the extraction buffer resulted in good amplification.

*Amplification of DNA from different soils*

One gram samples from five different soils were extracted by boiling in buffer, and after the soil was removed by centrifugation the supernatant was genecleaned. Aliquots of the final solution were added to PCR reactions containing DNA and amplified. The results show that of the six soils tested, amplification was obtained in only two soil types. This is a consequence of the different chemical composition of the different soil types.

*Amplification of zoospore DNA*

Zoospores were prepared according to (Ribeiro, 1978). Dilutions of the zoospore suspension were extracted for DNA, and aliquots of each dilution tested for amplification. The results show that zoospore DNA could be readily detected by the *P. cinnamomi* specific primers.

*Extraction of DNA from large soil samples.*

Attempts were made to extract large samples 5-50g of soil as this would increase the sensitivity of detection. The approach was to concentrate the mycelium in the buffer slurry by using magnetic beads coated with antibodies raised against the mycelium (Fig 3.5). The bead mycelium complexes can be immobilised using a magnet whilst the supernatant is poured off. The mycelium can then be eluted in a small volume of buffer for extraction. However, even with samples as small as 5g, no amplification was observed. Several ethanol wash steps were introduced into the process without any effect.

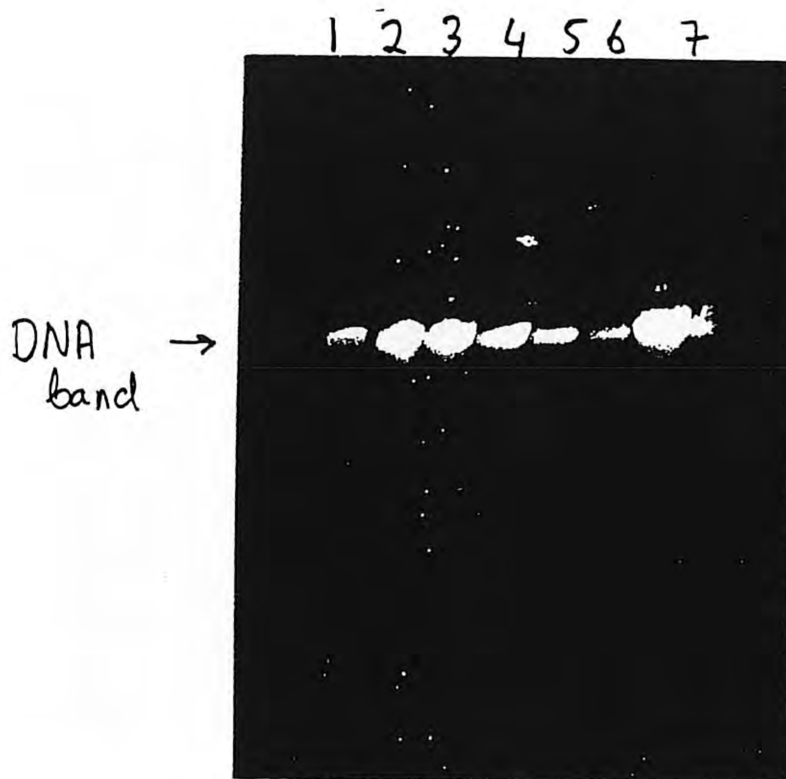



Figure 3.1: *Extraction of DNA from fungal mycelium by boiling in buffer.*
Mycelium of P. cinnamomi was mixed with soil and suspended in 10mMTris.HCl pH 8.0 and boiled for various times. The mixtures were centtrifuged to remove insoluble debris, and the DNA concentrated using glassmilk. Aliquots of the extract were electrophoresed on an agarose gel. The gel was stained with ethidium bromide and photographed under UV light. Lanes **1 - 6**, mixtures boiled for 5,10, 15, 20 30, and 35 min respectively; Lane **7** , DNA purified fron P. cinnamomi mycelium.

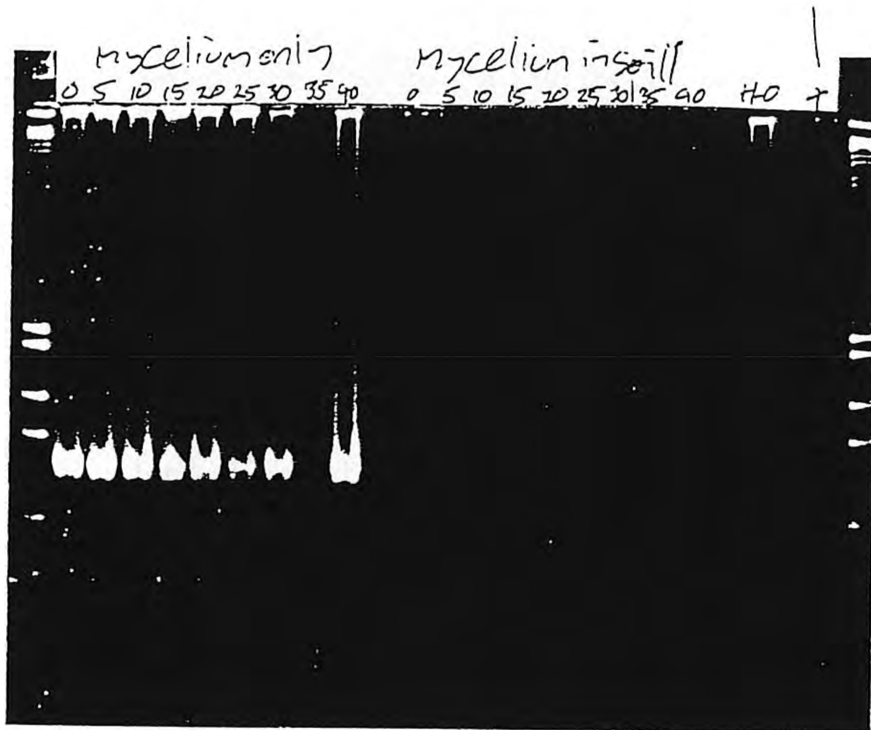



Figure 3.2: *Effect of extract on PCR.*
DNA was extracted from mycelium, or mycelium soil mixtures as described in the legend to Fig 3.1. Aliquots were added to PCR reactions containing P. cinnamomi DNA. The products of PCR amplification were electrophoresed on a 5% polyacrylamide gel. The gel was stained and photographed under UV light. The numbers above the lanes refer to the time the mixtures were extracted for.

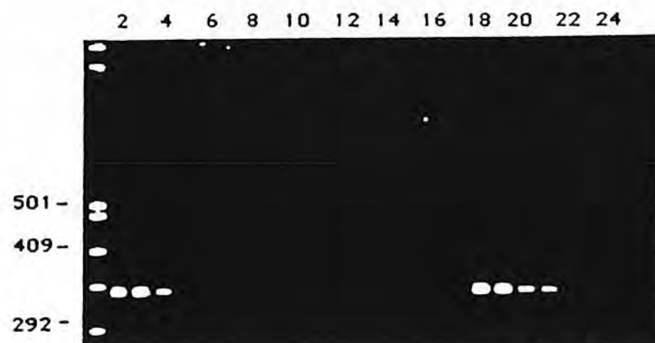



Figure 3.3. *Removal of inhibitors by chromatgraphy through Sephadex G-50.*
Soil extract prepared by boiling for 10 mins, followed by centrifugation to eliminate insoluble debris was chromatographed through a Sephadex G-50 column (5ml vol.) equilibrated with TE pH8.0 buffer and fractions of 0.25 ml collected. Fractions containing DNA were identified by spotting aliquots onto agarose ethidium bromide plates and viewing under UV. these fractions were tested for inhibitory activity by addition of aliquots to PCR reactions. Lanes (**1**) molecular size standards; (**2 - 7**) fractions from the column; (**9 - 14**), concentration of DNA in the fractions by Geneclean; (**16**) extract centrifuged through a Sephadex G-50 spin column; (**17**) as for 16 but concentrated by geneclean.

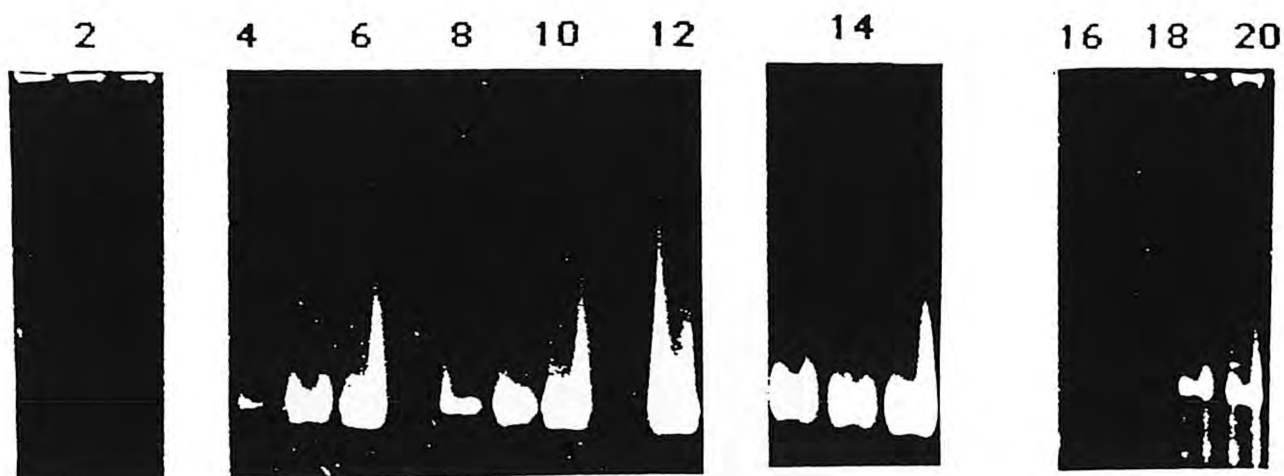
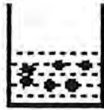



Figure 3.4. *The effect of extraction buffer composition on the PCR inhibitory acivity of soil extract.*

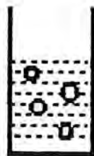
Additions were made to the extraction buffer, which was mixed with soil. The mixture was extracted by boiling for 10 mins. Insoluble debris was removed by centrifugation, and DNA in the supernatant was concentrated using glassmilk. Aliquots of the concentrated DNA were added to PCR reactions containing purified *P. cinnamomi* DNA.. Lanes (**1-3**) addition of 1μl of undiluted, 1/8 diluted, and 1/16 diluted soil extract respectively; (**4-6**) as for lanes 1-3 but with the addition of 1%PVP to the extraction buffer; (**7**) reaction with no DNA but with 1% PVP; (**8-10**) as for lanes 4-6 but with three cycles of freeze thaw in liquid nitrogen; (**11**) reaction without DNA ; (**12**) reaction without soil extract; (**13-15**)) as for lanes 1-3 but with the addition of 10% PVPP; (**16-20**) 1μl of soil extracted in the presence of 0.025, 0.125, 0.25, 0.625, and 1.25μM spermidine in the extraction buffer respectively.

Sample



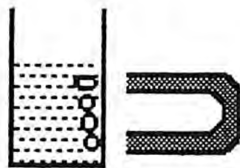
The sample is mixed with buffer.
This separates the mycelium from the soil.

The slurry is centrifuged to pellet the soil particles which are discarded.
The mycelium remains in the supernatant.



Antiserum is added and it binds to mycelium.

The antibody binds to the magnetic beads.



The beads are collected by using a magnet. The supernatant can be removed.
The mycelium can be resuspended in a smaller volume for analysis.

Figure 3.5: *Use of biomagnetic beads for recovery of mycelium from soil.*

## SECTION 4: COMPOSITION OF THE PCR REACTION

### Background

The composition of the PCR reaction can be varied to reduce the effects of phenolic and polysaccharide inhibitors in the DNA extract. Phenolic compounds may interact with the binding site on the DNA polymerase thereby reducing it's effectiveness, whereas polysaccharides coat the enzyme molecules and inhibit their action (Demeke and R.P.Adams, 1992; Wan and Wilkins, 1993).

### Experimental Results

Parameters that were tested for their effect on amplification were:

(a) The amount of enzyme
(b) The addition of Tween
(c) The addition of spermidine.

Inhibition of amplification could be overcome by increasing the amount of DNA polymerase in the PCR reaction (Fig 4.1). With purified DNA we would normally use 1 unit of enzyme activity per reaction. However with DNA extracted from soil a stronger signal was obtained when this was increased to 2-4 units per reaction.

The addition of Tween-20 or spermidine to the reaction also increased the strength of the signal.

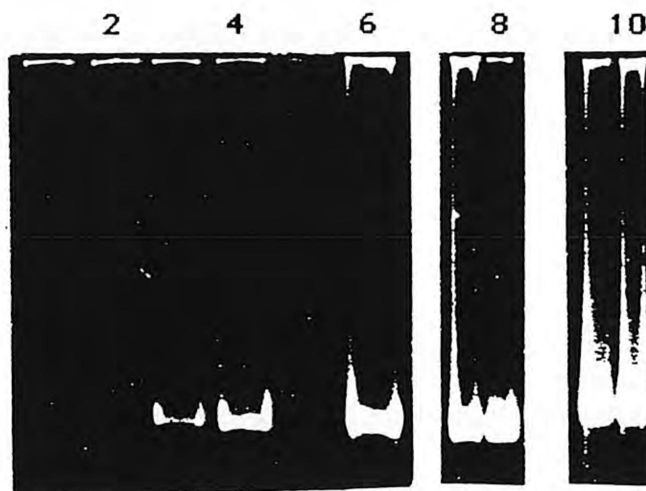



Figure 4.1. *The effect of PCR reaction composition on the inhibitory effect of soil extracts.*

1μl of soil extract was added to PCR reactions containing *P. cinnamomi* DNA. Lanes: (**1 - 5**), reactions containing 2, 2.5, 3, 3.5, and 4 units of Tth polymerase activity; (**5**) reaction without any DNA (**6**) reaction without any soil extract ; (**7**) reaction with 0.5% Tween 20; (**8**) reaction with 1% Tween 20; (**9**) 0.1mM spermidine; (**1 0**) 1mM spermidine.

## SECTION 5: SENSITIVITY OF DETECTION BY PCR

### Background

The sensitivity of detection of bacteria in soil and complex samples using PCR ranges from 1 cell / 100 g soil to 105 cells per gram. The sensitivity varies according to the target sequence, whether it is multicopy or single copy, and the type of material being sampled. There are no comparable estimates for filamentous fungi as most estimates of sensitivity are based on detection of purified DNA. To gain a more realistic estimate of sensitivity we have blended mycelium in buffer to create a slurry. Dilutions of this are added to soil samples, extracted and amplified.

### Experimental Results

When purified DNA extracted as described by (Panabieres *et al.*, 1989) was used as a substrate for amplification, as little as 10-15g DNA could be amplified. Similarly when mycelium was homogenised and dilutions of the slurry amplified, all samples down to 10-15 g mycelium could be detected. However, when the homogenised mycelium was mixed with soil and extracted, the level of detection decreased to 10-8 g mycelium (Fig 5.1). This difference reflects the presence of PCR inhibitors in the extract from the mycelium soil mixture. The amplification reactions in this series of experiments were carried out without the addition of Tween-20 or spermidine to the reaction and with only 1 unit of enzyme activity per reaction. Therefore considerable scope exists for increasing the detection sensitivity by changing these parameters (see Ch 3).

The detection level could be increased ten fold by blotting the DNA onto a nylon filter and probing this with a radioactively labelled probe.

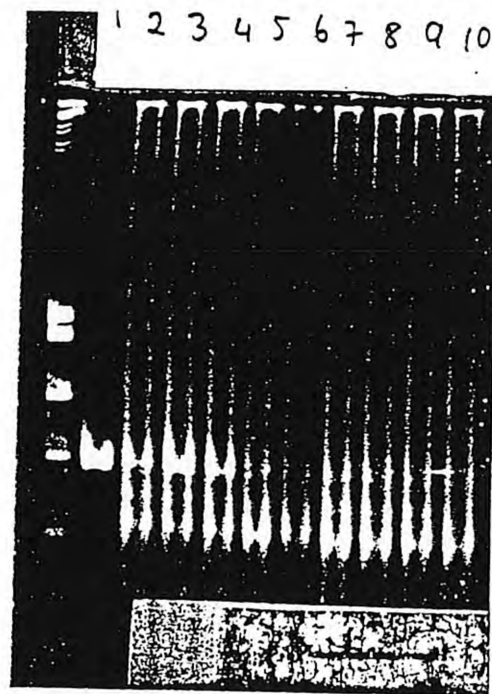



Figure 5.1: *Sensitivity of detection.*
Aliquots of mycelium were mixed with soil and the mixtures extracted, and amplified by PCR. Lanes **1-10** represent dilutions of mycelium ranging from 10mg to $10^{-8}$ mg.

# SECTION 6: DEVELOPMENT OF AN INTERNAL STANDARD FOR THE PCR REACTIONS

## Background

Soil samples are expected to be highly friable in composition and to affect the efficiency of amplification to different extents. In running a diagnostic facility it is essential to be able to differentiate real negative samples which do not contain the pathogen, from false negative samples which inhibit PCR because of the high level of inhibitors. Differentiation can be achieved by use of an internal standard fragment which is added to the reaction and which is amplified by the same primers as the target sequence. We should always observe the product from the internal standard, and it's absence indicates a false negative reaction. It is a prerequisite that the standard is different in size from the target sequence so that it separated electrophoretically, and that the sequence is different from the standard (except for the primer binding sites) so that they cannot anneal together.

The standard fragment can also be used to quantitate the reaction. This is achieved by running a series of reactions containing different amounts of standard with constant amounts of extracted DNA. When the ratio of the amplification products are equal, it is assumed that equal amounts of standard and target were present at the start of the reaction, and from the known amount of standard we can calculate the amount of target. This can be related to the amount of biomass present in the sample.

The strategy for developing a standard is to run RAPD-PCR reactions with heterologous DNA and with the *P. cinnamomi* primers. The products obtained are fragments that are able to be amplified with the primers but which will differ in internal sequence. In these experiments we used DNA from tobacco and sheep to run the RAPD-PCR reactions.

## Experimental Results

Tobacco and Sheep DNA was obtained from researchers at the SABC, Murdoch University and used to run RAPD-PCR reactions with the *P. cinnamomi* primers. Multiple products were obtained which were separated by electrophoresis. Some of the bands were extracted from the gel and electrophoresed alongside products obtained from *P. cinnamomi* with the same primers. The results show that a number of these can be differentiated from the *P. cinnamomi* products, and therefore would be useful as an internal standard. These fragments were cloned in the bacterial plasmid pGem-T for further evaluation as an internal standard.

*This evaluation is continuing throughout 1996.*

## SECTION 7: PROCEDURE FOR ANALYSIS OF SOIL SAMPLES FOR DETECTION OF *P. CINNAMOMI*

**Procedure for sample preparation and analysis**

The following procedure is developed from the results presented in this report, and would be sufficiently simple for operation of a diagnostic facility.

1. The soil sample[2] is mixed with 2 vols of extraction buffer (10mM Tris.HCl pH8.0, 10% PVPP, 2mMspermidine), and placed in a boiling water bath for 10 min.

2. The extract is centrifuged for 10 min to pellet the soil particles. The supernatant is transferred to a fresh tube.

3. DNA in the supernatant is concentrated and purified by using Bresaclean.

4. An aliquot of the DNA is amplified by the specific primers LPc2 RPc3. The rest of the extract is stored in the freezer.

5. An aliquot of the amplified DNA is analysed on an agarose gel. The gel is stained and photographed. The results are recorded and stored in a computerised database.

This procedure is technically simple to use and requires very little in the way of reagents. From sample to analysis takes several hours (Table 1).

***TABLE 1: Stages in analysis of samples by DNA detection test.***

| STAGE | PROCEDURE | TIME |
|---|---|---|
| 1 | Extraction of DNA from the sample | 30-60 min |
| 2 | Preparation of amplification reactions: (It is easier to prepare multiple reactions simultaneously). | 30-60 min |
| 3 | Amplification | 140 min |
| 4 | Agarose gel electrophoresis | 180 min |
| 3 | Staining of the gel and analysis of results | 10-20 min |

[2] Sample size should be limited to 1g.

It should be emphasised that what is being developed here is not just a test for a specific fungus, but a technology which can be readily applied to other species of pathogenic fungi. The techniques for extraction and analysis have already been worked out in this project. All that is needed to develop tests for other species is a DNA sequence specific to that species. Such sequences can be isolated relatively easily, and we now have isolated sequences for *P. cinnamomi*, and the ectomycorrhizal fungi *Laccaria*, and *Hydnangium*.

Validation of further tests can be easily carried out using samples stored in the freezer from this project. Instead of having to start fresh and collect samples from the field for validation.

## SECTION 8: APPLICATION AND ADOPTION

### Applications of the detection test

1. Measurement of fungal biomass. This enables us to assess the effect of management practices for disease control.

2. Quarantine

   i) Detection of *P. cinnamomi* in plant and soil that is being moved from one location to another,

   ii) in soil to be used for nurseries,

   iii) in plant material to be used for nursery stock.

3. Assessment of disease potential in nondisease sites. What is the level of fungal biomass and how does relate to disease threat.

### Adoption of the detection test

A decision needs to be made at some stage on whether or not to use the DNA detection test or continue using alternative methods. Points that need to be considered in making this decision are:

* What capital costs are involved in converting to the use of the DNA test?

* How does the cost of analysing a sample using the DNA test compare with the cost of analysing a sample by a) baiting or b) dipstick?

* Do we need the advantages to be gained from using a DNA test?

Capital Costs: The capital costs involved in setting up a testing service based on the DNA test are given in Table 2.

Running costs: The costs of analysing samples by the DNA test are given in Table 3. The actual cost/samples decreases as the number of samples increases. This is because some of the costs are fixed regardless of how many samples are analysed, e.g., the same agarose gel has to be poured, run, stained and photographed whether we are running 1 sample or 20 samples, and the labour costs in analysing 1 sample are the same as for 20 samples. Therefore it is more cost effective to analyse multiple samples.

*Table 2: Capital costs involved in setting up a PCR testing facility.*

| ITEM | COST |
|---|---|
| Thermocycler | $8,000 |
| Agarose Gel Box | $1,500 |
| Power pack | $1,500 |
| UV Transilluminator | $1,500 |
| Computer | $2,000 |
| Microcentrifuge | $4,200 |
| Camera and Stand assembly | $6872 |
| **TOTAL** | **$25,572** |

*Table 3: Costs of sample analysis by PCR.*

| No of Samples Analysed | Total Cost of Samples | Cost per sample |
|---|---|---|
| 1 | $244.63 | $244.63 |
| 10 | $369.32 | $36.93 |
| 20 | $508.08 | $25.40 |
| 40 | $785.52 | $19.64 |

These costs include the costs of the reagents used in all stages of the procedure and the plasticware. They also include labour, (GRA), and equipment depreciation. For estimating equipment depreciation it is assumed that we would be testing 500 samples per year, and writing off the equipment over a five year period. The costs do not include infrastructure such as lab and office costs, secretarial, stationery, or royalty payments to Roche for use of the PCR technology.

**Multiple use of PCR technology**

One consideration in deciding to develop a test facility is that the same technology can be used for multiple purposes and not just for detection of *P. cinnamomi.* Other uses might include:

* Identification of plant germplasm by PCR. This will become increasingly important as we move more towards the use of DNA markers for identification of varieties designated for specific purposes, e.g. varieties of eucalypts that are good for pulping, or which are disease resistant.

* Estimation of the relatedness of plant species by RAPD-PCR.

* Identification of other fungal pathogens. Although multiplex PCR detection tests can be developed there is a limit to the number of species that can be detected in a single test.

* Identification of mycorrhizal fungi and estimation of their biomass.

* Identification of insect pests.

* Detection of plant viruses.

Thus at the same time as analysing samples for the presence of *P. cinnamomi*, one might also be analysing samples with different primers for identification of plant germplasm, plant viruses, etc. All of the samples do not have to be analysed for the same purpose. This enables the running costs to be spread over multiple applications.

**Modes of Technology Adoption**

There are two modes of adopting this technology: (1) CALM can set up their own testing facility, or (2) contract with an outside group such as Murdoch to carry out the testing.

1. Mode 1: If CALM were to set up a test facility they would incur the capital costs outlined above. There would be a period of technology transfer during which personnel were trained in the use of the technology. We would be able to help in a consultative capacity with this. Our chief activities would be:

    a) To help design and equip the facility

    b) To train personnel in the techniques

    c) To act as a backup consultant to the test facility, should there be problems, and to transfer new technologies as they are developed.

2. Mode 2: An alternative possibility would be for CALM to contract with us to carry out the testing for them. We have the facilities, technical expertise and infrastructure to do this. Moreover we are abreast of new developments in PCR technology and can incorporate these developments into the testing procedures to make them more efficient.

## REFERENCES

Bunny, F., Shearer, B. and Hardy, G. (1996). *Biology and ecology of Phytophthora citricola in native plant communities affected by mining.* Minerals and Energy Research Inst. Western Australia, Perth.

Cahill, D. (1993). *Review of Phytophthora Diseases in Australia.* Australian National University, Canberra.

Demeke, T. and Adams, R.P. (1992). The effects of plant polysaccharides and buffer additives on PCR. *Biotechniques* **12**:332-333.

Duncan, S., Barton, J.E. and O'Brien, P.A. (1993). Analysis of variation in isolates of *Rhizoctonia solani* by random amplified polymorphic DNA assay. *Mycological Res.* **97**:1075-1082.

Ersek, T., Shoelz, J.E. and English, J.T. (1994). PCR amplification of species specific DNA sequences can distinguish among *Phytophthora* species. *Applied and Environmental Microbiology* **60**:2616-2621.

Jacobsen, C.S. and Rasmussen, O.F. (1992). Development and application of a new method to extract bacterial DNA from soil based on separation of bacteria from soil with cation exchange resin. *Applied and Environmental Microbiology* **58**:2458-2462.

Marks, G.C. and Kassaby, F.Y. (1974). Detection of *Phytophthora cinnamomi* in soil. *Aust. Forestry* **63**:198-203.

More, M.I., Herrick, J.B., Silva, M.C., Ghiorce, W.C. and Madsen, E.L. (1994). Extraction of microbial DNA from sediment. *Applied and Environmental Microbiology* **60**:1572-1580.

Panabieres, F., Marais, A., Trentin, F., Bonet, P. and Ricci, P. (1989). Repetitive DNA polymorphism analysis as a tool for identifying *Phytophthora* species. *Phytopathology* **79**:1105-1109.

Ribeiro, O.K. (1978). *A Source Book of the Genus Phytophthora.* J. Cramer, Vaduz.

Rochelle, P.A. and Olsen, B.H. (1991). A simple technique for electroelution of DNA from environmental samples. *Biotechniques* **11**:724-728.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988). Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**:487-494.

Schots, A., Dewey, F.M. and Oliver, R. (1994). *Modern Assays for Plant Pathogenic Fungi: Identification, detection and Quantification.* CAB International, Oxford.

Selenska, S. and Klingmuller, W. (1992). Direct recovery and molecular analysis of DNA and RNA from soil. *Microbial Releases* **1**:41-46.

Steffan, R.J. and Atlas, R.W. (1991). Polymerase chain reaction. Applications in environmental microbiology. *Ann. Rev. Microbiol.* **45**:137.

Tsai, Y. and Olsen, B.H. (1992). Rapid method for extraction of DNA from soil and sediments. *Applied and Environmental Microbiology* **50**:1070-1074.

Tsao, P.H. and Guy, S.O. (1977). Inhibition of Mortierella and Pythium in *Phytophthora* isolation medium using hymexazol. *Phytopathology* **67**:796-801.

Wan, C.Y. and Wilkins, T.A. (1993). Spermidine facilitates PCR amplification of target DNA. *PCR methods and Applications* **3**:208-210.

Young, C.C., Burghoff, R.L., Keim, L.G., Minak-Bernero, V., Lute, J.R. and Hinton, S.M. (1993). Polyvinylpyrrilidone agarose gel electrophoresis purification of polymerase chain reaction amplifiable DNA from soils. *Applied and Environmental Microbiology* **59**:1972-1974.

## PUBLICATIONS FROM THIS PROJECT

Dobrowolski M., and P.A. O'Brien. 1993. Isolation of a species specific DNA probe for *Phytophthora cinnamomi* by RAPD-PCR. FEMS Microbiol. Letts. 113: 43-47.

Farbey M., C. Nicholson and P.A. O'Brien. 1996. Methods for extraction of PCR amplifiable DNA from soil (submitted).

Dobrowolski, M. M. Farbey and P.A. O'Brien. 1996. Development of a PCR test for detection of *Phytophthora cinnamomi* (in preparation).

Nicholson C.J.M., M.D. Farbey, M.P. Dobrowolski, and P.A. O'Brien. Rapid detection of *Phytophthora cinnamomi* in soil samples by PCR. Combined Biological Sciences Meeting. Perth 19/8/1994.

Farbey M.D., M.P. Dobrowolski, C.J.M. Nicholson, and P.A. O'Brien. Fast detection of *Phytophthora cinnamomi* in soils by PCR. Symposium on Plant Pathology Research In Western Australia. Perth 22/3/1995

Farbey, M., Dobrowolski, M., Nicholson, C., and O'Brien, P.A. Detection of fungal pathogens by Polymerase Chain Reaction (PCR). 10th Australasian Plant Pathology Society Meeting. Lincoln University, New Zealand 28-30th Aug 1995.

C. Nicholson, M. Farbey, and P. A. O'Brien. New Methods for Detection of *Phytophthora cinnamomi*. 10th International Biotechnology Symposium. Sydney 25-30 August 1996.

## PROJECT 3

## DEVELOPMENT OF GIS-BASED DECISION-SUPPORT TOOLS AND THE DATABASING OF *PHYTOPHTHORA*-SENSITIVE TAXA

*FINAL REPORT*

G. Behn, A. Chapman, A. Conacher & R. Wills

### OBJECTIVE

To provide a reliable, graphical decision-support system for monitoring and controlling the spread of dieback disease.

The project has three elements:

1. database and automate access to Herbarium specimen records for *Phytophthora* susceptible taxa;
2. maintain, update and interrogate data sets to answer basic management questions;
3. develop a model with predictive capabilities and test validity of predictions.

### INTRODUCTION

This project uses a Geographic Information System (GIS) to tie together relevant spatial information about *Phytophthora* to provide both managers and researchers with a means of easily answering questions that were previously impossible or too time-consuming to attempt. In its development, the project targeted the Two People's Bay-Mt Manypeaks area in CALM's Albany District. This area is significant because of a long history of *Phytophthora* impact and a high conservation value due to the large representation of susceptible plant species, the presence of 22 plant taxa on CALM's Priority list, including 7 Declared Rare Flora (DRF) and four of which are highly susceptible to *Phytophthora cinnamomi* (*Adenanthos cunninghammi*, *Andersonia* sp. (TPB) G. Keighery 8229, *Banksia brownii* and *Banksia verticillata*), vegetation associations of special conservation interest, as well as the presence of a number of endangered fauna including the Noisy Scrub-bird, the Western Bristlebird, the Western Whipbird and the recently rediscovered Gilbert's Potoroo.

### DATA SYSTEMS

Two further study areas, Cobiac forest block and the Stirling Range National Park, were used to test the ability of the GIS-based decision-support tools to readily incorporate additional information, and to provide a data set to better test the value of the GIS system in presenting information to managers and scientists.

Data for the Cobiac forest block have been provided by G. Behn. Additional data sets for the Stirling Range National Park have been obtained from Department of Land Administration, but requests for key files for both of these areas are still to be met by the Department of Agriculture.

A collaborative project with the CSIRO to determine if identification of dieback impact can be derived from remote sensed imagery was completed and is briefly reported below.

Data sets now accessible as a result of this project include cadastre, roads, contour, elevation, slope, aspect, hydrology, distribution of dieback affected vegetation, landform, vegetation, and species distribution based on data accessed from WA Herbarium voucher records. For development purposes, the Herbarium data sets are limited to include the Proteaceae, Myrtaceae and Cyperaceae.

## RESULTS BASED ON ACTION PLAN

### 1. Databasing specimen records

The databasing of all Herbarium specimens of *Phytophthora* susceptible taxa has been completed with details provided in previous annual reports.

### 2. Develop predictive GIS tools

For details on data linkages, interface development and dieback modelling see the Final Report to ANCA (March 1995). A brief overview of the model is provided below. Work over the past year has focussed on the use of remote sensing for evaluating or monitoring vegetation health particularly in relation to *Phytophthora* dieback.

#### *2.1 Development of a predictive model*

Basically, the dieback model relies on GRIDs of aspect, slope, flow direction and flow accumulation. Basic assumptions of the model are that *Phytophthora cinnamomi* will move rapidly downhill with water movement and will grow uphill through the roots of hosts; spread and impact will vary depending upon landform, susceptibility of vegetation, aspect, and road networks in an area.

The model can be conceptualised as a broad scale model which utilises available data to provide an indication of possible dieback threats. The model relies on LANDSAT imagery to identify sources of infection, from which the algorithm predicts the direction and rate of spread. Hydrological modelling techniques have been facilitated through the raster-based GRID module of Arc/Info which provides the ability to combine numerous 'surfaces' within the same spatial context. The factors affecting dieback at higher resolutions, than LANDSAT imagery, require detailed knowledge of micro-hydrogeological processes and cannot be addressed using existing data.

## 2.2 *Use of remote sensing for monitoring vegetation health in relation to Phytophthora dieback.*

An evaluation of the current remote sensing systems and technologies available, based on multi-temporal satellite imagery, was conducted to assess their capacity to detect dieback occurrences within coastal heath and jarrah forest communities of the south west.

Based on 59 ground samples, the classification of vegetation health in the south coast study was 86%. This indicates that the technique can give a useful synoptic assessment of vegetation health in that range of plant communities. The particular communities exhibit a relatively simple physical structure and have few other agencies besides *Phytophthora* and fire that cause widespread plant deaths. In this type of vegetation, the results from analysis of satellite multispectral imagery provide a useful picture of the extent and location of dieback infections at a synoptic scale.

The studies in the jarrah forest also showed that analysis of satellite multispectral imagery could separate areas of healthy vegetation from areas of less healthy vegetation. However, the physical structure of the jarrah forest is more complex than heathland, leading to more complexity in the spectral responses requiring analysis. The utility of such a tool for assessing forest health is influenced by some major limitations.

- Plant stress and death in jarrah forest may be due to a variety of factors:

  ⇒ *Phytophthora cinnamomi*
  ⇒ other pathogens
  ⇒ drought
  ⇒ insect attack
  ⇒ fire

  Satellite imagery alone may be unable to differentiate these.

- The effect of disease may be present as subtle changes in the understorey with little effect on the dominant vegetation. Subtle disease expression is not likely to be detected.

- Resistant vegetation persists in areas of *Phytophthora cinnamomi* infection and show as healthy areas.

This work has shown that careful analysis combined with additional topographic information can be used to prepare maps of vegetation condition that are suitable for reconnaissance information prior to detailed ground disease surveys. In this instance, it can replace small-scale aerial photographs in highlighting general patterns of vegetation condition.

It does not, however, give an accurate estimate of the area nor precise location of *Phytophthora cinnamomi* infections. The reasons for this were outlined above. As examples, areas of healthy forest may appear as diseased on areas of rock or drought-

prone sites. Conversely, remnant patches of resistant species within broad areas infected by *Phytophthora cinnamomi* might be mistaken for healthy forest of susceptible species.

### 3. Transfer specimen records into a relational database

The establishment of a relational database of susceptible and threatened taxa, based on existing and new herbarium specimen sheets is continuing and is expected to be completed within the next 12 months. Under the original proposal it required the purchase of an ORACLE license, and substantial programming to implement the new database. This was delayed until project enhancements to the existing herbarium specimen database environment (TEXPRESS) (funded by CALM), in particular the availability of SQL and ODBC compatibility, enabled cost savings to be identified. By developing direct linkages between the existing TEXPRESS specimen database and the GIS modelling and Management Decision Support components in ARC/Info, using ODBC methodologies, additional costs involving ORACLE development can now be avoided.

The TEXPRESS ODBC driver is due to be delivered later in 1996 at which time it is envisaged that, once dynamic links are established between the Herbarium database and the GIS interface, a fully functional relational database will be established.

## *EVALUATION OF CURRENT PROJECT AND PROPOSALS FOR NEW DIRECTIONS*

Independent evaluation by CALM

### PREDICTIVE MODELLING

Although there appeared to be considerable merit in pursuing a process based modelling approach, to develop a decision-support tool for predicting *Phytophthora* spread, it has not proved successful. This is due in part to a number of unanticipated features in the study area. They are:

i. the slow movement upslope along infection fronts, ca. 1m per annum in what was originally thought to be areas of rapid spread of infection, a figure well below the sensitivity of landsat imagery minimum pixel size of 30m x 30m,

ii. the absence of a means of sensing variability in the diverse substrates of southwestern Australian landscapes and their effects on hydrology,

iii. the heuristic nature of the movement of animal vectors which introduces a strong element of unpredictability to estimates of dispersal.

The analysis of satellite multispectral imagery can provide useful synoptic analysis of vegetation condition and changes to that condition over time. The proviso is that any such analysis must include detailed knowledge of artificial disturbances and ground sampling of natural factors due to the complexity of vegetation over time and space. The best results will only be obtained with ground sampling as an integral part of the analysis and will then only give statistical estimates of vegetation parameters with a broad picture of patterns.

The danger is that half-completed image analysis could erroneously be shown as depicting large areas of *Phytophthora cinnamomi* infection when in fact it may be change in vegetation due to drought, harvesting or some other agency. Any project involving this type of data analysis would have to be managed to avoid this type of result.

It is proposed that the GIS based decision making element of the study be redirected toward an expert systems approach. This will exploit the gains made in databasing and the development of a relational database, and focus on the development of protocols for identifying those populations of threatened taxa which might be protectable.

## DATABASING *PHYTOPHTHORA* SUSCEPTIBLE TAXA

The initial data-basing of *Phytophthora* susceptible taxa element of this project has been finalised and reported on previously. On the basis of more recent and widespread experience in CALM a precautionary note needs to be sounded concerning the indicative rather than definitive nature of the data base.

It has not been possible to evaluate the susceptibility of all taxa in all families because of

i. the great diversity in many families which contain a significant number of taxa reliably known to be susceptible to *P. cinnamomi*
ii. the considerable variation in susceptibility between and within populations,
iii. the important effects of temporal and spatial variation in environmental factors in conditioning interactions between hosts and pathogen.

The conservative and precautionary approach therefore adopted ie. to assume likely susceptibility of taxa on the basis of taxonomic relatedness to known suscepts, carries with it a risk of significant error of classification. For this reason the data base cannot be taken to be always a reliable tool for evaluating the threat which *P. cinnamomi* poses for the health or continued existence of species or even a single population. Despite this, the database and the development of a relational database system will provide the basis for an extremely useful tool for monitoring and designing control strategies for preventing the spread of *Phytophthora*, particularly in threatened flora populations.

## PROJECT 4

## THE CONTROL AND MANAGEMENT OF *PHYTOPHTHORA. MEGASPERMA* IN THE NATIVE PLANT COMMUNITIES OF WESTERN AUSTRALIA

S.A. Carstairs, L.E. Newcombe, S.E. Bellgard and F.D. Podger

## INTRODUCTION

In an earlier report Bellgard *et al.* (1995) provided details of *Phytophthora megasperma* research conducted by CALM in the period February 1994 to February 1995. The research components of that report included:

- An investigation of isozyme variation in the *Phytophthora megasperma* complex in Western Australia.
- An assessment of the pathogenicity of *P. megasperma* from the Fitzgerald River National Park (FRNP).
- The effects of a phosphate trial on susceptible plant species in *P. megasperma* diseased areas.

In the current report the research presented in Bellgard *et al.* (1995) is developed further and a full account is given of a new scope item:

- to investigate the significance of oospores and variability within *P. megasperma*

## 1. *PHYTOPHTHORA MEGASPERMA* IN NATIVE PLANT COMMUNITIES OF WESTERN AUSTRALIA.

*P. megasperma* Drechs. was described by Drechsler (1931) as an oomycete with unusually large oogonia. Subsequently, the species concept has been broadened to include similar *Phytophthora* with smaller oospores, however there has been 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) believe that their interpretation of *P. megasperma*, with *P. sojae*, *P. medicaginis*, and *P. trifolii* removed, fits 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) retain three groups identifiable by their hosts as well as morphological and chemical characteristics:

| | | |
|---|---|---|
| Group 1. | Broad Host Range | (BHR) |
| Group 2. | Apple Cherry | (AC) |
| Group 3. | Douglas Fir | (DF) |

In an isozyme survey of seventy seven Western Australian isolates of *Phytophthora* that had been identified as *P. megasperma* on morphological grounds, Bellgard and Carstairs (1996) were able to match only 9 isolates with *P. megasperma* standards (Table 1). None of the other sixty eight isolates matched with any of the three legume pathogens examined.

**Table 1.** Isozyme affinities of seventy seven Western Australian isolates of *Phytophthora* which were identified as *Phytophthora megasperma* on morphological grounds.

| **Standard Isolates** | **Hosts** | **No. WA Field Isolates In Each Group** |
|---|---|---|
| *P. medicaginis* * | alfalfa ** | 0 |
| *P. sojae* * | soybean ** | 0 |
| *P. trifolii* * | clover ** | 0 |
| | | |
| *P. megasperma* | | |
| BHR * | many ** | 1 |
| AC * | fruit trees ** | 8 |
| DF * | Douglas Fir, herbaceous weeds ** | 0 |
| Isolates not matched | | |
| WA.1 | not known | 1 |
| WA.2 | many | 67 |

* Isolates obtained from C.M. Brasier U.K.
** Hansen and Marshall (1991).

The only BHR isolate found in the Western Australian sample was recovered near the town site of Cataby on the sand plain north of Perth. Five of the eight AC isolates identified in the survey were also recovered from the sand plain north of Perth, two were recovered from FRNP in the south west of Western Australia, and an isolate was recovered from the town site of Hopetoun near FRNP.

Among those sixty eight isolates that did not match with any of the standards, WA.1 was a single isolate obtained from near Cataby, and WA.2 was widely distributed and was recovered from many hosts. In WA.2 two isozyme types were identified, WA.2.1

and WA.2.2. WA.2.1 of which there were sixty two isolates, was widely spread, and the five WA.2.2 isolates were recovered from the sand plain north of Perth.

Subsequent to Hansen and Maxwells (1991) treatment of the *P. megasperma* complex, Forster and Coffey (1993) evaluated a worldwide collection of isolates of *P. megasperma* using mitochondrial and nuclear DNA polymorphisms. 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.

Three new standards identified in the Forster and Coffey (1993) study have been obtained by the Department of Conservation and Land Management from Michael Coffey of Riverside California USA. The results are yet to be determined.

The findings to date might be interpreted as analogous to recent interpretation of replacement in periodic selection in *P. infestans*. Analysis of "continental" populations of *Phytophthora infestans*, the late potato blight fungus, from Europe, USA/Canada and Japan suggested that there has been a recent and worldwide migration and genotype displacement in Europe (Spielman *et al.* 1990, Spielman *et al.* 1991). Almost total displacement in this pathogen has occurred within a few years. This phenomenon may be an example of "periodic selection" which is common to organisms, such as Phytophthoras, where clones of greater fitness displace those with lesser fitness (Hartl and Dykhuizen, 1984).

**Recommendations:**

1. With 87.3% of the isolates tested falling into the broad host range WA.2 taxon, it is recommended that research emphasis be placed on the control and management of this pathogen. The AC pathogen, which is usually associated with mortalities in fruit trees, accounted for 10.1% of the isolates assessed and need not receive the priority given to WA.2.

2. It is recommended that isozyme types WA.1 and WA.2 be compared with the new Coffey standards. Should the WA.2 taxon be matched with an isotype previously identified by Coffey we might obtain from the literature information about the original source of the pathogen, possible modes of introduction into Western Australia, information about its hosts, climate and soil type preferences, and possibly information relating to control measures.

3. Given that four *P. megasperma* taxa were recognised in only seventy seven WA isolates tested it is recommended that monitoring of isotypes associated with outbreaks of disease caused by *P. megasperma* continue. Regions requiring our particular attention include the sand plain north of Perth and the southern and south-eastern coastal regions. Should displacement of the WA.2.1 taxon occur by new WA.2 genotypes or other taxa, it will become apparent only by continued monitoring.

## 2. ON THE SIGNIFICANCE OF OOSPORES AND VARIABILITY WITHIN *PHYTOPHTHORA MEGASPERMA*.

In this section a series of three short research projects are presented which describe the techniques and analyses used to assess diversity and outcrossing rates, and hence the significance of oospores, in *P. megasperma* and other homothallic *Phytophthora* species in Western Australia.

In the first project (2.1) the inheritance of isozyme phenotypes of NADP-IDH enzymes in CIT 3 *Phytophthora citricola* is examined. In the second project (2.2) the competitive abilities of NADP-IDH-2 genotypes of CIT 3 *P. citricola* is examined, and in the final section (2.3) an estimate of outcrossing (sexual crossing in the field) is determined for *P. megasperma* (WA.2) and *P. citricola.*

Taken together the three studies described here lead to the novel and important conclusion that WA.2 *P. megasperma,* like other homothallic species of *Phytophthora* in Western Australia and *Phytophthora infestans* (ie. heterothallic species) in Mexico, may by sexual reproduction be producing new genetic strains of *Phytophthora.* Should existing field populations of WA.2 *P. megasperma* be displaced with new and fitter phosphonate resistant strains, then dieback managers may well be presented with yet another very serious problem.

### 2.1 An NADP-Isocitrate Dehydrogenase-2 ab heterozygote of CIT 3 *Phytophthora citricola* arose by oosporogenesis rather than by heterokaryogenesis; and evidence is adduced for separate sub-cellular localisations of two NADP specific IDH enzymes in an oomycete.

#### *Introduction*

The earliest work demonstrating multiple loci for coding of NADP-IDH using isozyme electrophoresis was that of Oudemans *et al.* (1994) who in *Phytophthora citricola* identified five electrophoretic subgroups, CIT 1-5, among a worldwide collection of 125 isolates. Commenting on their NADP-IDH results they reported that there were two loci coding for this enzyme in these Phytophthoras, and that one of the enzymes (IDH-1, their notation) was absent from some sub-groups.

Some field isolates of *P. citricola* from the south-west of Western Australia shared a high degree of isozyme fidelity with a CIT 3 standard isolate obtained from Michael Coffey of Riverside California USA (Carstairs, unpublished). In Western Australian (WA) CIT 3 isolates two NADP-IDH specific enzymes were consistently resolved by Cellulose Acetate Gel Electrophoresis (CAGE).

NADP-isocitrate dehydrogenase (NADP-IDH, EC 1.1.142) has been studied in several different higher plants (Omram and Dennis, 1971; Randall and Givan, 1981; Kiang and Gorman, 1985; Ni *et al.* 1987). Kiang and Gorman (1985) reported that there were four active NADP-IDH loci in soybean, two coding for cytosol - associated

enzymes and two coding for mitochondrial forms of the enzyme, whereas Ni *et al.* (1987) reported that there were two cytosolic NADP-IDH enzymes and a chloroplast specific enzyme in *Pisum sativum*. Oudemans *et al.* (1994) did not attempt to describe the subcellular compartmentalisation of the two NADP-IDH enzymes they reported for *P. citricola*.

Activity of NADP-IDH in the chloroplast fraction of *P. sativum* is much lower than that in the cytosol (Rundall and Givan, 1981; Ni *et al.*, 1987). In their summary table of the subcellular localisation of isozymes in plants Weeden and Wendel (1989) included a cytosolic NADP-IDH only arguing that plastid-specific isocitrate dehydrogenase is usually too faint to be observed under standard electrophoretic conditions.

In the case where two enzymes that are specific for the same substrate are located in the same subcellular compartment (eg. the two cytosolic NADP-IDH enzymes of *P. sativum*, and the polypeptides specified by all of the encoding alleles randomly associate into multimeric products), intergenic heteromultimers will be observed as well as homomultimers and intragenic heteromultimers (Wendel and Weeden, 1989).

With very few exceptions (Chou and Splittstoesser, 1972; Hock, 1948), analysis of the inheritance of cytosolic, mitochondrial, and plastid isozymes that are specific for the same substrate has shown that they are each encoded by a different locus (Newton, 1983; Weeden, 1983). While enzymes located in different subcellular compartments do not form intergenic heteromultimers, Wendel and Weeden (1989) caution that failure to do so suggests but does not prove different organelle locations for multigenic isozyme systems.

In this study we examined the CAGE isozyme patterns of the two NADP-IDH enzymes of CIT 3 *P. citricola* from WA with the aim of determining their subcellular compartmentalisation.

Shepherd and Pratt (1974) found that over 90% of the zoospores from eight isolates of *P. cinnamomi* were uninucleate. On the assumption that zoospores of all *Phytophthora* species are normally uninucleate, Layton and Khun (1988) used them to recover the parental phenotypes of synthesised interracial heterokaryons of *Phytophthora megasperma* f. sp. *glycinea*. Old *et al.* (1984) used them to show that the zoospore progeny from *Phytophthora cinnamomi* isolates that were putatively heterozygous for one or two of five loci, not including NADP-IDH, were also heterozygous for those loci.

Each group reasoned that because almost all zoospores are uninucleate, they could be used to distinguish between *Phytophthora* isolates that are heterozygotes and those that are heterokaryons. The zoospores from the mycelium of a heterokaryon would separate into two populations half of which would have the nuclei of one parent and half having the nuclei of the other. All of the zoospores from the mycelium of a putative diploid heterozygote, on the other hand, would be true breeding to the parent. In this study we used the same argument to determine whether a three banded NADP-

IDH-2 CIT 3 *P. citricola* isolate was a heterokaryon or was heterozygous for that locus thereby deducing its genesis.

### *Procedure*

To recover zoospore progeny we adopted a baiting technique whereby Phytophthoras are stimulated to produce sporangia and zoospores, and the zoospores are trapped with *Eucalyptus sieberi* cotyledon baits. The technique is an adaptation of that described by Marks and Kassaby (1972).

Ten 70% ethanol surface sterilised *E. sieberi* cotyledons were placed adaxial surface down onto the surface of each of six selective agar plates. To three of these plates 2 mm square cornmeal agar (CMA) inoculum plugs of a three banded NADP-IDH-2 CIT 3 isolate were placed alongside the Eucalypt cotyledons, and to the other three plates inoculum plugs of a single banded NADP-IDH-2 CIT 3 were placed alongside the cotyledons. The plates were sealed and allowed to incubate at 23° C in the dark for 48 hrs 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 then 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.

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 the diluted soil extract was added. Twenty five surface sterilised *E. sieberi* cotyledons were added to each container and floated adaxial surface down. The dishes were incubated in a constant temperature (24° C) room with constant fluorescent light. 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.

On the bottom of three plastic dishes and 8 cm apart were attached two *E. sieberi* cotyledons that had been infected with a three banded IDH-2 CIT 3 *P. citricola.* To each dish was added 250 mL of the diluted soil extract and 25 *E. sieberi* cotyledons which were floated adaxial surface down. As with the controls these dishes were incubated in a constant temperature room 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. citricola* colonies growing into the agar. From the *Phytophthora* colonies growing out from thirty Eucalypt baits half centimetre square inoculum plugs were removed 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 hyphae was harvested and assessed isozymically by the CAGE method for IDH (Hebert and Beaton, 1989). Five zoospore colonies from a second set of three replicate dishes inoculated with the same three banded IDH-2 CIT were also assessed isozymically for IDH. Three dishes to which single banded IDH-2 CIT 3 *P. citricola*

infected *E. sieberi* cotyledons were added were treated in the same manner as that described for the three banded IDH-2 CIT 3 inoculated dishes.

### *Results*

An example of a typical NADP-IDH gel is presented in figure 1. Two loci, IDH 1 and 2, were readily resolved. The most anodally migrating isozyme (hereafter referred to as IDH-1) stained intensely and could be observed within 15 seconds of the stain being applied to the gel. The more slowly migrating isozyme (hereafter referred to as IDH-2) stained less intensely and was only visible after five to ten minutes of the stain being applied to the gel.

**Figure 1.** A Cellulose Acetate Gel Electrophoresis (CAGE) plate stained for NADP-IDH.

*Het : Zoospore colony having the three banded phenotype of its mycelium parent.

H : Zoospore colony having the single banded phenotype of its mycelium parent.

No intergenic heteromultimers were formed between the most anodally migrating isozyme (IDH-1) and the less intensely staining isozyme (Figure 1). This is particularly evident from examination of the patterns obtained in positions 6 to 10 of Figure 1, where a two banded pattern was observed with no intermediate band occurring between the faster and more intensely stained isozyme (IDH-1) and the slower isozyme (IDH-2).

NADP-IDH-2 was polymorphic with both ab heterozygotes (positions 1 to 5 of Figure 1) and aa homozygotes being easily distinguished. IDH-2 ab heterozygotes produced an intermediate heterodimer band typical of NADP-IDH dimer molecules produced in plants (Weeden and Wendel, 1989).

No Phytophthoras were recovered from the three bait dishes to which no inoculum was added. There was no evidence that the soil extract was contaminated with CIT 3 *P. citricola* or any other *Phytophthora.* All of the baits that were exposed to either the single banded NADP-IDH-2 CIT 3 *P. citricola* inoculum or the three banded inoculum showed symptoms of infection (loss of anthocyanin pigment from the abaxial surface of the *E. sieberi* cotyledons) by *Phytophthora.* All of the baits plated from these dishes onto selective agar proved positive for *Phytophthora.* The five

zoospore colonies obtained from the dishes inoculated with the single banded IDH-2 CIT 3 isolate all produce the single band IDH-2 phenotype of the parent (Table 2 and see Figure 1 position 6-10). All 35 zoospore colonies obtained from the dishes inoculated with the three banded IDH CIT 3 isolate produce the three banded IDH-2 phenotype of their parent (Table 2 and see Figure 1 positions 1-5).

**Table 2.** Colony formation from zoospores of two CIT 3 *P. citricola* isolates that may be distinguished by their NADP-IDH-2 phenotypes.

| IDH-2 isolate | No. of zoospore colonies tested | Single banded (aa) | Three banded (ab) | Single banded (bb) |
|---|---|---|---|---|
| Single banded (aa) | 5 | 5 | 0 | 0 |
| Three banded (ab) | 35 | 0 | 35 | 0 |

### *Discussion*

CAGE identified two isozymes in Western Australian isolates of CIT 3 *P. citricola.* Either these enzymes are specified by the same subcellular compartment or they are localised to different compartments. In the paragraphs following, we argue that the data support the latter ie. that the two enzymes are localised in different compartments.

Analysis of the inheritance of enzymes specific to the cytosol, mitochondria or plastids in plants has shown that they are each encoded by different loci. Enzymes which are encoded by different loci and are specific to different subcellular compartments do not form intergenic heteromultimers. In contrast to this enzymes which are encoded by different loci and are specific to the same subcellular do form intergenic heteromultimers (Wendel and Weeden, 1989). The two NADP-IDH enzymes of WA Cit 3 *P. citricola* did not form intergenic heteromultimers and we accept this as prima facie evidence that they were localised in different subcellular compartments. The evidence is not taken as proof, but it is supportive.

Wendel and Weeden (1989) and others (Rundall and Givan, 1981; Ni *et al*, 1978) observed in plants that cytosolic-specific NADP-isocitrate dehydrogenase is very active, and that plastid-specific NADP-IDH is much less active such that it may not be visualised under standard electrophoretic conditions. The activity of the two NADP-IDH enzymes from WA CIT 3 *P. citricola* corresponded to that reported for plants. One of the enzymes (NADP-IDH-1) was extremely active and appears to correspond in action to what has been described for cytosolic-specific NADP-IDH in plants, and the less active enzyme (NADP-IDH-2) behaved as do plastid specific NADP-IDH enzymes in plants. Since, however, Phytophthoras do not have chloroplasts the plastids to which the NADP-2 enzyme may be associated could be protoplastids. Alternatively it may be associated with mitochondria or peroxisomes, although this is less likely (Ni *et al*, 1987).

The three banded (putative heterozygote) NADP-IDH-2 CIT 3 *P. citricola* may have arisen by oosporangenesis between two isozymically different homozygotes, a heterozygote and homozygote or two heterozygotes, or it may have originated by heterokaryogenesis. Heterokaryotes produce two types of uninucleate zoospores, one having the phenotype of one of the parents of the heterokaryon and the other type of zoospore having the phenotype of the second parent. The zoospores of a diploid heterozygote, on the other hand, would all have the phenotype of their parental mycelium. As all of the zoospore colonies from the three banded NADP-IDH-2 CIT 3 mycelium had the same three banded phenotype as their parent, we conclude that the parental isolate originated by oosporangenesis rather than heterokaryogenesis.

## 2.2 Allozymes discriminate between morphologically indistinguishable pathogens recovered in competition experiments between IDH-2 genotypes of the CIT 3 form of *Phytophthora citricola*.

- Using isozyme electrophoresis, Oudemans *et al.* (1994) identified five electrophoretic subgroups, CIT 1-5, among a worldwide collection of 125 isolates identified as *Phytophthora citricola.*

- Some field isolates of *Phytophthora* from the south-west of Western Australia shared a high degree of isozyme fidelity with a CIT 3 standard isolate obtained from Michael Coffey of Riverside California USA (Carstairs, unpublished data).

- In Western Australian (WA) CIT 3 isolates the IDH-2 (EC. No. 1.1.1.42) locus was found to be isozymically polymorphic, and had two alleles (isozymically distinguishable allozymes). The more electrophoretically (anodally) mobile allele will here-after be referred to as the 'a' allele, and the slower allele will be referred to as the 'b'.

- Among the WA CIT 3 isolates three IDH-2 genotypes were recognised, they being aa homozygotes, ab heterozygotes and bb homozygotes.

- The IDH-2 protein is a dimer molecule, and individuals that are heterozygotes for this locus typically produce three banded IDH-2 electrophoresis patterns. CIT 3 heterozygotes are readily distinguished from mixed cultures of aa and bb CIT 3 homozygotes which do not produce heterodimer bands (data not presented).

- The CIT 3 subgroup has been described as one which is recovered from diverse locations and hosts (Oudemans *et al.* 1994).

- In five field samples from the south-west of Western Australia which were infested with two species of *Phytophthora*, Carstairs (1995) found that one of the species was usually recovered in higher frequency ($X^2 = 10.53$ and 1 d.f.) than the other. The common species was recovered 4.05 (average) times more frequently than the uncommon species.

- In experiments designed to compare the abilities of *Phytophthora parasitica* and *Phytophthora palmivora* in mixed culture to infect fibrous roots of citrus, Zitko and Timmer (1994) used hyphal morphology of the colonies on a selective (PARPH) nutrient agar (NA) to distinguish between the pathogens.

- In experiments combining *P. parasitica* and *P. palmivora* Zitko and Timmer (1994) did not report the occurrence of multiple infections (ie. both species) in any of hundreds of 1 cm root pieces plated and visually assessed for the presence of the pathogens.

- The objective of this study was to explore the use of isozyme electrophoresis to discriminate between morphologically indistinguishable pathogens, in this case IDH-2 genotypes of the CIT 3 subgroup of *P. citricola*, in competition. To our knowledge this is the first time the electrophoresis technique has been used to discriminate between competitors in any kind of competition experiment.

***Procedure***

To assess the competitive abilities of IDH-2 genotypes of CIT 3 *P. citricola* to infect a host in combination, we employed a baiting technique whereby inoculum is stimulated to produce sporangia and zoospores and the zoospores are trapped with *Eucalyptus sieberi* cotyledon baits. The Phytophthoras thus recovered were identified by Cellulose Acetate Gel Electrophoresis (CAGE) of their IDH proteins (Hebert and Beaton, 1993).

Ten 70% ethanol surface sterilised *E. sieberi* cotyledons were 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 a heterozygous CIT 3 isolate were placed alongside the Eucalypt cotyledons, and to the other three plates inoculum plugs of an aa homozygous CIT 3 were placed alongside the cotyledons. The plates were sealed and allowed to incubate at 23$^{\circ}$ C in the dark for 48 hrs 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$^{\circ}$ C in the light for 24 hrs then 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.

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 the diluted soil extract was added. Twenty five surface sterilised *E. sieberi* cotyledons were added to each container and floated adaxial surface down. The dishes were incubated in a constant temperature (24$^{\circ}$ C) room with constant fluorescent light. 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.

On the bottom of three plastic dishes and 8 cm apart were attached two *E. sieberi* cotyledons that had been infected with an ab heterozygous CIT 3 *P. citricola*. To each dish was added 250 mL of the diluted soil extract and 25 *E. sieberi* cotyledons which were floated adaxial surface down. As with the controls these dishes were incubated in a constant temperature room 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. citricola* colonies growing into the agar. From the *Phytophthora* colonies growing out of five Eucalypt baits half centimetre square inoculum plugs were removed 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 hyphae was harvested and assessed isozymically by the CAGE method for IDH. Three dishes to which aa homozygous CIT 3 *P. citricola* infected *E. sieberi* infected cotyledons were added were treated in the same manner as that described for the ab heterozygote inoculated dishes.

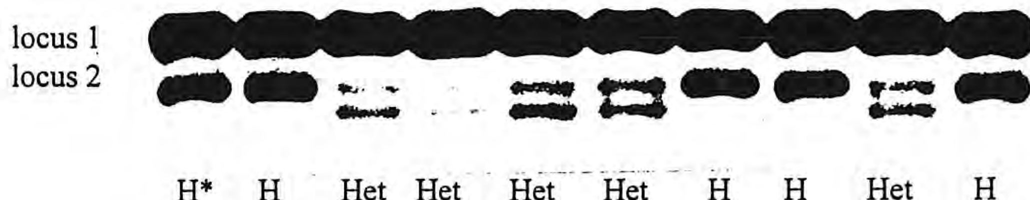
To each of three dishes both ab heterozygous and aa homozygous CIT 3 *P. citricola* infected *E. sieberi* cotyledons were added. On the bottom and toward one end of the dish were attached an aa and ab infected cotyledon and 8 cm from these and toward the other end of the dish was attached a second pair of cotyledons, one infected with the aa homozygote and one with the ab heterozygote CIT 3 *P. citricola.* Diluted soil extract and 25 surface sterilised *E. sieberi* cotyledons were added to each dish and the dishes were incubated in a constant temperature (24° C) room in the light for 48 hrs. Ten *E. sieberi* baits from each of the three dishes were then plated onto selective agar plates and the *Phytophthora* colonies were grown out for 24 hrs. Each of the baits from each of the three dishes were given an identification number and then three hyphal tip samples from the colonies growing from each of the baits were plated onto selective agar plates and grown out for 24 hrs. Each hyphal tip colony was given an identification number, and from these colonies half centimetre square inoculum plugs were removed and transferred to McCartney bottles containing V8/Pea broth. After being incubated at 23° C in the dark for 48 hrs the hyphae was harvested and assessed isozymically for IDH.

### *Results*

No Phytophthoras were recovered from the three bait dishes to which no inoculum was added. There was no evidence that the soil extract was contaminated with CIT 3 *P. citricola* or any other *Phytophthora.* All of the baits that were exposed to either aa homozygous CIT 3 *P. citricola* inoculum or ab heterozygous inoculum showed symptoms of infection (loss of anthocyanin pigment from the abaxial surface of the cotyledons) by *Phytophthora.* All of the baits plated from these dishes proved positive for *Phytophthora,* and those colonies tested for their IDH isozymes proved to be positive for either aa homozygous CIT 3 *P. citricola* or ab heterozygotes (data not presented). There was no evidence of the aa homozygote inoculated dishes being contaminated with ab heterozygote inoculum or *vice versa.*

An example of a typical IDH gel is presented in Figure 2. Two loci, IDH-1 and 2, were readily resolved. The intensely stained IDH-1 locus was monomorphic, whereas the IDH-2 locus was polymorphic with both aa homozygotes and ab heterozygotes being easily distinguished. The samples depicted in Figure 2 were taken at random from hyphal tip colonies derived from baits exposed to aa homozygous inoculum and ab heterozygous inoculum in combination.

**Figure 2.** A Cellulose Acetate Gel Electrophoresis (CAGE) plate stained for IDH. The ten samples depicted in this figure were obtained from hyphal tip samples from baits plated from dishes to which aa homozygous and ab heterozygous CIT 3 *P. citricola* infected *E. sieberi* cotyledon inoculum was added in combination.




* H : Sample homozygous at the IDH-2 locus.
Het : Sample heterozygous at the IDH-2 locus.

Contingency Chi Square analysis of the raw scores in Table 3 of baits infected indicated that there was no significant difference ($X^2_{obs}$ = 9.83 with 6 d.f., and $X^2_{0.05}$ = 12.59) in the recoveries of the Phytophthoras between replicate dishes inoculated with aa homozygous and ab heterozygous CIT 3 isolates in combination.

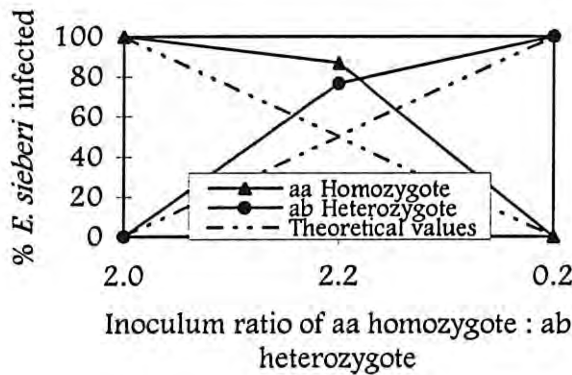
**Table 3.** Raw score recoveries of aa homozygotes and ab heterozygotes of CIT 3 *P. citricola* in combination from three hyphal tip samples from each of ten *E. sieberi* cotyledon baits from each of three replicate dishes.

| **aa homozygote : ab heterozygote inoculum ratio** | **Possible recovery outcomes per bait** | **No. baits infected with Phytophthora** | | | **Observed Totals** | **Expected Totals** |
|---|---|---|---|---|---|---|
| | | **Dish No.** | | | | |
| | | **1** | **2** | **3** | | |
| 2:2 | 3 aa Homs | 2 | 0 | 5 | 7 | 5.85 |
| | 2 Homs + Het | 5 | 6 | 1 | 12 | 12.69 |
| | Hom + 2 Hets | 2 | 2 | 3 | 7 | 9.18 |
| | 3 ab Hets | 1 | 2 | 1 | 4 | 2.22 |
| **Totals** | | 10 | 10 | 10 | 30 | 29.94 |

The frequency of hyphal tip colonies that proved positive for the aa homozygotes CIT 3 (P) was significantly higher (0.58 with S.E. 0.059) than that which proved positive for ab heterozygotes (H) which was 0.42 (S.E. 0.059). Using these values for P and H the expected totals (Table 3) of possible recovery outcomes were determined and found not to be significantly different from that observed ($X^2_{obs}$=2.21 with 3 d.f., and $X^2_{0.05}$=7.81).

The frequency of *E. sieberi* baits that proved positive for the aa homozygote CIT 3 (0.87 and S.E.=0.03) was not significantly different from that which proved positive for the ab heterozygote CIT 3 which was 0.77 (S.E.=0.14), and see Figure 3. In Figure 3, the observed percentage of *E. sieberi* that proved positive for the pathogens singly and in combination (entire lines) are compared with the expected theoretical values (dotted lines).

**Figure 3.** Relative percentage of infection of *E. sieberi* baits by aa homozygous and ab heterozygous CIT 3 *P. citricola* inoculum singly or in combination.




When in combination the frequencies of *E. sieberi* baits infected by the aa homozygote and ab heterozygote CIT 3 were significantly lower (87% and 77% respectively) than when either pathogen was treated singly (100% and 100%), see Figure 3.

Using the values of P and H it is possible to determine the confidence of detecting either the aa homozygote or ab heterozygote from a bait when in combination and where three hyphal tip samples from each bait are tested using the formula:

$$100\text{x}[1-(1-P \text{ or } H)^3] \qquad \ldots\ldots 1$$

The percent confidence of retrieving the aa homozygote from a bait, if the bait had been infected by it, would be 92.6% (from formula 1 above). The percent confidence of retrieving the ab heterozygote would be 80.5%.

### *Conclusions*

The IDH-2 aa homozygote and ab heterozygote allozyme markers were used successfully to distinguish between two morphologically indistinguishable CIT 3 *P. citricola* genotypes singly and in combination.

When the aa homozygote and ab heterozygote CIT 3 isolates were tested in combination the frequency of *E. sieberi* baits infected by each of them was significantly lower than when the pathogens were treated singly. Given that the baits were exposed to the pathogens in combination for a short time (48 hrs), it may be that this observed reduction in baits infected by them is due to the short exposure time used in this study. A different result (higher frequencies of baits infected) may be obtained by exposing the baits to the inoculum for longer periods.

There was no significant difference between the frequency of baits infected by the aa homozygote and that infected by the ab heterozygote when both pathogens were treated in combination. This suggests that under the experimental conditions employed the zoospores of both CIT 3 genotypes were equally able to access the baits offered. However when the frequency of recoveries of the two pathogens was determined by identifying each of the hyphal tip colonies obtained from the infected baits it was found that there was a significantly higher frequency of aa homozygote colonies recovered than there was ab heterozygote colonies. So, although there was no significant difference in the number of baits infected by the pathogens tested in combination, one of them (the aa homozygote) was recovered in higher frequency in the hyphal tip samples from those baits. It may be that the aa homozygote grew more aggressively in the host, or that it grew out into the agar more rapidly than the ab heterozygote, such that the aa homozygote was sampled significantly more often when the hyphal tip samples were taken. Post host infection the aa homozygote seemed to have a selective advantage.

Due to the success in which allozyme analysis discriminated between the two morphologically indistinguishable pathogens used in these competition experiments, we feel that, there is a newly identified place for isozyme electrophoresis in the field of competition studies.

## 2.3 MEASURES OF OUTCROSSING RATES IN FIELD POPULATIONS OF HOMOTHALLIC SPECIES OF *PHYTOPHTHORA* IN THE SOUTH-WEST OF WESTERN AUSTRALIA.

### *Introduction*

The considerable interest researchers have about modes of reproduction in, and the significance of sexually derived perenating spores (oospores) of species of *Phytophthora* is due to the influence that sex, and recombination in particular, must have on the epidemiology of disease caused by Phytophthoras and the consequences this has for their control.

An expected outcome of sexually reproducing populations, relative to asexually reproducing ones, is elevated genetic diversity as a consequence of recombination. Tooley *et al.* (1985) used isozyme electrophoresis to compare populations of *P. infestans* from Central Mexico with those from USA and Canada. In the Mexico populations, in which the two mating types (A1 and A2) of *P. infestans* occurred in a ratio of 1:1, four loci were polymorphic, and fifteen multilocus genotypes were observed. The observed genotype frequencies at two polymorphic loci (GPI and PEP) were not significantly different from those expected for populations in Hardy-Weinberg equilibrium. Up to 1985 only the A1 mating type occurred in USA and Canada. Fewer (four) multilocus genotypes occurred in these populations, and they were not in Hardy-Weinberg equilibrium.

Oospores of the heterothallic *Phytophthora infestans* it seems, are important long-term perenating spores (Fry *et al.* 1992; Drenth *et al.* 1995), and in some species of *Phytophthora* oospores can survive for years in soil (Duncan 1980; Duncan and Cowan 1980). Oospores may permit Phytophthoras to survive harsh environmental conditions and start epidemics when weather conditions are once again favourable.

By coculturing genetically different isolates of the homothallic *Phytophthora sojae* several researchers (Bhat and Schmitthenner 1993; Forster *et al.* 1994; Whisson *et al.* 1994 and Whisson *et al.* 1995) have demonstrated that hybrid oospores can be generated in vitro. Some crosses resulted in few (*circa* 4-10%) hybrid oospores being produced relative to selfed oospores (Forster *et al.* 1994; Whisson *et al.* 1994), while other crosses produced high frequencies (76%) of hybrid oospores (Bhat and Schmitthenner 1993). This demonstration of in vitro outcrossing in a homothallic species immediately raises the possibility that outcrossing may be a feature of field populations of homothallic species of *Phytophthora* in Western Australia. If outcrossing occurs, it is to be expected that diversity levels will be elevated, further complicating control strategies.

Using isozyme electrophoresis Oudemans *et al.* (1994) identified five electrophoretic subgroups, CIT 1-5, among a worldwide collection of 125 isolates identified as the homothallic species *Phytophthora citricola*. Some field isolates from the south-west of Western Australia shared a high degree of isozyme fidelity with a CIT 3 standard

isolate obtained from Michael Coffey of Riverside California USA (Carstairs, unpublished).

Using Cellulose Acetate Gel Electrophoresis (CAGE) Newcombe and Carstairs (1996) consistently resolved two NADP-IDH specific enzymes in Western Australian (WA) CIT 3 *P. citricola* isolates, and argued that the isozymes were localised in different subcellular compartments. One enzyme was localized in the cytosol and the other in protoplastids. The isozyme associated with protoplastids (NADP-IDH-2) was polymorphic, and it was shown that an isolate which produced a three banded NADP-IDH-2 phenotype was a heterozygote which originated by oosporogenesis rather than by heterokaryogenesis (Newcombe and Carstairs, 1996).

Using isozyme electrophoresis Bellgard and Carstairs (1996) identified a homothallic taxon, WA.2, which had morphological affinities with the *Phytophthora megasperma* complex. In this taxon, three putative isozyme loci were polymorphic, each having two alleles. Assuming that the genotypes identified in this taxon originated by oosporangenesis, as was the case for CIT 3 *P. citricola*, it should be possible to use the relative frequencies of these genotypes to derive an estimate of the outcrossing rate in this *Phytophthora.*

### *Procedure*

Fry *et al.* (1992) defined populations of *Phytophthora infestans* as being those which include isolates from a geographically defined unit. The population of WA.2 *P. megasperma* used in this study differed considerably from that of the CIT 3 *P. citricola*. The WA.2 *P. megasperma* population range extended from the sand plain north of Perth Western Australia to east of FRNP on the south eastern coast. Sixty seven isolates were used in this study, and usually a single isolate from any one site was assessed. In contrast to this the CIT 3 *P. citricola* population range was restricted to a small area *circa* forty kilometres east and north of the d'Entrecasteaux National Park. Forty seven sites were positive for CIT 3 *P. citricola* and on average 2.96 (range 1-29) samples from each site were tested for the pathogen. Single samples were taken from twenty eight sites.

Polymorphic isozyme loci were identified by Cellulose Acetate Gel Electrophoresis (CAGE) using the procedures of Hebert and Beaton (1993).

The allozyme data was analysed with the aid of BIOSYS (Swofford and Selander 1981), a computer program for the analysis of allelic variation. Outcrossing rates were estimated using the following formula:

$$t = \frac{Ho}{4pq - Ho} \qquad \text{1.}$$

where t is an estimate of the outcrossing rate, Ho is the observed heterozygote frequency, p is the frequency of one of the allozymes at a locus, and q is equal to 1-p.

*Results*

Fifty three CIT 3 *P. citricola* isolates were recovered from forty seven sites. In this sample of isolates one polymorphic locus (IDH-2) was identified, it having two alleles. Three multilocus genotypes (electromorphs) were observed for the IDH-2 (Table 1), and this was to be expected given that for one polymorphic locus with two alleles the expected number of electromorphs may be determined from the following formula:

$$3^{n} = \text{the No. of EM's} \qquad \text{.......} \quad 2.$$

where n is the number of polymorphic loci with two alleles and electromorphs is given by EM's. So for CIT 3 with one polymorphic locus the expected number of electromorphs is three.

In the sample of 67 WA.2 *P. megasperma* isolates three loci were polymorphic, each having two alleles. So the number of electromorphs in this species would be twenty seven. Only two electromorphs were observed however (Table 4). A Chi Square test to compare the frequencies of the multilocus genotypes expected between the GPI and MDH-1 loci of WA.2 and those observed indicated that there was a significant difference between them ($X^2_{obs} > 25.85$, $X^2_{0.05} = 7.815$ with 3 df), suggesting that the loci were linked.

**Table 4.** Multilocus genotypes (electromorphs) of CIT 3 *P. citricola* and WA.2 *P. megasperma* as determined by CAGE.

| Species/ Population | Electromorph | Locus GPI | IDH-2 | MDH-1 | MDH-2 | Number Observed |
|---|---|---|---|---|---|---|
| P. citricola (CIT 3) | | | | | | |
| | CIT 3.1 | aa | aa | aa | cc | 28 |
| | CIT 3.2 | aa | ab | aa | cc | 1 |
| | CIT 3.3 | aa | bb | aa | cc | 24 |
| P. megasperma (WA.2) | | | | | | |
| | WA.2.1 | cc | N/A | bb | bb | 62 |
| | WA.2.2 | bb | N/A | cc | aa | 5 |

N/A : not available

Chi Square analysis of observed genotype frequencies of the three polymorphic loci of WA.2 *P. megasperma* indicated that in all cases they departed significantly from expected, and had a deficiency of heterozygotes (Table 5). Further examination of Table 5 reveals that this was also the case for the IDH-2 locus of CIT 3 *P. citricola*, which like WA.2 is a homothallic species. The heterothallic species *P. infestans*

(Mexico), used for comparative purposes in this study, gave quite a different result. The GPI locus was at Hardy-Weinberg equilibrium, and the PEP locus appeared to have a significant excess of heterozygotes (Table 2).

**Table 5.** Summary of genotypic frequencies of loci found to be polymorphic in two Western Australian homothallic species of *Phytophthora* and a heterothallic species from Mexico.

| Species/ Population | Locus | Genotype | Observed frequencies | Expected frequencies | Chi Square |
|---|---|---|---|---|---|
| P. citricola (CIT 3) | | | | | |
| | IDH-2 | aa | 28 | 15.2 | |
| | | ab | 1 | 26.6 | |
| | | bb | 24 | 11.2 | |
| | | | | | 50.045*** |
| P. infestans (Mexico)* | | | | | |
| | GPI** | aa | 2 | 1.2 | |
| | | ab | 12 | 13.44 | |
| | | bb | 36 | 35.28 | |
| | | | | | 0.574 |
| | PEP | aa | 32 | 38.52 | |
| | | ab | 15 | 10.73 | |
| | | bb | 3 | 0.75 | |
| | | | | | 9.554*** |
| P. megasperma (WA.2) | | | | | |
| | GPI | bb | 5 | 0.34 | |
| | | bc | 0 | 9.32 | |
| | | cc | 5 | 57.34 | |
| | | | | | 73.93*** |
| | MDH-1 | bb | 62 | 57.34 | |
| | | bc | 0 | 9.32 | |
| | | cc | 5 | 0.34 | |
| | | | | | 73.93*** |
| | MDH-2 | aa | 5 | 0.34 | |
| | | ab | 0 | 9.32 | |
| | | bb | 62 | 57.34 | |
| | | | | | 73.93*** |

* Results obtained from Tooley *et al.* 1985.

** Rare allele frequencies were pooled.

*** Observed frequencies were significantly different from expected at the 0.05 level and for 1 degree of freedom.

In both homothallic species, WA.2 *P. megasperma* and CIT 3 *P. citricola*, the estimated outcrossing rates were low, zero and 0.019 respectively (Table 6). Compared with this the estimated mean outcrossing rate in *P. infestans* (Mexico), the heterothallic species, was high at 0.816. The zero estimate of outcrossing rate for WA.2 *P. megasperma* is likely to be an underestimate. When it is assumed that the outcrossing rate in this species is the same as that in CIT 3 *P. citricola* it is possible to determine the expected number of heterozygotes for any of the three polymorphic loci in WA.2 given that:

$$\text{Expected No. of Hets.} = N \times \frac{t \times 4pq}{1 + t} \qquad \text{......................... 3.}$$

where N is the number of isolates tested, t is the estimated outcrossing rate, p is the frequency of one of the two alleles at a locus, and $q = 1-p$. When t is estimated to be 0.019 for WA.2 the expected number of heterozygotes in a sample of 67 isolates would be 0.347. So it is not surprising that no WA.2 heterozygotes were detected in this study.

**Table 6.** Estimates of outcrossing (t) of two homothallic species of *Phytophthora* in Western Australia and a heterothallic species from Mexico.

| Species/ Population | Locus | Heterozygote frequencies | t |
|---|---|---|---|
| P. citricola (CIT 3) | | | |
| | IDH-2 | 0.019 | 0.019 |
| P. infestans (Mexico) | | | |
| | GPI | 0.24 | 0.806 |
| | PEP | 0.3 | 0.825 |
| mean | | | 0.816 |
| | | | |
| P. megasperma (WA.2) | | | |
| | GPI | 0 | 0 |
| | MDH-1 | 0 | 0 |
| | MDH-2 | 0 | 0 |

### *Discussion*

The estimates of outcrossing determined in this study are to our knowledge the first reported for species in the Oomycetes.

A high mean estimate of outcrossing, 0.816, was determined for the heterothallic species *P. infestans* (Mexico). In order for this species to cross and produce oospores the two A1 and A2 mating types have to be present in the same host. Several

observations: high outcrossing; that some loci (GPI) are in Hardy-Weinberg equilibrium; and that oospores most probably persist in the soil for may years (Drenth *et al.* 1994), when taken together, suggest that in the Mexico population of *P. infestans* oospores serve the important function of presenting nature with new and novel genotypes. Field populations of *P. infestans* are ephemeral, and Fry *et al.* (1992) have described the biology of this species as fitting the characteristics of a metapopulation (Olivieri *et al.* 1990), i.e. a large number of sites can support a single population, that each population has a given probability of extinction, and that vacant sites are recolonized from within the metapopulation. This concept of the biology of *P. infestans* would also account for the observed rapid displacement in Europe of 'old' genotypes with fitter new ones (Spielman *et al.* 1990, Spielman *et al.* 1991).

Estimated outcrossing rates in WA.2 *P. megasperma* and CIT 3 *P. citricola* were very low, zero and 0.019 respectively, by comparison with *P. infestans* (Mexico). The zero estimate for WA.2 is likely to be an underestimate given that the frequencies of rare alleles at each of three polymorphic loci reported in this study were low (0.075). So the probability of recovering a heterozygote, when outcrossing is low (in the order of 0.019), is very unlikely. Presumably these measures of outcrossing are low because the incidence of selfing (s) in these homothallic species, where $s = 1 - t$, is high. Alternatively, the estimates may be low because the heterozygotes are disadvantaged in some way relative to homozygotes, and are being overlooked. Carstairs and Newcombe (1996) explored this second possibility by comparing the competitive ability of a CIT 3 *P. citricola* IDH-2 ab heterozygote to infect host baits with that of an aa homozygote. What they found was that while there was no significant difference in the abilities of the zoospores of the two IDH-2 genotypes to access the host baits, there appeared to be some post host infection selection in favour of the homozygote, although certainly not enough to explain the deficiency of heterozygotes reported in this study. The most likely explanation for the low outcrossing rates in these homothallic species, and intuitively this is to be expected, is that the intensity of selfing by them would be high relative to outcrossing. This is in contrast to diecious or heterothallic species such as *P. infestans* which must cross in order to complete the sexual phase and produce oospores.

Even though outcrossing in these homothallic species is low, the significance of outcrossing to the species should not be under estimated. Obviously the potential to generate new variants, as a consequence of recombination and the union of otherwise novel genomes, is a feature of field populations of these species in Western Australia. If the metapopulation concept applies to homothallic species, as it seems to apply to *P. infestans*, whereby field populations become extinct only to be displaced with new genotypes, it may be that field populations of WA.2 *P. megasperma* in the south-west of Western Australia will at some stage be displaced by new and fitter genotypes. Given that the genotypes that displaced the 'old ones' in Europe were fitter and more resistant to fungicides (Fry *et al.* 1992), should existing field populations of WA.2 *P. megasperma* be displaced with new and fitter phosphonate resistant genotypes, then dieback managers may well be presented with yet another very serious problem.

## 3. CONTROL OF *PHYTOPHTHORA MEGASPERMA* IN NATIVE PLANT COMMUNITIES OF WESTERN AUSTRALIA.

This scope item can be subdivided into two discrete research areas:

1. To compare the relative pathogenicities of taxa within the *P. megasperma* complex and other field occurring *Phytophthora*; and

2. To investigate strategies for managing:
   a. the disease caused by these pathogens; and
   b. the spread of the pathogens into uninfected plant communities.

In relation to item 1 above, which is a new initiative, we give an account of field inoculation trials funded jointly by Science and Information Division of CALM (with financial support from the Australian Nature Conservation Agency), and Forest Resources Division of CALM.

### 3.1 On the relative pathogenicities of taxa within the *P. megasperma* complex and other field occurring *Phytophthora*.

The purpose of this research was to determine the pathogenicity of six Phytophthoras found in the karri forest region, and to refine techniques for investigating this new initiative in the conservation estate.

In an earlier section Bellgard and Carstairs (1996) used isozymes to discriminate between four taxa which on morphological grounds have been placed in the *P. megasperma* complex. Two of the taxa (AC and WA.2) were prominent in a sample of seventy seven isolates from the south-west of Western Australia. The AC taxon, which comprised 10.45% of the sample of isolates tested, is usually associated with the death of fruit trees in orchards (Hansen and Maxwell 1991), whereas WA.2, which comprised 87% of the sample of isolates tested, was associated with the deaths of a wide range of host species in native plant communities in the south-west of Western Australia (Bellgard *et al.* 1995). The prominence of WA.2 isolates in the sample was taken as prima facie evidence that this taxon was the most significant pathogen among those identified among the *P. megasperma* isolates, and it was subsequently recommended that research efforts be directed toward the control of WA.2 (Bellgard and Carstairs Section 2 of this report).

Both WA.2 and the AC taxon have been recovered from FRNP. In a comparative stem inoculation trial Bellgard *et al.* (1995) determined that WA.2 isolates were significantly more pathogenic on five year old *Banksia baxteri* stems than AC isolates.

The scope of this research has now been extended and efforts are being made to determine the comparative pathogenicities of other field occurring Phytophthoras as

well as *P. megasperma*. The new research includes stem inoculation trials and experiments in which the soil is deliberately inoculated. Unlike stem inoculation experiments, soil inoculation is an analogue of a natural process given that infection by Phytophthoras in Western Australian plant communities is soil born.

Four forest types in a confined area *circa* 40 km east and north of d'Entrecasteaue National Park were selected for a deliberate soil inoculation experiment. Six isozymically distinguishable Phytophthoras:

*Phytophthora cinnamomi*
CIT 3 *P. citricola* 1
CIT 3 *P. citricola* 2
*P.* aff. *citricola* JF
*P. drechsleri*, and
*P.* sp. Jeffers No.2

were the subjects of this comparative study against nine higher plant species known to be susceptible to *P. cinnamomi* (four in each forest type).

At one of the sites *B. grandis* stems were inoculated with the six Phytophthoras.

In a multi-stem inoculation experiment, each of four plants were inoculated with the six pathogens, one of the plants with two replicates. The results, after six weeks of this experiment, are presented in Figure 4 and indicate that *P. cinnamomi* was significantly more pathogenic than the other Phytophthoras. CIT 3 *P. citricola* 1 and 2 were not significantly different from each other and after *P. cinnamomi* were the next most pathogenic Phytophthoras. Compared with *P. cinnamomi* the other Phytophthoras appeared to be inconsequential pathogens.

In a separate experiment the capacity of taxa of *Phytophthora* to kill stem inoculated *B. grandis* plants was measured and *P. cinnamomi* was found to cause optimum mortality after five months (Figure 5). The only other deaths were recorded for CIT 3 *P. citricola* 1 and *P.* sp. *Jeffers* No. 2.

At one of the deliberate soil inoculation sites, symptoms of dieback were apparent after five months. Three dead or dying *Pultenaea* sp. plants, to which *P. cinnamomi* had been introduced into the soil adjacent to their stems, were returned to the laboratory and tested for the presence of *Phytophthora* in their tissues. *P. cinnamomi* was recovered from 42.1 to 83.3% of tissue samples tested from these apparently diseased plants. No other species of *Phytophthora* was recovered from their tissues.

None of the plants that were soil inoculated with any of the other five Phytophthoras used in this experiment were showing signs of dieback after five months.

The results of the experiments being conducted east of d'Entrecasteaue National Park indicate that of the Phytophthoras being compared for pathogenicity, *P. cinnamomi* is having the greatest impact. *B. grandis* is less resistant to lesion extension by *P. cinnamomi* than it is to any of the other Phytophthoras tested, and *P. cinnamomi* is the

most efficient killer of this host. In addition to this, after five months it is the only pathogen to demonstrate the capacity to infect plants through the soil.

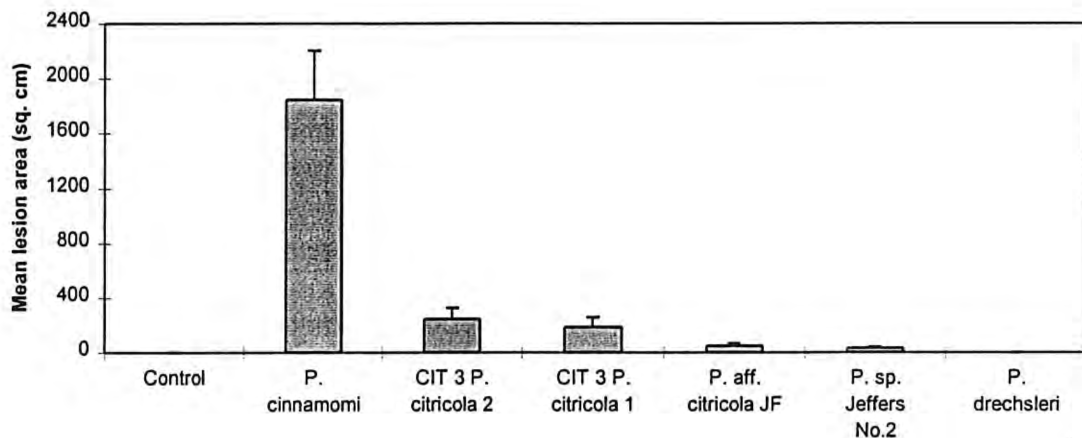



**Figure 4.** Mean lesion area (± s.e, n=5) developed on *B. grandis* stems after six weeks of being inoculated with six isozymically distinguishable *Phytophthoras*.

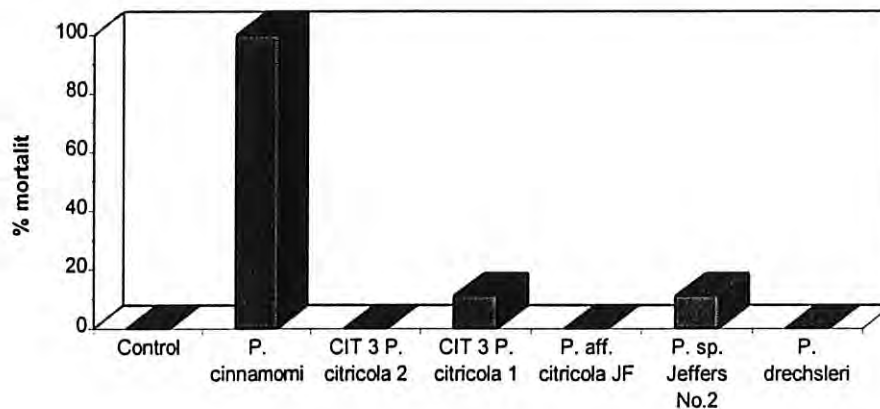



**Figure 5.** Percent mortality of *B. grandis* plants being stem inoculated after five months with six isozymically distinguishable Phytophthoras.

### *Recommendations*

1. It is recommended that stem and soil inoculation pathogenicity experiments, similar to those described here, be extended to include three new centres in the vicinity of:

   FRNA
   Stirling Range National Park, and
   Mt. Lesueur National Park.

These centres have been selected because they fit certain important criteria:

a. There is a high diversity of higher plant species in these centres;
b. A substantial existing occurrence of pathogens has been recorded for these centres; and
c. Several species of *Phytophthora* have been reported to co-occur in each of these centres.

2 The pathogenicity test locations should be restricted to healthy communities, adjacent to the parks, and in the immediate vicinity of severe existing damage associated with *Phytophthora* species. The benefits of extending the research to these centres include:

a. resolution of questions concerning variability in pathogenicity among field occurring species of *Phytophthora*; and

b. the identification of significant pathogens, other than *P. cinnamomi*, which is essential for development of rational strategies for management.

## REFERENCES


Bellgard, S.E. and Carstairs, S.A. (1996). Isozyme variation of *Phytophthora megasperma* retrieved from diseased vegetation in some national parks of south-western Australia. Unpublished manuscript.

Bellgard, S.E, Crane, C., Shearer, B. and Smith, B. (1995). The control and management of *Phytophthora megasperma* in the native plant communities of s-w Australia. Department of Conservation and Land Management annual report to the Australian Nature Conservation Agency.

Bhat, R.G. and Schmitthienner, A.F. (1993). Genetic crosses between physiologic races of *Phytophthora sojae*. *Exp. Mycol.* **17**:122-129.

Carstairs, S.A. (1995). Rapid identification of species of *Phytophthora*. Department of Conservation and Land Management Quarterly Report No. 5.

Carstairs, S.A. and Newcombe, L.E. (1996). Allozymes discriminate between morphologically indistinguishable pathogens recovered in competition experiments between IDH-2 genotypes of the CIT 3 form of *Phytophthora citricola*. Unpublished manuscript.

Chou, K.H. and Splittstoesser, W.E. (1972). Glutamate dehydrogenase from pumpkin cotyledons; characterisation and isoenzymes. *Pl. Physiol.* **49**:550-554.

Drechsler, C. (1931). A crown rot of holly hock caused by *Phytophthora megasperma* n. sp. *J. Wash. Acad. Sci.* **21**:513-526.

Drenth, A., Janssen, E.M. and Govers, F. (1995). Formation and survival of *Phytophthora infestans* under natural conditions. *Plant Path.* **44**:86-94.

Duncan, J.M. (1980). Persistence of mycelium of *Phytophthora fragarial* in soil. *Tran. Brit. Mycol. Soc.* **75**:383-387.

Duncan, J.M. and Cowan, J.B. (1980). Effect of Temperature and soil moisture content on persistence of infectivity of *Phytophthora fragariae* in naturally infested field soil. *Tran. Brit. Mycol. Soc.* **75**:133-139.

Forster, H. and Coffey, M.D. (1993). Molecular taxonomy of *Phytophthora megasperma* based on mitochondrial and nuclear DNA polymorphisms. *Mycol. Res.* **97**:1101-1112.

Forster, H., Tyler, B.M. and Coffey, M.D. (1994). *Phytophthora sojae* races have arisen by clonal evolution and by rare outcrosses. *Mol. Plant-Microbe Int.*. **7**:780-791.

Fry, E.W., Goodwin, S.B., Matuzak, J.M., Spielman, L.J., Milgroom, M.G. and Drenth, A. (1992). Population genetics and intercontinental migrations of *Phytophthora infestans*. *Annu. Rev. Phytopath.* **30**:107-129.

Hansen, E.M. and Maxwell, D.P. (1991). Species of the *Phytophthora megasperma* complex. *Mycologia* **83**:376-381.

Hartl, D.L. and Dykhuizen, D.E. (1984). *E. coli* population genetics. *Annu. Rev. Genet.* **18**:31-68.

Hebert, P.D.N. and Beaton, M.J. (1993). Methodologies for allozyme analysis using Cellulose Acetate Electrophoresis. A Practical Handbook. Helena Laboratories Publication.

Hock, B. (1984). Processing and organelle import of malate dehydrogenase isoenzymes : is there a common precursor for the glyoxysomal and mitochondrial forms? *Physiol. Veg.* **22**:333-339.

Kiang, Y.T. and Gorman, M.B. (1985). Inheritance of NADP-active isocitrate dehydrogenase isozymes in soybeans. *J. Hered.* **76**:279-284.

Layton, A.C. and Kukn, D.N. (1988). The virulence of interracial heterokaryons of *Phytophthora megasperma* f. sp. *glycinea*. *Phytopath.* **78**:961-966.

Marks, G.C. and Kassaby, F.Y. (1974). Detection of *Phytophthora cinnamomi* in soils. *Aust. For.* **36**:199-203.

Newcombe, L.E. and Carstairs, S.A. (1996). An NADP-isocitrate dehydrogenase-2 ab heterozygote of CIT 3 *Phytophthora citricola* arose by oosporogenesis rather than by hetrokaryogenesis; and evidence for separate sub-cellular localisations of two NADP specific IDH enzymes in an oomycete. Unpublished manuscript.

Newton, K.J. (1983). Genetics of mitochondrial isozymes. In: Tanksley, S.D. and Orton, T.J. (eds.), *Isozymes in plant genetics and breeding*, Part A. pp 157-174. Elsevier, Amsterdam.

Ni, W., Robertson, E.F. and Reeves, H.C. (1987). Purification and characterisation of cystolic NADP specific isocitrate dehydrogenase from *Pisum sativum*. *Plant Physiol.* **83**:785-788.

Old, K.M., Moran, G.F. and Bell, J.C. (1984). Isozyme variability among isolates of *Phytophthora cinnamomi* from Australia and Papua New Guinea. *Can. J. Bot.* **62**:2016-2022.

Olivieri, J., Couvet, D. and Gouyoun, P.H. (1990). The genetics of transient populations. Research at the metapopulation level. *Trends Ecol. Evol.* **5**:207-210.

Omram, R.G. and Dennis, D.T. (1971). Nicotinamide adenine dinucleotide phosphate-specific isocitrate dehydrogenase from a higher plant. *Plant Physiol.* **47**:43-47.

Oudemans, Forster, P.H. and Coffey, M.D. (1994). Evidence for distinct isozyme subgroups within *Phytophthora citricola* and close relationships with *P. capsici* and *P. citrophthora*. *Mycol. Res.* **98**:189-199.

Randall, D.D. and Givan, C.V. (1981). Subcellular localisation of NADP-isocitrate dehydrogenase in *Pisum sativum* leaves. *Plant Physiol.* **68**:70-73.

Shepherd, C.J. and Pratt, B.H. (1974). Temperature-growth relations and genetic diversity of A2 mating-type isolates of *Phytophthora cinnamomi* in Australia. *Aust. J. Bot.* **22**:231-249.

Spielman, L.J., Drenth, A., Davidse, L.C., Sujkowski, L.J., Gu, W.K., *et al.* (1991). A second worldwide migration (and population displacement) of the potato late blight fungus? *Plant Pathol.* **40**:422-430.

Spielman, L.J., Gu, W.K. and Fry, W.E. (1990). Genetic relationships among *Phytophthora infestans* populations from Europe, North America, and Japan. *Phytopath.* **80**:1006 (Abstr.).

Swofford, D.L. and Selander, R.K. (1981). A computer program for the analysis of allelic variation in genetics. *J. of Heredity.* **72**:281-283.

Tompkinson, C.M., Tucker, C.M. and Gardner, M.W. (1936). *Phytophthora* root rot of cauliflower. *J. Agric. Res.* **53**:685-692.

Tooley, P.W., Fry, W.E. and Villarreal Gonzalez, M.J. (1985). Isozyme characterisation of sexual and asexual *Phytopthora infestans* populations. *J. Hered.* **76**:431-435.

Weeden, N.F. (1983). Plastid isozymes. In: Tanksley, S.D. and Orton, T.J. (eds.), *Isozymes in plant genetics and breeding*, Part A. pp 139-156. Elsevier, Amsterdam.

Weeden, N.F. and Wendel, J.F. (1989). Genetics of plant isozymes. In: Soltis, E.E. and Soltis, P.S. (eds.), *Isozymes in Plant Biology*. pp 46-72. Chapman and Hall, London.

Whisson, S.C; Drenth, A., Maclean, D.J. and Irwin, J.A.G. (1994). Evidence for outcrossing in *Phytophthora sojae* and linkage of a DNA marker to two avirulent genes. *Curr. Genet.* **27**:77-82.

Whisson, S.C., Drenth, A., Maclean, D.J. and Irwin, J.A.G. (1995). *Phytophthora sojae* avirulent genes, RAPD, and RFLP markers used to construct a detailed genetic linkage map. *Mol. Plant-Microbe. Interact.* **8**:988-995.

Zitko, S.E. and Timmer, L.W. (1994). Competitive parasitic abilities of *Phytophthora parasitica* and *P. palmivora* on fibrous roots of citrus. *Phytopath.* 84:1000-1004.

# PROJECT 5

# IDENTIFYING, GERMPLASM STORAGE AND *IN VITRO* PROPAGATION OF *PHYTOPHTHORA* AND CANKER THREATENED TAXA

**A. Cochrane, A. Kelly and D. Coates**

## 1. IDENTIFICATION OF RARE AND THREATENED FLORA AT RISK FROM DIEBACK DISEASE

The Department of Conservation and Land Management Declared Rare (Threatened) and Priority Flora List for Western Australia (Ken Atkins 14/09/95) is currently being used as the basis for all collections. Recently collections have focussed on WA's critically endangered taxa (38 species in total) as well as continued collections of *Phytophthora* and canker dieback susceptible species in the south-west of the State. Consultation with Kings Park and Botanic Gardens has highlighted the gaps in the *ex-situ* conservation of the critically endangered taxa and additional effort will be made to secure germplasm of these species in the face of declining populations and possible extinction.

## 2. IN VITRO PROPAGATION

Discontinued ( see Year 1 report)

## 3. CRYOSTORAGE

Discontinued (see Year 1 report)

## 4. SEED COLLECTION

Three hundred and thirty-two accessions of rare or priority taxa have now been incorporated into the Threatened Flora Seed Centre as of 28th February, 1996 (See Appendix 1). This represents 145 taxa in 37 genera spanning 14 families.

Good genetically representative seed collections of most if not all known populations of 12 out of the 38 critically endangered taxa have been made by staff at the TFSC. A further 6 critically endangered taxa have seed from at least some populations held in long term storage. Of the remaining 12 critical taxa, 3 have been visited but no seed was collected due to either timing, and/or low or sporadic fruit set. Six taxa have not been dealt with due to known poor seed set, existing projects covered by Kings Park and Botanic Gardens, no locality details and doubtful taxonomy. Three taxa are still to be covered. The 8 orchid taxa on the critically endangered flora list are not being

considered as Kings Park and Botanic Gardens are better equipped to deal with the collection and holding of the associated fungi that is vital for the propagation of these plants. Of the critically endangered species that are now held at the TFSC over 70% of these were incorporated into long term storage over the past collecting season.

Thirty-one endangered and vulnerable taxa are well represented in long term storage and a further 30 declared rare flora have seed from at least some of their populations stored in the TFSC. Over 50% of these taxa are presumed susceptible to *Phytophthora* dieback disease. Fifty-one taxa on the priority flora list are represented in storage, with 15 of these well represented and 36 with at least some populations represented. Of these 51 taxa, 6 are currently being recommended for declaration as rare flora, and an additional 2 taxa have not yet been listed as priority taxa but are nevertheless being considered for gazettal. A further 10 geographically restricted or otherwise "interesting" taxa are also represented in the long term storage facility.

Although only 28% of the total number of declared rare flora for the State have seed represented in the TFSC this in fact equates to approximately 63% of taxa in dieback susceptible genera which occur in known or likely dieback prone sites. Many of the remaining taxa have not been collected due to reasons such as low or sporadic seed set, immaturity of populations, difficult access to sites or simply due to lack of time and resources. These species will begin to be addressed in the coming year.

A small proportion of the time spent in the field has been devoted to collecting germplasm from a variety of species for research into their population biology. The germinants have been used for genetic work at the WA Herbarium to determine the differences within and between population of these species and to assess the status of the taxonomy of some of the flora.

This past field season has served to consolidate our knowledge and understanding of some of the variables that affect phenology, pollination, seed set, and seed ripeness as well as the identification of seed of a wide variety of species. New populations of threatened taxa and extensions of previously known populations continue to be discovered on routine collection trips. Communication between CALM districts and the TFSC remains excellent with assistance being given for collections and the provision of field advice. Whilst in the field staff of the TFSC have frequently collected additional germplasm material from critically endangered and other taxa for Kings Park and Botanic Garden.

## 5. SEED STORAGE, VIABILITY TESTING AND INVENTORY SYSTEM

In the laboratory research into a variety of techniques to promote germination has resulted in a better understanding of the biology of some of the threatened taxa. The use of growth hormones, smoke and heat treatment and scarification have provided useful information for assessing viability and determining the optimum methods for germination of seed collected.

New techniques such as the use of smoke for promoting germination have produced mixed results. In some cases, smoke has aided germination, although research has also shown that high concentrations of smoke can, in many cases, inhibit germination. This variation in the amount of smoke used has confounded some of our results and will only be alleviated when the chemical or chemicals that trigger germination are identified.

Almost 100 seedlots have be retested for viability after the first year in storage at -18°C. The majority of the accessions continue to respond positively to storage conditions with little or no loss in germinability and once again in some instances with improved results. A number of accessions have shown a reduction in germinability after storage. Several of these have been due to errors in the technique used, for example the order of application of sterilisation and pre-treatment has caused seed death on one or two occasions. Protocols are now in place to ensure that the sequence of treatments prior to germination is standard. Fungal contamination and small sample size render comparison of pre and post storage germination results of some accessions impossible.

Two species in two genera have been used as examples of the differences experienced in germinability with the use of varying pre-treatments. Figure 1 shows the differences in germination for *Verticordia staminosa* ssp. *staminosa* when seed is soaked in a 100% solution of smoked water for 24 hours and when full strength smoked water is added to the agar medium. The former treatment has produced a far superior result and reiterates the premise that smoke as a treatment can at times inhibit germination. Figure 2 shows the variation in germination using four different pre-treatments on *Andersonia echinocephala*. Two concentrations of the growth hormone Gibberellic Acid and two concentrations of smoked water in the agar solutions have been compared. It is easy to see the enhanced germination of this species in the higher concentration of growth hormone. The seed from all four treatments was leached in running tap water for four hours prior to commencement of the trial.

Three provenances of *Daviesia megacalyx* have been used in Figure 3 to illustrate the high germinability of this species and the positive response to low temperature storage for one year. Figure 4 shows seven provenances of *Banksia brownii*, a rare and highly dieback susceptible species. Despite a range in population size and condition, germination results for all provenances is similar. The response of this species to storage has been in the main positive with initial germination of seed being high.

Research into using liquid nitrogen (-196°C) as a storage medium has shown some interesting results. *Banksia brownii* germination after storage at ultra-low temperature exhibited a slower germination rate than for that seed not stored at -196°C, although germinability was comparable within the constraints of the small sample size (see Figure 5). Tests on *Acacia tenuissima*, a desert dwelling species, showed that pre-treatment in boiling water prior to plunging into liquid nitrogen was vital to prevent loss of seed due to shattering of the endosperm (see Figure 6).

Due to the unusually busy field season of 1995/96 the final completion of the WASeed database was delayed. It is expected to be completed soon and full scale

data entry will begin mid year. The phenology database continues to be updated as new information and new species are added to the list of flora being targeted by the TFSC.

The large reprint collection of articles on seed germination and dormancy, and storage techniques continues to be updated. Plans for the preparation and future publication of an annotated bibliography on these topics is in progress.

The final results of research into the after-ripening processes in *Dryandra* using ethylene to emulate dry storage after-ripening have been disappointing. It appears that ethylene is no substitute for dry storage.

## 6. FUTURE COLLECTIONS AND PROPOSED DEVELOPMENTS

The coming year will see continued collections of threatened flora from the south-west and resampling of previously collected taxa. Additional populations of targeted flora will be added to the accessions where necessary to consolidate the gene pool of that taxon.

The development of a training course in the identification of seed and in collecting techniques is proposed for relevant CALM district and research staff.

It is proposed to begin testing the effects of a variety of growth media on the germinability of a number of rare species. Already preliminary investigation on the critically endangered *Daviesia microcarpa* has shown a variation in germination results for seed grown on agar as compared to filter paper over vermiculite.

Research into seed set in rare and poorly known *Verticordia* continues as more species are collected, tested and placed in storage. From preliminary observations it appears that the timing of collections of some species is very important. Repeat collection of several species of *Verticordia* have been made over a period of 6-8 weeks and tested for viability, with results showing that seed collected later in the season has a higher % germination than that collected earlier in the season. Seed set was not found to differ despite fruits appearing ripe at both times. This is vital information for the planning of field collections. We aim to investigate this variability in maturity for other species.

Scarification is considered to be necessary as a pre-treatment to promote germination of some hard fruited species and a small lapidary tumbler has recently been purchased to investigate this method of pre-treatment in a range of species of *Eremophila, Grevillea, Astroloma* and other hard seeded genera.

Staff of the TFSC attended the 4th International Botanic Gardens Congress in Perth last year and presented 2 poster papers on germplasm storage and documentation. A training session was given on Developing a Germplasm Bank. Several members of the Seed Conservation Section of Wakehurst Place, Kew Botanic Gardens in Perth for the Congress visited the TFSC. This visit proved useful in the formulation of new

ideas for seed storage protocols and facilities. Continued liaison with Kew is proving to be most beneficial. A second attempt to obtain a Churchill Fellowship has been made to visit the Kew Seed Bank to work in collaboration with scientists on genebanking and to attend a Plant Conservation Techniques course held at Kew Botanical Gardens.

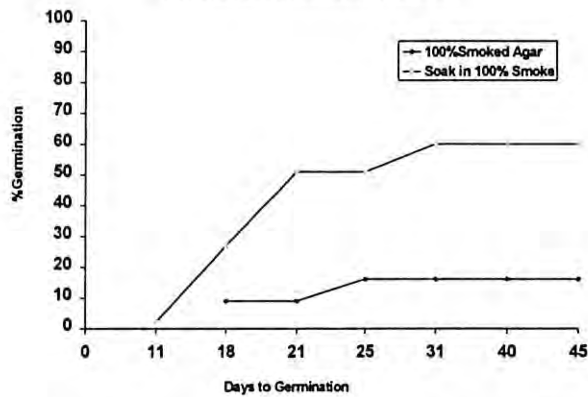



Figure 1: Percentage germination for Verticordia staminosa ssp. staminosa 00240E subjected to different smoke treatments.

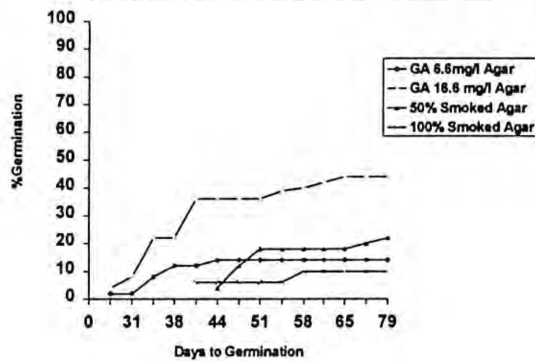



Figure 2: Percentage germination for Andersonia echinocephala 00129TD on varying concentrations of growth hormone and smoke media.

Figure 3: Pre and post storage (-18C) germination for three provenances of Daviesia megacalyx.

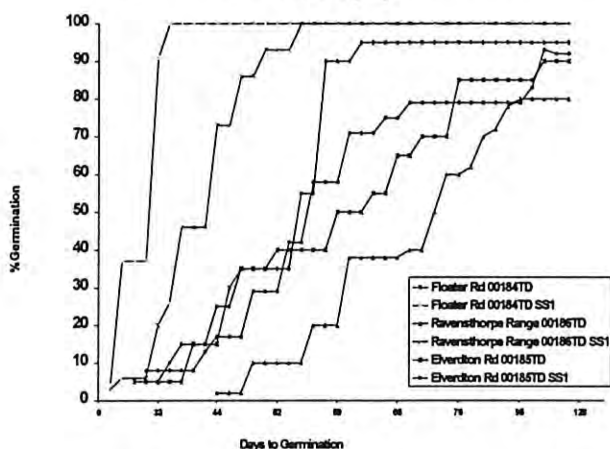
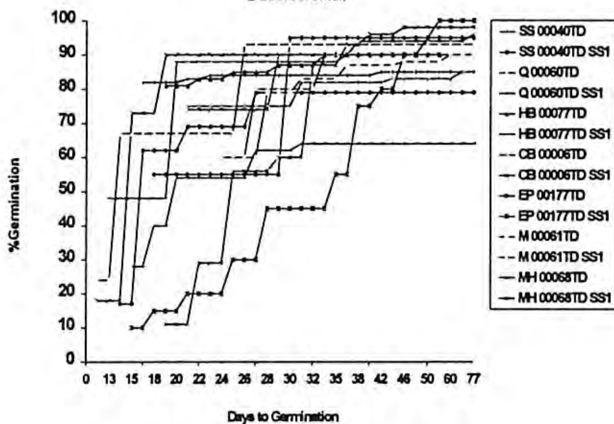



Figure 4: Pre and post storage (-18C) germination for seven provenances of Banksia brownii.



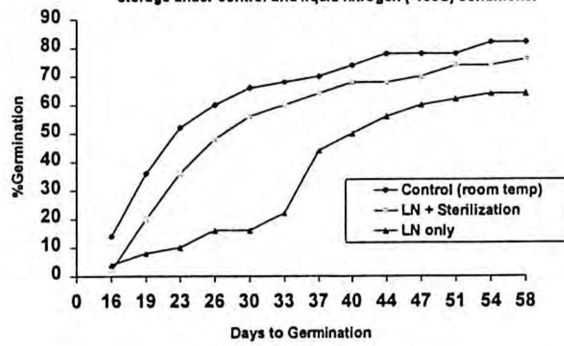


Figure 5: Percentage germination for Banksia brownii after storage under control and liquid nitrogen (-196C) conditions.

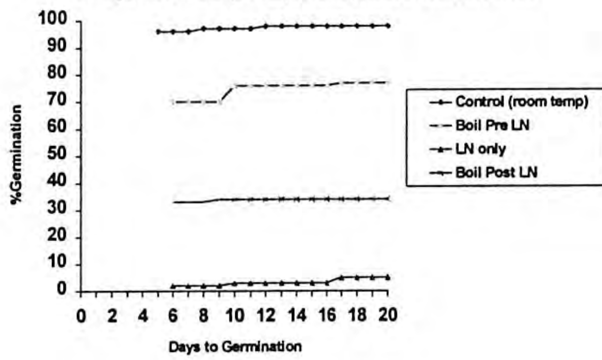


Figure 6: Percentage germination for Acacia tenuissima subjected to boiling water treatment pre and post storage in liquid nitrogen (-196C).

# APPENDIX 1: ACCESSIONS LIST

| Seedlot | Collection no | Date | Species | Location | Type |
|---|---|---|---|---|---|
| 00001 TD | DJC | 9/08/87 | *Banksia brownii* | Mt Hassell | I/4 |
| 00002 TD | DJC | 2/11/88 | *Banksia brownii* | Bluff Knoll | I/14 |
| 00003 TD | DJC | 9/02/88 | *Banksia cuneata* | Lake Mears Road | I/6 |
| 00004 TD | DJC | 9/02/88 | *Banksia cuneata* | McCooke Road | I/10 |
| 00005 TD | DJC | 9/02/88 | *Banksia cuneata* | Badjaling NR | I/13 |
| 00006 TD | DJC | 21/04/89 | *Banksia brownii* | Cheyne Beach | I/38 |
| 00007 TD | DJC | | *Banksia brownii* | Quaranup | I/29 |
| 00008 TD | DJC | 24/08/89 | *Banksia brownii* | Cheyne Bch Road | I/49 |
| 00009 P2 | DJC | 26/04/88 | *Banksia epica* | Toolina Cove | I/45 |
| 00010 TD | DJC | 9/02/88 | *Banksia cuneata* | Water Res. 12397 | I/30 |
| 00011 TD | DJC | 9/02/88 | *Banksia cuneata* | Simpsons Farm | I/15 |
| 00012 TD | DJC | 8/02/88 | *Banksia cuneata* | Bruce Rock Road | I/30 |
| 00013 TD | DJC | 23/05/88 | *Banksia cuneata* | Quairading | I/23 |
| 00014 TD | DJC | 24/09/90 | *Banksia cuneata* | Laze Away | I/29 |
| 00015 TD | DJC | 1/06/86 | *Banksia verticillata* | Mt Hopkins | I/6 |
| 00016 TD | DJC | 10/05/86 | *Banksia verticillata* | The Gap,Torndirrup | I/11 |
| 00017 TD | JAC1016 | 15/03/94 | *Verticordia attenuata* | Elgin Rd, Capel | B/20 |
| 00018 TD | DJC | 8/03/88 | *Banksia oligantha* | Wongeling N | I/10 |
| 00019 TD | DJC | 8/03/88 | *Banksia oligantha* | Wongeling W | I/20 |
| 00020 TD | DJC | 8/03/88 | *Banksia oligantha* | Wongeling E | I/10 |
| 00021 TD | DJC | 26/11/86 | *Banksia verticillata* | Mermaid Point Tk | I/20 |
| 00022 TD | DJC | 1987 | *Banksia verticillata* | Poison Hill | I/9 |
| 00023 TD | DJC | 10/05/86 | *Banksia verticillata* | Stony Hill Creek | I/13 |
| 00024 TD | DJC | 28/11/90 | *Dryandra ionthocarpa* | Kambellup S | I/19 |
| 00025 TD | DJC | 9/08/90 | *Banksia oligantha* | Marribank site 1 | I/14 |
| 00026 TD | DJC | 9/08/90 | *Banksia oligantha* | Marribank site 2 | I/11 |
| 00027 TD | DJC | 9/08/90 | *Banksia oligantha* | Marribank site 3 | I/10 |
| 00028 TD | DJC | 9/08/90 | *Banksia oligantha* | Marribank site 4 | I/30 |
| 00029 TD | DJC | 7/06/85 | *Lambertia orbifolia* | Narrikup 1 | I/9 |
| 00030 TD | MM1490 | 13/12/92 | *Lambertia orbifolia* | Narrikup 2 | B/15 |
| 00031 TD | MM1453-1469 | 12/12/92 | *Isopogon uncinatus* | Muttonbird | I/16 |
| 00032 TD | MM1473-1482 | 12/12/92 | *Banksia verticillata* | Stony Hill Creek | I/10 |
| 00033 TD | MM1484 | 13/12/92 | *Andersonia* sp. Two Peoples Bay | Boulder Hill | B/50 |
| 00034 TD | MM1489 | 14/12/92 | *Andersonia echinocephala* | Mondurup | B/30 |
| 00035 TD | MM1492-1503 | 15/12/92 | *Andersonia grandiflora* | Toolbrunup | I/12 |
| 00036 TD | MM1507 | 18/12/92 | *Adenanthos pungens ssp effusa* | Tambellup | B/10 |
| 00037 TD | MM1528-1540 | 22/01/93 | *Dryandra seneciifolia* | Red Gum Pass | I/13 |
| 00038 TD | MM1541-1550 | 22/01/93 | *Andersonia grandiflora* | Stirling Drive | I/10 |
| 00039 TD | MM1551-1560 | 23/01/93 | *Dryandra ionthocarpa* | Kambellup N | I/10 |
| 00040 TD | MM1575-1596 | 25/01/93 | *Banksia brownii* | South Sister | I/22 |
| 00041 TD | MM1612-1614 | 29/01/93 | *Lambertia echinata ssp. echinata* | Lucky Bay | I/3 |
| 00042 TD | MM1615-1624 | 30/01/93 | *Isopogon alcicornis* | Mt Burdett | I/10 |
| 00043 TD | MM1638 | 31/01/93 | *Dryandra serra* | South Sister | B/20 |
| 00044 TD | RWills | 18/09/92 | *Banksia verticillata* | Jimmy Newhills Harbour | B/6 |
| 00045 P3 | MM1565-1569 | 24/01/93 | *Thomasia solanacea* | Limeburners Lane | I/5 |
| 00046 P3 | MM1573 | 24/01/93 | *Eucalyptus acies* | South Sister | B/5 |
| 00047 P2 | MM1625 | 30/01/93 | *Melaleuca coccinea ssp. eximia* | Mt. Burdett | B/10 |
| 00048 V | MM1626-1630 | 30/01/93 | *Eucalyptus merrickiae* | Kent Road | I/5 |
| 00049 TD | JAC1047 | 16/03/94 | *Verticordia attenuata* | Bussel Hwy | B/30 |
| 00050 TD | JAC1045 | 16/03/94 | *Verticordia attenuata* | Ruabon Rd, Tutunup | B/50 |

| | | | | | |
|---|---|---|---|---|---|
| 00051 V | MM | 19/05/93 | *Banksia goodii* | Millbrook NR | I/5 |
| 00052 V | DJC | 9/05/86 | *Banksia goodii* | Millbrook NR | I/12 |
| 00053 TD | JAC1020-1029 | 16/03/94 | *Verticordia plumosa var ananeotes* | Ambergate NR | I/10 |
| 00054 G | DJC | 1/06/87 | *Banksia semi-nuda ssp remanens* | Mt. Hopkins | I/7 |
| 00055 TD | MM1654-1678 | 4/04/93 | *Banksia verticillata* | Channel Point | I/25 |
| 00056 TD | MM1680-1694 | 6/04/93 | *Banksia verticillata* | Stony Hill | I/15 |
| 00057 TD | MM1695-1719 | 7/04/93 | *Banksia verticillata* | Woolbale Hills | I/25 |
| 00058 TD | MM1720 | 15/05/93 | *Lambertia orbifolia* | Dennis Road | B/15 |
| 00059 TD | MM1721 | 15/05/93 | *Lambertia orbifolia* | Brennans Ford | B/21 |
| 00060 TD | MM1724-1739 | 18/05/93 | *Banksia brownii* | Quaranup Road | I/16 |
| 00061 TD | MM1740-1780 | 19/05/93 | *Banksia brownii* | Millbrook Nat. Res. | I/41 |
| 00062 TD | JAC251-260 | 20/07/93 | *Dryandra squarrosa ssp. argillacea* | Ruabon/Tutunup | I/10 |
| 00063 TD | JAC261-277 | 21/07/93 | *Dryandra squarrosa ssp. argillacea* | Smith Rd, Wicher R. | I/17 |
| 00064 G | JAC278-294 | 22/07/93 | *Banksia semi-nuda ssp remanens* | Thompsons Cove | I/17 |
| 00065 G | JAC295-308 | 22/07/93 | *Banksia semi-nuda ssp remanens* | Aldridge Cove SW | I/14 |
| 00066 G | JAC309-311 | 23/07/93 | *Banksia semi-nuda ssp remanens* | Long Point carpark | I/3 |
| 00067 TD | JAC312-329 | 23/07/93 | *Banksia seminuda ssp remanens* | Poison Hill SW face | I/18 |
| 00068 TD | JAC330-354 | 24/07/93 | *Banksia brownii* | Mt. Hassell | I/25 |
| 00069 TD | JAC363 | 6/08/93 | *Isopogon scaber* | 44mp Brookton Hwy | B/16 |
| 00070 TD | JAC368 | 18/08/93 | *Dryandra anatoma* (sp. 48) | Mt. Tayluberlup | B/15 |
| 00071 TD | JAC372-387 | 19/08/93 | *Dryandra seneciifolia* | Lookout-Stirling Range Dr | I/16 |
| 00072 TD | JAC388-404 | 19/08/93 | *Dryandra sp. 49* (aff.ferruginea) | Lookout-Stirling Range Dr | I/17 |
| 00073 TD | JAC405 | 20/08/93 | *Andersonia sp. nov* (Mt.Lindesay) | Mt. Lindesay | B/10 |
| 00074 TD | JAC406 | 21/08/93 | *Dryandra serra* | Hunwick Rd(East) | B/32 |
| 00075 TD | JAC409-419 | 21/08/93 | *Dryandra serra* | Hunwick Rd(West) | I/11 |
| 00076 TD | JAC421-424 | 21/08/93 | *Banksia brownii* | Hazzard Rd | I/4 |
| 00077 TD | JAC425-439 | 22/08/93 | *Banksia brownii* | Hassell Beach Rd | I/15 |
| 00078 TD | JAC441-448 | 23/08/93 | *Banksia verticillata* | Mermaid/Channel Pt | I/8 |
| 00079 TD | JAC449-464 | 23/08/93 | *Banksia verticillata* | Mermaid Pt Track | I/16 |
| 00080 TD | JAC466-475 | 24/08/93 | *Dryandra seneciifolia* | Hamilla Hills | I/10 |
| 00081 TD | JAC477-488 | 14/09/93 | *Dryandra acanthopoda* (sp.1) | Carter Rd, Woodanilling | I/12 |
| 00082 TD | JAC489 | 14/09/93 | *Dryandra lepidorhiza* (sp. 41) | Orchard/Dinwoodie Rds, Woodanilling | B/20 |
| 00083 TD | JAC490 | 15/09/93 | *Dryandra sp. 45* | Katanning-Nyabing Rd | B/10 |
| 00084 TD | JAC491-510 | 15/09/93 | *Dryandra octotriginata* (sp. 38) | Nyabing-Kukerin Rd | I/20 |
| 00085 TD | JAC512-532 | 16/09/93 | *Dryandra erythrocephala var. inopinata* (sp. 44) | Hopkins NR | I/21 |
| 00086 TD | JAC533-551 | 16/09/93 | *Dryandra epimicta* (sp. 37) | Hopkins NR | I/21 |
| 00087 TD | JAC552-568 | 17/09/93 | *Dryandra sp. 31* | Corrigin-Quarading Rd | I/17 |
| 00088 TD | JAC569 | 21/09/93 | *Lambertia fairallii* | Stirling Range Drive, SRNP | B/100 |
| 00089 TD | JAC572 | 9/11/93 | *Dryandra mimica* | Old Kent Rd, Wicher Range | B/1? |
| 00090 TD | JAC574 | 9/11/93 | *Lambertia orbifolia* | Dennis Road | B/10 |
| 00091 TD | JAC577-596 | 10/11/93 | *Astroloma* sp. Nannup | Scott River Rd. Scott River | I/20 |
| 00092 TD | JAC598-612 | 11/11/93 | *Dryandra ionthocarpa* | Kambellup N | I/16 |
| 00093 TD | JAC613-628 | 11/11/93 | *Dryandra ionthocarpa* | Kambellup S | I/15 |
| 00094 TD | JAC630-639 | 11/11/93 | *Lambertia fairallii* | Stirling Range Drive, SRNP | B/50 |
| 00095 E | BHaberley | 3/11/93 | *Daviesia microcarpa* | Norseman | B/12 |
| 00096 TD | JAC644-654 | 25/11/93 | *Daviesia pseudaphylla* | Site 1, East Pillenorup Track, SRNP | I/11 |
| 00097 TD | JAC655 | 25/11/93 | *Daviesia pseudaphylla* | Site 2, East Pillenorup Track, SRNP | B/25 |
| 00098 TD | JAC643 | 25/11/93 | *Daviesia pseudaphylla* | Site 1/2, East Pillenorup Track, SRNP | B/20 |
| 00099 TD | JAC656-677 | 26/11/93 | *Andersonia echinocephala* | Stirling Range Dr., SRNP | I/22 |
| 00100 TD | JAC679-698 | 28/11/93 | *Banksia laevigata ssp.laevigata* | Dorrie Hill, Twertup, FRNP | I/20 |
| 00101 TD | JAC702 | 30/11/93 | *Adenanthos dobagii* | Quoin Head, FRNP | B/12 |
| 00102 TD | JAC700 | 30/11/93 | *Adenanthos ellipticus* | East Mt. Barren | B/8 |
| 00103 TD | JAC704 | 1/12/93 | *Daviesia megacalyx* | Elverdton Rd. | B/30 |
| 00104 TD | JAC706-718 | 1/12/93 | *Dryandra corvijuga* (sp. 36) | Elverdton Rd. | I/13 |
| 00105 TD | JAC719-728 | 1/12/93 | *Dryandra folisissima* | Mt. Desmond | I/10 |

| | | | | | |
|---|---|---|---|---|---|
| 00106 TD | JAC729-738 | 2/12/93 | *Dryandra folisissima* | Ravensthorpe 2 | I/10 |
| 00107 TD | JAC739-748 | 2/12/93 | *Dryandra corvijuga* (sp. 36) | Ravensthorpe 2 | I/10 |
| 00108 TD | JAC749-762 | 2/12/93 | *Daviesia megacalyx* | Ravensthorpe 2 | I/14 |
| 00109 TD | JAC763/1-5 | 2/12/93 | *Daviesia megacalyx* | Floater Rd. | I/5 |
| 00110 P3 | JAC767-789 | 3/12/93 | *Dryandra viscida* (sp. 16) | Hatter Hill | I/23 |
| 00111 V | JAC791-810 | 3/12/93 | *Banksia sphaerocarpa var. dolichostyla* | South Ironcap | I/20 |
| 00112 TD | JAC811-818 | 4/12/93 | *Daviesia oxylobium* | Wamenusking NR | I/8 |
| 00113 V | DWO | 1/11/90 | *Eucalyptus crucis ssp. crucis* | Sandford Rocks NR | B/? |
| 00114 TD | BHammersley | 8/09/93 | *Dryandra serra* | Mt. Hallowell | I/10 |
| 00115 R | SVL | 1/10/92 | *Lepidium catapycnon* | Garden Gorge, Hammerslay Range | I/1 |
| 00116 TD | JAC905 | 6/01/94 | *Adenanthos pungens ssp effusa* | Tambellup | B/3 |
| 00117 TD | JAC906 | 6/01/94 | *Adenanthos pungens ssp pungens* | Hamilla Hill, SRNP | B/12 |
| 00118 TD | JAC907-916 | 6/01/94 | *Adenanthos pungens ssp pungens* | Hamilla Hill, SRNP (traps) | I/10 |
| 00119 TD | JAC821-824 | 7/01/94 | *Adenanthos velutinos* | Geekabee Hill | I/4 |
| 00120 TD | JAC828-840 | 9/01/94 | *Banksia verticillata* | Mermaid Point | I/13 |
| 00121 TD | JAC841 | 10/01/94 | *Adenanthos ellipticus* | East Mt Barrren | B/40 |
| 00122 TD | JAC842-851 | 11/01/94 | *Adenanthos dobagii* | Quoin Head Track | I/10 |
| 00123 TD | JAC852-861 | 11/01/94 | *Adenanthos dobagii* | Quoin Head T/O (traps) | I/10 |
| 00124 TD | JAC865-867 | 14/01/94 | *Lambertia echinata ssp echinata* | Lucky Bay | I/3 |
| 00125 E | JAC868-877 | 14/01/94 | *Myoporum turbinatum* | Heywood Rd | I/10 |
| 00126 TD | JAC880 | 15/01/94 | *Astroloma* sp. Grass Patch | Ridley Rd, Grass Patch | B/40 |
| 00127 TD | JAC884-904 | 16/01/94 | *Daviesia campophylla* | Griffith/Edwards Rd | I/21 |
| 00128 TD | | 7/07/93 | *Banksia cuneata* | Johnston's property (Pop10) | B/? |
| 00129 TD | JAC925-944 | 9/02/94 | *Andersonia echinocephala* | Baby Barnett SRNP | I/20 |
| 00130 TD | JAC948-957 | 11/02/94 | *Dryandra sp. Fitzgerald* | Drummond Track west FRNP | I/10 |
| 00131 TD | JAC958-969 | 13/02/94 | *Dryandra sp. Fitzgerald* | Hammersley Dr east FRNP | I/12 |
| 00132 TD | JAC973-984 | 15/02/94 | *Andersonia* sp. Two Peoples Bay | Boulder Hill, Two Peoples Bay | I/12 |
| 00133 TD | JAC985-994 | 15/02/94 | *Andersonia sp. nov* (Mt. Lindesay) | Mt Lindesay | I/10 |
| 00134 TD | JAC996-1013 | 16/02/94 | *Banksia occidentalis ssp. formosa* | Black Point, Scott River | I/17 |
| 00135 TD | TSwarten | 12/93-1/94 | *Daviesia spiralis* | Wongan Hills | B/25 |
| 00136 P4 | ABrown? | 1/09/93 | *Eucalyptus carnabyi* | York | B/5 |
| 00137 TD | JAC1018 | 15/03/94 | *Petrophile latericola* | Williamson Rd, Wicher | B/40 |
| 00138 TD | JAC1056-1068 | 20/04/94 | *Banksia verticillata* | Stony Hill | I/13 |
| 00139 TD | JAC1069-1073 | 20/04/94 | *Dryandra montana* | Bluff Knoll, SRNP | I/5 |
| 00140 TD | JAC1074-1089 | 21/04/94 | *Verticordia harveyi* | South Stirling | I/16 |
| 00141 G | JAC1090-1099 | 22/04/94 | *Lambertia echinata ssp. propinqua* | Cheyne Beach Rd. | I/10 |
| 00142 G | JAC1100-1105 | 22/04/94 | *Lambertia echinata ssp. propinqua* | Boulder Hill | I/6 |
| 00143 TD | JAC1106 | 26/04/94 | *Dryandra aurantia* ("Little Darkin Swamp") | Little Darkin Swamp | B/15 |
| 00144 TD | JAC1108 | 12/05/94 | *Lambertia fairallii* | Below Ellen Peak | B/8 |
| 00145 V | JFS1 | 1/05/85 | *Eucalyptus rhodantha* | Three Springs 1 | I/6 |
| 00146 V | JFS3 | 1/04/85 | *Eucalyptus rhodantha* | Watheroo 3 | I/21 |
| 00147 V | JFS4 | 1/04/85 | *Eucalyptus rhodantha* | Watheroo 4 | I/32 |
| 00148 V | JFS5 | 1/08/85 | *Eucalyptus rhodantha* | Watheroo 5 | I/14 |
| 00149 V | JFS6 | 15/04/85 | *Eucalyptus rhodantha* | Watheroo 6 | I/8 |
| 00150 V | AK1 | 12/07/93 | *Eucalyptus rhodantha* | Three Springs 1 | I/11 |
| 00151 V | AK2 | 13/07/93 | *Eucalyptus rhodantha* | Three Springs 2 | I/6 |
| 00152 V | AK4 | 2/09/93 | *Eucalyptus rhodantha* | Watheroo 4 | I/35 |
| 00153 V | AK5 | 23/06/93 | *Eucalyptus rhodantha* | Watheroo 5 | I/34 |
| 00154 V | AK6 | 13/07/93 | *Eucalyptus rhodantha* | Watheroo 6 | I/4 |
| 00155 V | AK1(92) | 17/12/92 | *Stylidium coroniforme* | Wongan Hills 1 | B/6 |
| 00156 V | AK1(93) | 16/12/93 | *Stylidium coroniforme* | Wongan Hills 1 | B/9 |
| 00157 V | AK2(93) | 16/12/93 | *Stylidium coroniforme* | Wongan Hills 2 | I/13;B/1 |
| 00158 V | AK3(93) | 15/12/93 | *Stylidium coroniforme* | Maya 3 | I/11 |
| 00159 V | AK4(93) | 15/12/93 | *Stylidium coroniforme* | Maya 4 | I/25 |
| 00160 V | AK5(93) | 16/12/93 | *Stylidium coroniforme* | Wongan Hills 5 | I/14;B/2 |

| | | | | | |
|---|---|---|---|---|---|
| 00161 TD | DJC | 1/06/87 | *Banksia verticillata* | Mt. Hopkins | I/19 |
| 00162 TD | JAC1131-1144 | 26/07/94 | *Dryandra fraseri var. oxycedrus* (sp. 23) | Kadathinni NR | I/14 |
| 00163 TD | JAC1146 | 26/07/94 | *Dryandra borealis ssp. elatior* (sp .20) | Kadathinni NR | B/20 |
| 00164 TD | JAC1148-1153 | 27/07/94 | *Leucopogon obtectus* | Beekeeper Rd | I/6 |
| 00165 TD | JAC1157-1167 | 27/07/94 | *Dryandra stricta* (sp 15) | Willis Rd | I/11 |
| 00166 TD | JAC1127 | 25/07/94 | *Dryandra serratuloides* | Gillingara Pop9A | B/12 |
| 00167 TD | JAC1170-1184 | 28/07/94 | *Dryandra serratuloides* | Marchagee Track (Pops5) | I/15 |
| 00168 TD | JAC1186-1195 | 28/07/94 | *Dryandra pteridifolia ssp. vernalis* (sp. 22) | Marchagee Track | I/10 |
| 00169 TD | JAC1196-1208 | 28/08/94 | *Banksia verticillata* | Isthmus Hill, Torndirrup | I/13 |
| 00170 TD | JAC1210 | 1/09/94 | *Dryandra anatoma* (sp.48) | Mt. Talyuberlup | B/6 |
| 00171 V | AK3 | 1/06/94 | *Eucalyptus rhodantha* | Watheroo 3 | I/5 |
| 00172 TD | JAC1480-1489 | 28/09/94 | *Lambertia echinata ssp. citrina* | Cape Riche | I/10 |
| 00173 V | Jens Olesen | Sept-Dec 93 | *Anigozanthus humilis ssp. chrysanthus* | Mogumber | I/44 |
| 00174 TD | JAC1493-1502 | 18/11/94 | *Melaleuca ordinifolia* | Salt River RD, SRNP | I/10 |
| 00175 TD | JAC1503 | 19/11/94 | *Daviesia pseudaphylla* | E. Pillenorup/Sth Bluff Knoll Tracks | B/30 |
| 00176 E | Ray Smith | 16/11/94 | *Rulingia* sp. Trigwell Bridge | Loc 3271 Arthur Shire | B/4 |
| 00177 TD | JAC1508 | 20/11/94 | *Banksia brownii* | SE of Ellens Peak | B/10 |
| 00178 TD | JAC1512-1524 | 20/11/94 | *Andersonia echinocephala* | SE of Ellens Peak | I/13 |
| 00179 TD | JAC1526 | 21/11/94 | *Darwinia lejostyla* | Mt. Trio | B/100 |
| 00180 TD | JAC1528 | 21/11/94 | *Darwinia oxylepis* | Below Baby Barnett | B/100 |
| 00181 TD | JAC1530 | 21/11/94 | *Darwinia wittwerorum* | Below Mt. Talyuberlup | B/100 |
| 00182 TD | JAC1533 | 22/11/94 | *Verticordia helichrysantha* | Cape Riche | B/200 |
| 00183 P2 | JAC1534-1544 | 22/11/94 | *Melaleuca sculponeata* | Mallee Rd, Jerramungup | I/11 |
| 00184 TD | JAC1545 (1-9) | 23/11/94 | *Daviesia megacalyx* | Floater Rd. | I/9 |
| 00185 TD | JAC1546 | 23/1194 | *Daviesia megacalyx* | Elverdton Rd. | B/10 |
| 00186 TD | JAC1214 | 23/11/94 | *Daviesia megacalyx* | Sth Ravensthorpe Range (new) | B/50 |
| 00187 TD | JAC1221 | 14/12/94 | *Grevillea mccutcheonii* | Princefield Rd, Wicher | B/5 |
| | JAC1294 | 28/12/94 | | | |
| | JAC1383 | 13/02/95 | | | |
| 00188 TD | JAC1222-1229 | 14/12/94 | *Hakea aff. varia* | Princefield Rd, Wicher | I/8 |
| 00189 TD | JAC1231-1240 | 14/12/94 | *Dryandra squarrosa ssp. argillacea* | Tutunup Rd, Wicher | I/10 |
| 00190 TD | JAC1247-1255 | 15/12/94 | *Dryandra aff. nivea* | Williamson Rd, Wicher | I/9 |
| 00191 TD | JAC1257 | 15/12/94 | *Petrophile latericola* | Williamson Rd, Wicher | B/25 |
| 00192 TD | JAC1260 | 15/12/94 | *Hakea aff. varia* | Williamson Rd, Wicher | B/30 |
| 00193 TD | JAC1265 | 16/12/94 | *Andersonia aff. latiflora* | Smith Rd, Wicher | B/100 |
| 00194 TD | JAC1272-1283 | 16/12/94 | *Grevillea elongata* | Princefield Rd, Wicher | I/22 |
| | JAC1284-1293 | 28/12/94 | | | |
| 00195 TD | JAC1243 | 15/12/94 | *Brachysema papilio* | Williamson Rd, Wicher | B/5 |
| | JAC1297 | 28/12/94 | | | |
| 00196 C | MRoddy+DP017 | 4/12/94 | *Acacia awestoniana* | Chester Pass Rd, SRNP | B/7 |
| | DP017 | 12/12/94 | | | |
| 00197 TD | JAC1301 | 28/12/94 | *Grevillea elongata* | Tutunup Rd, Wicher | B/8 |
| 00198 TD | DP009 | 14/12/94 | *Andersonia* sp. Two Peoples Bay | Boulder Hill - south face | B/10 |
| 00199 TD | DP010 | 14/12/94 | *Andersonia* sp. Two Peoples Bay | Boulder Hill - east face | B/10 |
| 00200 TD | DP011 | 14/12/94 | *Andersonia* sp. Two Peoples Bay | Goodga River | B/10 |
| | JAC1308 | 11/01/95 | | | |
| 00201 TD | Seed traps | 14/11/94-29/01/95 | *Adenanthos pungens ssp. pungens* | Hamilla Hills | I/10 (tra |
| 00202 TD | JAC1303 | 9/01/95 | *Lambertia fairallii* | Stirling Range Drive, SRNP | B/250 |
| | JAC1393 | 15/02/95 | | | |
| 00203 TD | JAC1304 | 9/01/95 | *Andersonia grandiflora* | Below Mt. Gog | B/100 |
| 00204 TD | JAC1322 | 12/01/95 | *Isopogon uncinatus* | Torndirrup (CJR pop B+C) | B/20 |
| 00205 TD | JAC1324 | 12/01/95 | *Isopogon uncinatus* | Torndirrup (CJR popD) | B/50 |
| 00206 TD | JAC1325 | 13/01/95 | *Andersonia* sp. Mt Lindesay | Mt. Lindesay | B/20 |
| 00207 TD | JAC1327 | 13/01/95 | *Verticordia fimbrilepis ssp. australis* | Kent River | B/200 |

| | | | | | |
|---|---|---|---|---|---|
| 00208 R | JAC1328 | 11/01/95 | *Hibbertia* sp. Porongorups | Devils Slide | B/6 |
| 00209 G | JAC1310-1320 | 11/01/95 | *Lambertia propinqua* | WAWA reserve Two Peoples Bay | I/11 |
| 00210 V | AK7 | 14/12/94 | *Eucalyptus rhodantha* | Watheroo 7 | I/8 |
| 00211 V | AK8 | 14/12/94 | *Eucalyptus rhodantha* | Watheroo 8 | I/9 |
| 00212 V | AK9 | 14/12/94 | *Eucalyptus rhodantha* | Watheroo 9 | I/11 |
| 00213 V | AK11 | 14/12/94 | *Eucalyptus rhodantha* | Watheroo 11 | I/23 |
| 00214 TD | DP023 | 3/01/95 | *Verticordia albida* | Three Springs | B/8 |
| 00215 TD | JAC1342 | 28/01/95 | *Verticordia bifimbriata* | Dryandra State Forest | B/12 |
| 00216 TD | JAC1340 | 28/01/95 | *Andersonia bifida* | Dryandra State Forest | B/35 |
| 00217 TD | JAC1343-1369 | 13/02/95 | *Verticordia plumosa var pleiobotrya* | Mundijong | I/27 |
| 00218 TD | JAC1370 | 13/02/95 | *Verticordia attenuata* | Elgin Rd, Capel | B/30 |
| 00219 TD | JAC1373-1382 | 13/02/95 | *Dryandra* aff. *nivea* | Tutunup Rd, Wicher | I/10 |
| 00220 TD | JAC1384 | 14/02/95 | *Verticordia plumosa var ananeotes* | Ambergate NR, Busselton | B/30 |
| 00221 TD | JAC1389 | 15/02/95 | *Verticordia endlicheriana var angustifolia* | Mt Barker Hill | B/50 |
| 00222 R | JAC1395 | 17/02/95 | *Kunzea pauciflora* | Mt Melville, Cape Riche | B/50 |
| 00223 TD | JWilliams | 21/04/94 | *Banksia cuneata* | Bruce Rock Rd | B/57 |
| 00224 TD | JWilliams | 21/04/94 | *Banksia cuneata* | Badjaling Nature Reserve | B/6 |
| 00225 TD | JWilliams | 13/03/94 | *Banksia cuneata* | Water Reserve 12397 | B/10 |
| 00226 TD | JWilliams | 18/05/94 | *Banksia cuneata* | McCooke Rd, NE of Brookton | B/9 |
| 00227 TD | JWilliams | 20/04/94 | *Banksia cuneata* | Quairading Town Site | B/78 |
| 00228 TD | JWilliams | 30/07/94 | *Banksia cuneata* | Simpsons Farm | B/50 |
| 00229 TD | JWilliams | 8/08/94 | *Banksia cuneata* | Lazeaway | B/90 |
| 00230 TD | JAC1397<br>JAC1449 | 25/04/95<br>15/06/95 | *Lambertia orbifolia* | Spencer Rd, Narrikup | B/20 |
| 00231 TD | JAC1398-1403 | 26/04/95 | *Banksia verticillata* | Waychinicup west | I/6 |
| 00232 TD | JAC1406 | 27/04/95 | *Verticordia harveyi* | East Pillenorup Track, SRNP | B/200 |
| 00233 TD | JAC1407-1418 | 28/04/95 | *Banksia verticillata* | Ben Dearg, Gull Rock NP | I/12 |
| 00234 TD | JWilliams | 11/05/95 | *Banksia cuneata* | Wally Mills Place | B/3 |
| 00235 TD | JAC1451 | 15/06/95 | *Lambertia orbifolia* | Sleeman Rd, Narrikup | B/10 |
| 00236 TD | JAC1419 | 13/06/95 | *Dryandra lepidorhiza* | Orchard/Dinwoodie Rds, Woodanilling | B/60 |
| 00237 TD | JAC1422-1431 | 13/06/95 | *Dryandra acanthopoda* | Carter Rd, Woodanilling | I/10 |
| 00238 TD | DJC | 26/09/90 | *Banksia meisneri var ascendens* | Scott River NP | B/? |
| 00239 TD | JAC1560-1569 | 24/08/95 | *Dryandra serratuloides ssp. serratuloides* | Gillingarra Pop 2a+2b | I/10 |
| 00240 TD | JAC1571-1585 | 10/10/95 | *Verticordia staminosa ssp. staminosa* | Mocardy Hill, Wongan Hills | I/15 |
| 00241 TD | JAC1588-1597 | 10/10/95 | *Melaleuca sciotostyla* | Wongan Hills Pistol Club | I/10 |
| 00242 TD | JAC 1598 | 15/10/95 | *Andersonia* sp. Mt. Lindesay | Mt. Lindesay | B/20 |
| 00243 E | JAC 1601 | 18/10/95 | *Eremophila denticulata ssp denticulata* | Philips River crossing on Moir Track, FRNP | B/20 |
| 00244 E | JAC 1603 | 19/10/95 | *Eremophila denticulata ssp denticulata* | Rawlinson Rd | B/200 |
| 00245 P1 | JAC 1606 | 19/10/95 | *Eucalyptus preissiana ssp lobata* | Farrells Rd, Quagi Beach | B/200 |
| 00246 C | BH/1-15 | 18/10/95 | *Daviesia microcarpa* | Norseman | I/15 |
| 00247 V | JAC 1608 | 20/10/95 | *Eucalyptus insularis* | Cape Le Grand | B/10+ |
| 00248 V | JAC 1613 | 20/10/95 | *Eucalyptus insularis* | Mt. Le Grand | B/5+ |
| 00249 TD | JAC 1614-1616 | 20/10/95 | *Lambertia echinata ssp echinata* | Lucky Bay | I/3 |
| 00250 C | JAC 1619 | 21/10/95 | *Prostanthera carrickiana* | Mt Buraminya | B/40 |
| 00251 V | JAC 1622-1638 | 22/10/95 | *Eucalyptus platydisca* | Jimberlana Hill | I/17 |
| 00252 TD | JAC 1647 | 24/10/95 | *Chamelaucium* sp. Hamersley | Hamersley Inlet Shire Reserve | B/60 |
| 00253 V | JAC 1649 | 26/10/95 | *Myoporum cordifolium* | Corackerup/Holden Rd | B/20 |
| 00254 V | JAC 1651 | 26/10/95 | *Myoporum cordifolium* | Toompup South Rd | B/100 |
| 00255 TD | JAC 1653 | 26/10/95 | *Verticordia helichrysantha* | Cape Riche | B/1000 |
| 00256 TD | JAC 1657-1669 | 27/10/95 | *Grevillea maxwellii* | Barrs Rd. Reserve | I/12 + B |
| 00257 C | JAC 1670 | 6/11/95 | *Chamelaucium griffinii* | Yandin Nature Reserve | B/20 |
| 00258 E | JAC 1671 | 6/11/95 | *Grevillea calliantha* | Moora-Caro Rd (Pop 6) | B/14 |
| 00259 E | JAC 1674 | 6/11/95 | *Grevillea calliantha* | Minyulo Brook Rd (Pop 3) | B/5 |
| 00260 E | JAC 1675 | 6/11/95 | *Grevillea calliantha* | Minyulo Brook Rd (Pop 2) | B/10 |
| 00261 P1 | JAC 1681 | 8/11/95 | *Grevillea kenneallyi* | Wongan-Piawanning Rd | B/40 |

| | | | | | |
|---|---|---|---|---|---|
| 00262 E | JAC 1684 | 8/11/95 | *Gastrolobium hamulosum* | Craig Rd, Wongan Hills | B/9 |
| 00263 V | JAC 1689 | 9/11/95 | *Microcorys eremophiloides* | Mt Matilda Nature Reserve | B/10 |
| 00264 C | JAC 1688 | 9/11/95 | *Acacia pygmaea* | Mt Matilda Nature Reserve | B/6 |
| 00265 E | JAC 1673 | 6/11/95 | *Grevillea calliantha* | Minyulo Brook Rd (Pop 5) | B/4 |
| 00266 P1 | JAC 1690 | 22/11/95 | *Chamelaucium sp. Gin Gin* | Ioppolo Rd | B/15 |
| 00267 E | JAC 1693-1702 | 22/11/95 | *Darwinia acerosa* | Bulbarnett Rd | I/10 |
| 00268 V | JAC 1704-1727 | 30/11/95 | *Allocasuarina fibrosa* | Charles Gardner NR | I/24 |
| 00269 C | JAC 1729 | 1/12/95 | *Eremophila caerulea ssp. merrallii* | Bruce Rock | B/10 |
| 00270 P1 | JAC 1731 | 1/12/95 | *Jacksonia sp Quairading* | Quairading townsite | B/100 |
| 00271 C | JAC 1733 | 4/12/95 | *Darwinia carnea* | Narrogin | B/10 |
| 00272 E | JAC 1735-1744 | 5/12/95 | *Acacia leptalea* | Chinocup Rd | I/10 |
| 00273 E | JAC 1746-1758 | 5/12/95 | *Acacia leptalea* | Chinocup Rd reserve | I/13 |
| 00274 V | JAC 1760-1766 | 5/12/95 | *Vert stam ssp. cylind var. cylind* | Pingaring Rock | I/7 |
| 00275 V | JAC 1767-1776 | 6/12/95 | *Vert stam ssp. cylind var. erecta* | McGlin Rd | I/10 |
| 00276 V | JAC 1779-1788 | 6/12/95 | *Allocasuarina tortiramula* | Lake King NR (2) | I/10 |
| 00277 V | JAC 1789-1799 | 6/12/95 | *Allocasuarina tortiramula* | Lake King NR (1) | I/11 |
| 00278 V | JAC 1802 | 7/12/95 | *Billardiera mollis* | near Elverdton Rd | B/20 |
| 00279 P1 | JAC 1803-1812 | 9/12/95 | *Chamelaucium sp. Gin Gin* | Breera Rd | I/10 |
| 00280 E | JAC 1814-1823 | 9/12/95 | *Acacia sp. Dandaragan* | Mullering Rd | I/10 |
| 00281 ? | JAC 1825-1835 | 11/12/95 | *Calothamnus aff. quadrifidus* | Oates Rd | I/11 |
| 00282 E | JAC 1836 | 12/12/95 | *Brachysema papilio* | Williamson Rd west | B/10 |
| 00283 TD | JAC 1837-1846 | 12/12/95 | *Hakea aff. varia* | Williamson Rd west | I/10* |
| 00284 TD | JAC 1849-1858 | 12/12/95 | *Dryandra nivea ssp. uliginosa* | Williamson Rd west | I/10* |
| 00285 TD | JAC 1861 | 12/12/95 | *Petrophile latericola* | Williamson Rd west | B/10 |
| 00286 TD | JAC 1862 | 12/12/95 | *Lambertia echinata ssp. nov* | Williamson Rd east | B/6 |
| 00287 TD | JAC 1863 | 12/12/95 | *Petrophile latericola* | Williamson Rd east | B/15 |
| 00288 TD | JAC 1864 | 13/12/95 | *Lambertia orbifolia* | Brennans Ford | B/15 |
| 00289 TD | JAC 1867 | 13/12/95 | *Lambertia orbifolia* | Dennis Rd | B/20 |
| 00290 TD | JAC 1874 | 14/12/95 | *Lambertia orbifolia* | Adelaide Springs | B/50 |
| | | 18/01/96 | | | |
| 00291 V | JAC 1871 | 14/12/95 | *Kennedia macrophylla* | Turners Cellar | B/? |
| 00292 V | JAC 1873 | 14/12/95 | *Kennedia macrophylla* | Leeuwin Rd | B/20 |
| 00293 C | JAC 1876 | 9/01/96 | *Verticordia spicata ssp squamosa* | Simpson Rd | B/2 |
| 00294 C | JAC 1879-1888 | 9/01/96 | *Eremophila nivea* | Glen Avon Farm | I/10 |
| 00295 C | JAC 1890 | 9/01/96 | *Eremophila nivea* | Three Springs-Morawa Rd | B/50+ |
| 00296 C | JAC 1891 | 9/01/96 | *Verticordia spicata ssp squamosa* | Three Springs-Morawa Rd | B/15 |
| 00297 C | JAC 1892 | 9/01/96 | *Verticordia spicata ssp squamosa* | Colgate/Yandanooka-Morawa Rd | I/1 |
| 00298 C | JAC 1894 | 9/01/96 | *Verticordia spicata ssp squamosa* | Colgate rd | B/5 |
| 00299 C | JAC 1897 | 10/01/96 | *Verticordia albida* | Cnr Wilton Wells/Three Springs-Eneabba Rd | B/500 |
| 00300 C | JAC 1898 | 10/01/96 | *Verticordia albida* | Three Springs-Eneabba Rd | B/10 |
| | | 22/01/96 | | | |
| 00301 C | JAC 1900 | 10/01/96 | *Verticordia albida* | Sweetman Rd | B/10 |
| | | 22/01/96 | | | |
| 00302 C | JAC 1902 | 10/01/96 | *Verticordia albida* | Lynch Rd | B/16 |
| | | 22/01/96 | | | |
| 00303 TD | JAC 1911 | 18/01/96 | *Lambertia orbifolia* | Snake Springs | B/100 |
| 00304 TD | JAC 1914 | 19/01/96 | *Lambertia orbifolia* | Beenup Minesite | B/50+ |
| 00305 C | JAC 1919 | 22/01/96 | *Eremophila nivea* | Campbell Rd | B/13 |
| 00306 C | JAC 1921 | 23/01/96 | *Acacia sciophanes* | Wundowlin Well Nature Reserve | B/150 |
| 00307 C | JAC 1922-1931 | 23/01/96 | *Eremophila caerulea ssp merrallii* | Bruce Rock | I/10 |
| 00308 C | JAC 1933 | 24/01/96 | *Verticordia fimbrilepis ssp fimbrilepis* | Narrogin-Harrismith Rd | B/10 |
| 00309 C | JAC 1934 | 24/01/96 | *Verticordia fimbrilepis ssp. fimbrilepis* | Jingaring Rd (Pop 4) | B/30 |
| 00310 C | JAC 1935 | 24/01/96 | *Verticordia fimbrilepis ssp. fimbrilepis* | Jingaring Rd (Pop 7) | B/150 |
| 00311 C | EHolland | 9/12/95 | *Eremophila caerulea ssp. merrallii* | Hunt Range (Pop 1) | B/15 |
| 00312 C | EHolland | 9/12/95 | *Eremophila caerulea ssp. merrallii* | Hunt Range (Pop 2/3) | B/20 |

| | | | | | |
|---|---|---|---|---|---|
| 00313 C | EHolland | 9/12/95 | *Eremophila caerulea ssp. merrallii* | Hunt Range (Pop 4) | B/42 |
| 00314 C | GRichmond | 18/01/94 | *Eremophila viscida* | Geelokin Rd, Westonia | I/10 |
| 00315C | GRichmond | 17/01/94 | *Eremophila viscida* | English Rd, Westonia | I/10 |
| 00316C | JAC 1939-1946 | 20/02/96 | *Verticordia fimbrilepis ssp. fimbrilepis* | Aldersyde townsite | I/8 |
| 00317C | JAC1950 | 20/02/96 | *Eremophila veneta* | Nth boundary Hopkins NR | B/10 |
| 00318V | JAC 1952 | 21/02/96 | *Darwinia collina* | Bluff Knoll | B/10 |
| 00319C | JAC 1953-1960 | 21/02/96 | *Dryandra montana* | Bluff Knoll | I/8 |
| 00320E | JAC 1962 | 22/02/96 | *isopogon uncinatus* | Torndirrup (CJR popD) | B/20 |
| 00321E | JAC 1963 | 22/02/96 | *Isopogon uncinatus* | Torndirrup (CJR pop B+C) | B/20 |
| 00322V | JAC 1964 | 22/02/96 | *Verticordia fimbrilepis ssp. australis* | Kent River | B/200 |
| 00323V | JAC 1965 | 23/02/96 | *Lambertia orbifolia* | Sleeman Rd, Narrikup | B/10 |
| 00324V | JAC 1966 | 23/02/96 | *Lambertia orbifolia* | Spencer Rd, Narrikup | B/40 |
| 00325x | EMB 5911 | 14/11/95 | *Hemigenia exilis* | HE1 Minara station | I/10 |
| 00326x | EMB 5910 | 14/11/95 | *Hemigenia exilis* | HE3 Glenorn/Minara station | B/? |
| 00327x | EMB 5909 | 14/11/95 | *Hemigenia exilis* | HE4 Glenorn/Minara station | B/15 |
| 00328x | EMB 5907 | 14/11/95 | *Hemigenia exilis* | HE5 Glenorn station | I/10 |
| 00329x | EMB 5908 | 14/11/95 | *Hemigenia exilis* | HE6 Glenorn station | B/15 |
| 00330x | EMB 5912 | 14/11/95 | *Hemigenia exilis* | HE7 Rio Tinto Mine on Minara station | B/? |
| 00331x | EMB 5906 | 13/11/95 | *Hemigenia exilis* | HE8 Poison Creek, Leonora-Agnew Rd | B/15 |
| 00332x | EMB 5913 | 15/11/95 | *Hemigenia exilis* | HE9 Copperfield Base station, Mt Ida | I/5 |

*Status refers to:

I= seedlot comprised of seed from individual parent plants kept separate;

B= seed from individual parent plants bulked as one seedlot due to sporadic or sparse seed production

/number indicates plants represented in the seedlot.

## PROJECT 6

# CONTROL AND MANAGEMENT OF *CRYPTODIAPORTHE MELANOCRASPEDA* CANKER THREATENING *BANKSIA COCCINEA*

L. McCaw


## SUMMARY

Canker caused by the ascomycete *Cryptodiaporthe melanocraspeda* has been implicated in the decline of *Banksia coccinea* stands in the south coastal region of Western Australia. Serious decline of *B. coccinea* was first reported in 1989, and since then the disease has been recorded throughout the range of this species. *B. coccinea* occurs in shrubland plant communities which are prone to periodic fire, and previous work by Bathgate and Shearer (1995) has identified the potential importance of fire in the management of canker affected stands. The project described in this report is intended to build on earlier work by investigating the feasibility and wider implications of using fire to manage canker disease in *B. coccinea* stands.

The project is oriented towards three major outcomes which are important in the management of canker-affected stands. The first of these is to quantify the extent of regeneration in severely degraded stands in the absence of fire. Clearly, if sufficient regeneration occurs without fire, then an appropriate strategy for ensuring the persistence of *B. coccinea* on a canker-affected site may be to exclude fire for a period while seed accumulates on maturing plants. Preliminary observations at a range of sites have indicated that some regeneration does occur without fire, but more detailed information on the growth rate and survival of regeneration is necessary to confirm whether stand replacement will ultimately be successful. A series of permanent quadrats is being established in which recrutiment, survival and growth can be monitored over the longer term. Plots are being established across the geographic range of *B. coccinea.*

The second outcome for the study is to develop criteria to assist decision-making as to the most appropriate time to introduce fire to canker-affected stands. Important criteria include the extent of branch decline and the status of the on-plant seed store on *B. coccinea.* As *B. coccinea* often occurs in species-rich shrublands, patterns of seed accumulation and storage on co-occurring plant species are also important. Detailed information about post-fire regeneration, flowering and seed set is available for a shrubland community containing *B. coccinea* at the Stirling Range National Park. This is being supplemented by data on the life history characteristics of plants occurring with *B. coccinea* across its geographic range, gathered from both field studies and surveys of published literature and unpublished reports. Height growth, flowering and seed production of *B. coccinea*, *Banksia baxteri* and two *Hakea* species are being monitored in a field study at the Stirling Range National Park. *B. coccinea* flowered for the first time at age 60 months in spring 1994, with about 60% of plants in flower. However, none of the plants in the sample population flowered in the

subsequent year. Variable flowering will introduce errors into the estimation of plant age using the node-counting technique, leading to a tendency to underestimate true plant age. This factor needs to be taken into account when studying the development of disease severity across stands of differing ages.

The third outcome is to confirm the relationship between the scale of fire events and the development of canker in *B. coccinea* stands. Previous work by Bathgate and Shearer has demonstrated a gradient of reduced disease severity with increasing distance from remnants of an older, disease-affected stand, suggesting that large, even-aged patches of *B. coccinea* may provide the greatest scope for conserving the species at a landscape scale. The opportunity to test this hypothesis exists in the Stirling Range National Park where an extensive fire occurred in 1983. A field survey to locate stands of the species at different distances from the burn perimeter and from older, potentially canker-affected sites is to be undertaken, and the severity of disease impact will then be assessed at each site. The study area has been stratified on the basis of landform, soil type and fire history in preparation for the survey. In addition, several long unburnt sites (> 40 years since last fire) which may contain *B. coccinea* have been identified and will be visited to assess the structure and condition of stands. These long unburnt sites are of considerable interest and significance as they may provide information as to whether or not decline due to canker is an inevitable consequence of increasing stand age on all sites.

## INTRODUCTION

*Banksia coccinea* R. Br. is a distinctive species characteristic of shrubland plant communities on the southern sandplain of Western Australia, occuring within the region bounded approximately by Albany, the Stirling Range and the Young River in the east. Serious decline of *B. coccinea* stands was first reported in 1989 (Shearer and Fairman 1991), and subsequent work has implicated the ascomycete *Cryptodiaporthe melanocraspeda* as the causal agent for a destructive canker disease affecting the species (Bathgate and Shearer 1995). Previous work has investigated various aspects of the disease and its impact on *B. coccinea* populations including seed bank dynamics of *B. coccinea,* sources of inoculum and conditions favouring spore release, the infection process, factors influencing disease intensity, and possible management strategies to minimise disease impact (Bathgate and Shearer 1995). The shrubland plant communities in which *B. coccinea* occurs are prone to periodic fire and the potential role of fire in the management of canker-affected stands has been recognised. Bathgate and Shearer (1995) demonstrated a pattern of increasing disease severity with increasing stand age, and recorded canker present in all surveyed stands over 14 years of age. The importance of unburnt remnants of old stands as foci for infection of adjacent, younger regeneration was also confirmed, suggesting that fire regimes which create mosaics of small patches of unburnt vegetation could exacerbate disease development.

The project described in this report is intended to build on this prior work by investigating the feasibility and wider implications of using fire to manage canker-

affected stands of *B. coccinea*. Goals of the project are (1) to determine the role of fire in the management and rehabilitation of *B. coccinea* stands affected by canker, and (2) to recommend measures that may assist in maintaining viable stands of *B. coccinea* throughout its current geographic range. Work on the project commenced in October 1995 with the preparation and submission of a Science Project Plan (SPP) within the Science and Information Division of CALM which was subsequently approved as SPP 96/0002.

## PROJECT OUTCOMES

The current project is oriented towards three outcomes, which are as follows:

1. **To quantify the extent of regeneration in severely degraded stands in the absence of fire.**

   Stands in a degraded condition may remain unburnt for considerable periods because they are localised in extent, or cannot be burnt because of practical constraints or conflicts with other land management objectives. If sufficient regeneration occurs without fire, then a realistic strategy for ensuring the persistence of *B. coccinea* on such sites may be to exclude fire for a period while the seed store on plants accumulates. Although such stands may not reflect the full potential of *B. coccinea* on the site, they would at least maintain the occurrence of the species across its current geographic range.

2. **To provide criteria for determining the appropriate stage at which to apply prescribed fire to manage disease within particular stands.**

   Bathgate and Shearer (1995) proposed that canker-affected stands should be burnt when the proportion of dead branches exceeds 50%, as beyond this stage seed loss by spontaneous release and by cone incineration during fire would be so great as to threaten the ultimate regenerative capacity of the stand. They proposed that the lower age limit for applying prescribed fire should be flexible but should take account of the need to have sufficient seed for stand replacement. Given that fire is applied at a landscape level, it is also critical to understand the patterns of flowering and seed store accumulation of co-occurring plant species in order that the broader requirements for plant community conservation are met by any proposed fire regime that may be applied.

3. **To determine whether canker disease develops more slowly in extensive even-aged stands than in mixed age stands.**

   Bathgate and Shearer (1995) demonstrated that within a regenerating stand of *B. coccinea* there was a gradient of reduced disease severity with increasing distance from remnants of an older, disease-affected stand. They went on to recommend that patch sizes of regeneration created by fire should at least be of

the order of a few hectares to limit the rate of infection into the younger regenerating stand, and that fires should be sufficiently intense to scorch the shrub layer so that inoculum potential is significantly diminished. The implication of this finding for fire management in areas containing stands of *B. coccinea* is that fires should be relatively large in scale and contain few unburnt remnants of this species. There is a need to validate this hypothesis at a landscape scale by examining disease severity in stands at varying distance from the boundary of a burnt area.

## WORK UNDERTAKEN

### Outcome (1): Regeneration within disease-affected stands in the absence of fire

Observations of the extent of regeneration in stands exhibiting a range of impacts were made during a field trip in January 1996 as a preliminary step to the establishment of a series of permanent quadrats in which the recruitment, survival and growth of regenerating plants will be monitored. Sites were inspected at Waychinicup, Hassell Highway, Kojaneerup and in the Stirling Range National Park, with some regeneration observed at all sites. Quadrats are to be established in autumn and spring 1996. Current stand structure and disease status will be recorded, together with details of the height, condition and reproductive status of regenerating individuals. It is envisaged that these quadrats will be re-assessed periodically to monitor the development of regeneration and will provide an important benchmark against which the longer term prognosis for canker-affected stands can be gauged.

### Outcome (2): Criteria for appropriate application of prescribed fire to disease-affected stands

Post-fire regeneration of a mallee-heath community near Two Mile Lake at the south-eastern corner of the Stirling Range National Park has been studied since 1991, and the study has subsequently been expanded to address issues of specific relevance to the regeneration of *B. coccinea.* Life history characteristics including primary regeneration response, time of first flowering and time of first seed set have been recorded for 161 species of vascular plants which occur within a series of twelve 10 x10 m permanent quadrats. Regeneration from soil stored seed is the most common response mechanism, followed by resprouting from lignotubers, and then regeneration from seed stored in capsules on the plant (Table 1).

**TABLE 1:** Regeneration response following fire for 161 plant species in mallee-heath.

| Response mechanism | No. of species | Percent of total |
|---|---|---|
| Capsule stored seed | 20 | 12 |
| Soil stored seed | 71 | 44 |
| Lignotubers | 28 | 17 |
| Corms | 18 | 11 |
| Other mechanisms | 8 | 5 |
| No data | 16 | 10 |

Most of the plant species recorded in the quadrats flowered within 48 months of fire (90%), but a small number of woody shrubs including *B. coccinea* had a longer juvenile period at this site. Such species can fullfil an important role as indicators of the minimum interfire period necessary to maintain the species composition of the plant community.

Height and stem diameter growth, flowering and seed production are being examined in detail for a number of woody shrubs with extended juvenile periods at this site. The species being studied are *B. coccinea, B. baxteri, Hakea crassifolia* and *Hakea cucculata*. Samples of 20 seedlings of each species have been tagged, measured for stem diameter and height to growing tip, and assessed for flowering and fruit set. Sample populations of the two *Banksia* species have been established in adjacent areas burnt in March 1989 and November 1990 respectively, which will permit evaluation of the effect of different seasonal conditions on the length of the juvenile period for these species. Two sample populations of 20 *Hakea crassifolia* have been established in an area burnt in April 1986.

*B. coccinea* and *H. cucculata* flowered for the first time in spring 1994 at 60 months after fire, while *B. baxteri* flowered for the first time in autumn 1994 at 56 months after fire (Table 2), with the exception of a solitary plant which flowered during the previous autumn at 44 months. An interesting feature of the data was the absence of flowers on all three species the following year (1995). It is not clear whether this was due to adverse seasonal conditions in the form of a protracted summer drought which extended from October 1994 to May 1995, or to some other factor. Monitoring of the adjacent populations of younger plants should confirm whether the failure to flower in the year following initial flowering is a characteristic of these species, or is determined by environmental factors. *H. crassifolia* has a considerably longer juvenile period, and does not flower until at least 96 months after fire. However, in contrast to the other three species studied it appears that *H. crassifolia* does set seed following its first flowering event.

Variable flowering will introduce errors into the estimation of plant age using the node-counting technique, leading to a tendency to underestimate true plant age. This

factor needs to be taken into account when studying the development of disease severity across stands of differing ages.

**TABLE 2:** Height, flowering status and seed set for four woody shrub species in mallee-heath. Data are for *H. crassifolia* burnt in March 1986 and other species burnt in March 1989. *Banksia* seedlings regenerated following fire in November 1990 had not flowered at the time of assessment in October 1995.

| Species | Year | Plant age (months) | Height (cm) | % plants in flower | % plants with seed set |
|---|---|---|---|---|---|
| *B. coccinea* | 1994 | 60 | 76 | 60 | 0 |
| | 1995 | 72 | 83 | 0 | 0 |
| *B. baxteri* | 1994 | 60 | 55 | 60 | 0 |
| | 1995 | 72 | 64 | 0 | 0 |
| *H. cucculata* | 1994 | 60 | 71 | 15 | 5 |
| | 1995 | 72 | 84 | 15 | 10 |
| *H. crassifolia* | 1994 | 96 | 135 | 40 | 40 |
| | 1995 | 108 | 152 | 88 | 67 |

These results illustrate the need to consider the requirements of co-occurring plant species when developing fire regimes that may be applied to manage canker-affected populations of *B. coccinea.*

Additional information about the species composition of plant communities in which *B. coccinea* occurs will be collected in association with quadrats established to monitor regeneration in canker-affected stands (Outcome 1) and in the quadrats to be established to address Outcome 3. Particular attention will be paid to woody shrubs with extended juvenile periods which can be used as indicators of the minimum interfire period necessary to maintain plant community composition across a range of sites and environmental gradients, particularly rainfall.

Data from unpublished survey reports and from published sources will also be reviewed to compile a data base of plant life history information for species which occur with *B. coccinea.*

**Outcome (3): Development of disease in extensive even-aged stands**

The opportunity to test the hypothesis that the scale of fire events may affect the spread and development of canker exists in the western section of the Stirling Range National Park where an extensive area (>20 000 hectares) was burnt in 1983. The vegetation in this area is now 13 years old and approaching the stage of maturity at which a moderate to high incidence of canker would be expected, based on the results of previous work. However, Bathgate and Shearer (1995) did not assess sufficient sites within the area of the 1983 fire to determine whether a relationship between disease incidence and distance to older, unburnt stands of *B. coccinea* did in fact exist. Reconnaissance within the 1983 fire area in January 1996 revealed a number of *B. coccinea* stands exhibiting little if any evidence of canker, suggesting that a more comprehensive evaluation is warranted.

As a basis for this evaluation, the Park has been stratified on the basis of landform and soil information to identify areas likely to support stands of *B. coccinea*. This information is being used to select stands at varying distance from adjacent areas which have remained unburnt, and which could have provided inoculum for re-infection by airborne spores of *Cryptodiaporthe*. Sites are to be visited in autumn 1996 to assess stand structure and disease status.

Areas outside the boundary of the 1983 fire have been further stratified on the basis of time since last fire. This has identified several long unburnt sites (> 40 years since last fire) at which *B. coccinea* is likely to occur, and its presence has been confirmed by further field inspection at one of the sites. Long unburnt sites will be visited this autumn to assess condition and disease status.

## REFERENCES


Bathgate, J. and Shearer, B. (1995). Control and management of *Cryptodiaporthe melanocraspeda* canker threatening *Banksia coccinea*. Report to Endangered Species Unit, Australian Nature Conservation Agency, March 1995.

Shearer, B. and Fairman, R. (1991). Aerial canker fungi threaten *Banksia coccinea*. Abstract 85/C16. Proceedings of the Conservation Biology in Australia and Oceania Conference, University of Queensland.